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BOOK OF ABSTRACTS

OR001

IL-1B+ MACROPHAGES FUEL PATHOGENIC INFLAMMATION IN PANCREATIC CANCER

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Pancreatic ductal adenocarcinoma (PDAC) is a lethal disease with high resistance to therapies. Inflammatory and immunomodulatory signals typically co-exist in the tumor microenvironment (TME), leading to dysregulated reparative and cytotoxic responses. Tumor-associated macrophages (TAMs) control immune dynamics in the TME, but their heterogeneity and plasticity have hampered our understanding of the underlying mechanisms.

Here, we combined single-cell and spatial genomics in primary PDAC human samples with functional experiments in mouse models to elucidate macrophage functions in pancreatic cancer.

We uncovered an inflammatory loop between tumor cells and interleukin (IL)-1b+ TAMs, a subset of macrophages elicited by a local synergy between prostaglandin E2 (PGE2) and tumor necrosis factor (TNF)-a. Highly multiplexed in situ gene expression analyses showed accumulation of IL-1b+ TAMs within spatial niches that are distinct from those occupied by other macrophage types. In human PDAC, IL-1b+ TAMs co-localize with specific subsets of tumor cells characterized by elevated expression of inflammatory response, epithelial-to-mesenchymal transition (EMT), and oncogene activation gene programs. Interfering with the PGE2-IL-1b axis elicited reprogramming of TAMs and efficiently counteracted tumor cell-intrinsic and -extrinsic inflammation, leading to PDAC control in vivo. IL-1b+ TAMs are conserved across human cancers and negatively correlate with patient survival, in a context-dependent manner.

Our study uncovers IL-1b+ TAMs as key players in tumor-promoting inflammation, it elucidates the underlying molecular and niche determinants, and it provides a rationale for targeting the PGE2-IL-1b axis in pancreatic cancer – a disease whose treatment remains among the greatest challenges in immuno-oncology.

Ref. Caronni, La Terza, Vittoria et al., under invited revision, Nature

OR002

TCR-INDEPENDENT CD137 (4-1BB) SIGNALING PROMOTES CD8+ EXHAUSTED T CELL PROLIFERATION AND TERMINAL DIFFERENTIATION

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Targeting T cell exhaustion is a promising therapeutic strategy against cancer as illustrated by the clinical success achieved with monoclonal antibodies (mAbs) blocking PD1 and CTLA-4. Still, these immune checkpoint blockers (ICB) may only reinvigorate a subset of Tex cells with a progenitor or stem-like signature and the clinical responses are observed only in a fraction of cancer patients. Therefore, finding alternatives to increase ICB efficacy is being actively pursued. Most current strategies target additional inhibitory receptors, but activating receptors also impact the efficacy of cancer immunotherapy as we recently evidenced for CD226 (DNAM-1) (Weulersse et al, Immunity. 2020; Braun et al, Immunity. 2020).

CD137 (TNFRSF9, 4-1BB) is a costimulatory member of the TNFR superfamily whose stimulation by agonists mAbs promote tumor regression in diverse mouse models. yet, despite being a promising immunotherapeutic target, anti-CD137 mAbs had limited efficacy with frequent liver toxicity in humans. Thus, there is a clear need to understand the cellular program driven by CD137 and its role in cancer immune surveillance.

Through single cell transcriptomic, epigenomic and functional assays, we recently demonstrated the importance of T cell-specific CD137 signaling in CD8+ T cell exhaustion program.

Using T cell-specific CD137-deficient mice and agonists antibodies, we found that CD137 modulates tumor infiltration of CD8+ exhausted T (Tex) cells. We demonstrated that T cell-intrinsic, TCR-independent, CD137 signaling involving the RelA and cRel NF-kB subunits induced the proliferation and the terminal differentiation of CD8+ T cells already committed into Tex cell program. CD137 was specifically expressed by Tex cells and induced the Tox-dependent chromatin remodeling and expression of PD1, Lag-3 and Tim-3 immune checkpoints. While Tex cell accumulation induced by prophylactic CD137 agonists favored tumor growth, anti-PD-1 efficacy was improved with subsequent CD137 stimulation in pre-clinical mouse models.

Prolonged TCR signaling due to persistent antigen exposure seems to represent a cardinal cause of exhaustion in chronic infection and cancer. However, additional signals may also participate in exhaustion program since the mechanisms impacting Tex cell differentiation are still poorly understood. Our results (Pichler et al, Immunity. In press) that identify CD137 as a TCR independent regulator of Tex cell expansion and differentiation, could have broad therapeutic applications for the treatment of cancer and infectious diseases.

OR003

NONRECIPROCAL PLASTICITY AND DIVERGENT DEVELOPMENT OF TISSUE RESIDENT MEMORY AND EXHAUSTED CD8+ T CELLS

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Cancer and chronic infections promote the development of exhausted CD8+ T (TEX) cells that fail to combat disease progression. While TEX cells are distinct from conventional central and effector circulating memory T (TCIRC) cells, they share many overlapping features with non-circulating tissue-resident memory (TRM) cells developing after acute infection. CD8+ T cells that co-express markers of terminal exhaustion and the hallmark tissue-residency markers CD69 and CD103 are abundant in many solid cancers where they correlate with favorable immunotherapy responses and are often classified as TRM cells. However, similarities between TEX and TRM cells make it difficult to interpret CD8+ T cell identity and lineage dynamics in the context of persisting antigen.

Here, we used the lymphocytic choriomeningitis (LCMV) model of acute-resolving and chronic infection in mice to interrogate the developmental relationships between committed TCIRC, TRM and TEX cells. We combined lineage tracing experiments with a recently developed sequencing approach enabling concurrent trimodal protein, RNA and ATAC sequencing analysis in the same single cell (TEA-seq) to assess the developmental relatedness and interchangeability of these CD8+ T cell states and compare the transcriptional and epigenetic networks operating in tissue-localizing CD8+ T cells responding to acute or chronic stimulation.

We found that TCIRC cells, non-epithelial CD103– TRM cells and epithelial CD103+ could all become exhausted and give rise to heterogeneous subsets of TEX cells including TCF1+ progenitor cells and KLR-hi effector-like cells during chronic antigen stimulation. Conversely, established TEX cells persisted in the circulation but inefficiently upregulated markers of tissue-residency including CD103 and CD49a in peripheral tissues after antigen withdrawal. Nevertheless, chronic antigen stimulation drove TEX progenitors to seed a distinct subpopulation of terminally exhausted CD39+CD69+CD103+ CD8+ T cells resembling bona fide TRM cells in most peripheral tissues. Simultaneous cell-surface, transcriptional and epigenetic profiling of CD8+ T cells from matched tissues during acute and chronic infection revealed these peripheral TEX cells were fundamentally distinct from their TRM counterparts and from TEX subpopulations residing in secondary lymphoid organs.

Our results highlight unappreciated heterogeneity in the terminal TEX cell pool and imply CD69+CD103+ TRM and TEX cells are fundamentally distinct cellular lineages with unilateral plasticity. These findings have implications for interpreting the immune landscape of solid tumors, determining the relative contributions of CD8+ TEX and TRM cells to cancer immunotherapy responses and understanding how CD8+ T cell states can be manipulated to improve cellular therapies.

OR004

IMMUNE DETERMINANTS OF RESPONSE TO NEOADJUVANT CHEMOTHERAPY IN ESOPHAGEAL ADENOCARCINOMA

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Esophageal adenocarcinoma (EAC) is a highly aggressive malignancy. Patients with locally advanced disease undergo neoadjuvant chemotherapy (nCT), alone or in association with radiotherapy, before surgery. However, less than 30% of treated patients achieve a pathological complete response associated with increased 5-year overall survival. Understanding the mechanisms of response to nCT is pivotal to better stratify patients and inform the design of more efficacious therapies. The response of EAC to nCT may be associated to an active tumor immune microenvironment, given the ability of chemo/radiation agents to stimulate anti-tumor immune responses.

We investigated the immune mechanisms underlying the response to nCT by multi-dimensional profiling of treatment-naïve tumor biopsies and blood samples from 68 EAC patients, comparing pathological complete responders versus non responders to therapy.

At the tumor level, we found that complete response to nCT associated with baseline molecular signatures of immune response and proliferation, increased putative anti-tumor tissue-resident memory CD39+CD103+CD8+ T cell infiltration and reduced immunosuppressive T regulatory cells and M2-like macrophages. Tumor and immune cells subsets were also characterized in situ in n=6 EAC cases by spatial single-cell transcriptomics profiling. Systemically, before starting nCT complete responders showed higher frequencies of immunostimulatory CD14+CD11c+HLA-DR^{high} cells and reduced PDL1+ monocytic myeloid-derived suppressor cells, alongside with high GM-CSF (pro-inflammatory) and low IL-4, CXCL10, C3a, and C5a (suppressive) plasma levels. Plasma pro-inflammatory and suppressive cytokines directly and inversely correlated with the frequency of tumor infiltrating CD39+CD103+CD8+ T cells, respectively.

These results suggest that pre-existing immunity in baseline tumors drives the clinical activity of nCT in locally advanced EAC. Furthermore, it may be possible to stratify patients based on predictive immune signatures, enabling tailored neoadjuvant and/or adjuvant regimens.

OR005

EARLY AND LATE T CELL DYNAMICS UPON NEOADJUVANT IMMUNE CHECKPOINT BLOCKADE IN CUTANEOUS SQUAMOUS CELL CARCINOMA

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The presence of T cells in the tumor microenvironment (TME) correlates with response to immune checkpoint blockade (ICB), but the contribution of distinct T cell states to this response remains unclear. Moreover, recent studies have described two distinct phenomena following ICB: (i) tumor-resident T cells are, at least transiently, reactivated by ICB in human cancer lesions, and (ii) new T cell clones are recruited to the tumor from the periphery. Based on these two observations, we propose a model, in which the early reactivation of pre-existing T cells that is accompanied by increased production of T cell chemoattractants may be a driver of the clonal replacement that is observed at later timepoints. Clinical trials of neoadjuvant ICB provide the opportunity to acquire matched tumor biopsies before and on-treatment to assess immune dynamics upon therapy. However, they commonly include biopsies taken weeks to months after treatment initiation, making it impossible to differentiate primary and secondary effects, and to understand which T cell subsets are reinvigorated by this treatment. To this end, we set up a unique study in which we perform parallel high-dimensional analysis of pre- and on-treatment biopsies, taken during the very first days of neoadjuvant ICB treatment.

The MATISSE trial (NCT04620200) has recruited 46 stage I-IVa cutaneous squamous cell carcinoma patients who received neoadjuvant ICB with either Nivolumab or Nivolumab and Ipilimumab. Matched biopsies and blood samples were collected at 4 timepoints, including at baseline and at weeks 1, 2 and 4 before curative surgery. Samples at all four timepoints were successfully collected for 29 patients (63%). For this study, 12 patients were selected based on material availability, balance of treatment arms and either complete or no pathological response. Single-cell RNA- & TCRseq was performed on CD45+ sorted cells from matched tumor biopsies and blood samples for all timepoints.

Focusing on CD8 T cells, we successfully mapped 13 CD8+ states and their dynamics at four timepoints. In brief, we identified naïve, effector, dysfunctional and proliferative states. As expected, clonal expansion, a hallmark of tumor reactivity, was highest within the dysfunctional-proliferative axis, and so was the clonal sharing among these states. Temporal intra-patient dynamics revealed an early wave of response, initiated in the first week after ICB treatment, with induction of proliferation and activation within the pre-existing pool of dysfunctional T cells. Notably, these clones were highly expanded already at baseline and persisted through the course of ICB. This early activation was followed by a later wave of response carried by effector-like T cell states that included transition from pre-existing dysfunctional clones as well as newly recruited clones. Interestingly, in a small group of patients, a dysfunctional pool was absent in the tumor. Instead, they harbored effector-like states that were clonally expanded and increased early upon treatment, as well as newly recruited clones, suggesting different mechanisms by which antitumor immunity is reinvigorated upon ICB.

Our findings show an early reinvigoration of pre-existing immunity upon ICB, with clonal expansion of either dysfunctional or effector-like states. Notably, the reactivation of dysfunctional states may occur in two waves, with reinvigoration of pre-existing clones in the first days after ICB treatment and a more profound remodeling including also newly recruited clones after a few weeks. Altogether, these observations underline that timing of samples is crucial, with assessment of early timepoints

being key to understand the temporal remodeling of the T cell landscape upon ICB treatment. By dissecting the early dynamics upon ICB we can identify distinct responsive TME subtypes with different involvement of CD8 states.

OR006

HARNESSING INNATE IMMUNITY FOR IMMUNO ONCOLOGY IN BREAST CANCER

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Despite improved outcomes in patients with localized disease, metastatic breast cancer remains a major clinical challenge with limited effective therapies. Targeted therapies may achieve initial responses; however, these strategies are invariably limited by intratumoral heterogeneity leading to disease recurrence.

Immunotherapies overcome these limitations by targeting restraining mechanisms in T cells, producing unparalleled effective and durable responses in a subset of refractory cancers. However, these therapies critically rely on abundant neoantigens and robust immune responses in the tumor microenvironment (TME). These dependencies pose formidable limitations, as abundant neoantigens are found only in a small subset of human cancers, and tumors are immunosuppressed by robust accumulation of tumor-associated macrophages (TAMs). Thus, most breast cancer patients do not benefit from immunotherapy. While TME immune suppression is a significant clinical hurdle, it also serves as a therapeutic vulnerability, highlighting macrophages as a promising target for therapeutic intervention.

Macrophages are antigen-presenting cells capable of regulating diverse immune responses through exceptional phenotypic plasticity. Shaped by complex microenvironmental signals, TME macrophages adopt wound-healing phenotype (M2-like) and provide a significant source of immunosuppressive signals.

To date, most macrophage-based therapies focus on survival or recruitment mechanisms. Notably, while TAM depletion may alleviate the immunosuppressive pressure, it raised safety concerns as macrophages are crucial for homeostasis, and cessation of recruitment intervention leads to a metastatic overshoot in tumor models. We posit that rather than eliminating macrophages or blocking their recruitment, forcing a complete phenotypic conversion into the immune-stimulatory state will have more substantial effects.

While the transcriptional profiles of TAMs are well-annotated across cancer types, modulating their tumor-supporting functions has proved exceptionally difficult, primarily due to the TME complexity and the lack of scalable TME models that enable high throughput target discoveries.

To overcome these limitations and identify targets for therapeutic interventions, we developed a scalable organotypic TME (oTME) platform that recapitulates the direct and indirect cell-cell interactions in the TME with high phenotypic fidelity. This model contains the major TME constituents, including macrophages, tumor epithelial cells, and their supporting fibroblasts, while preserving the functions, transcriptional programs, and typical spatial organization in mammary tumors. Notably, macrophages in this model closely recapitulate the human gene signatures that predict shorter patients' survival.

Leveraging the platform's scalability, we performed a genome-wide CRISPRi screen in primary macrophages to map key regulators of their immunosuppressive phenotype in TME settings. Our screening strategy has identified gene targets that convert TAMs into M2-resistant, immunostimulatory macrophages, overriding the TME signals.

We prioritized gene targets with clinical indications, specifically in patients that harbor somatic mutations and present hyper-inflammatory myeloid cells or autoimmunity symptoms.

Mechanistically, the genetically-reprogrammed macrophages display robust and self-propelled Th1 inflammatory profiles. Adoptive transfer of reprogrammed macrophages into tumor-bearing mice confirmed the “M2-resistance” and led to the abrogation of established tumors and phenotypic shifts in the host’s TME macrophages.

Our work represents a transformative vision to provide an actionable roadmap for developing new therapeutic strategies in breast cancer patients. The macrophage-reprogramming targets are poised to accelerate the development of effective interventions to unleash anti-tumor immune responses and define a novel framework for innate immunotherapy.

OR007

TISSUE RESIDENT CD8+ T CELL CLONAL EXPANSION IN TRIPLE NEGATIVE BREAST CANCER IS ASSOCIATED WITH RESPONSE TO IMMUNOTHERAPY – EXPLORATORY BIOMARKER STUDY FROM THE SYNERGY TRIAL.

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Chemotherapy combined with immune checkpoint blockade has shown clinical activity in breast cancer. Response, however, only occurs in a low proportion of patients. How the immune landscape of the tumor dictates the immune and clinical responses to chemoimmunotherapy is not well understood.

Using a combination of scRNAseq and scTCRseq we characterize the dynamics of tumor-infiltrating immune cells in triple-negative breast cancer (TNBC) patients treated with chemotherapy (carboplatin/paclitaxel) and anti-PD-L1 (durvalumab) alone or in combination with anti-CD73 (oleclumab) in a phase II randomized clinical trial (SYNERGY, NCT03616886). Tissue biopsies were collected PRE- and ON-treatment from a total of 27 TNBC patients.

Our results show that response to chemo + aPD-L1 was associated to an upregulation of interferon-signaling pathways; whereas, response to the addition of aCD73 was associated with early T cell activation and TCR signaling. Responder patients (R) displayed significantly higher fractions of clonally expanded tissue-resident CD8+ T cells with increased expression of genes linked with both T cell exhaustion and cytotoxicity, as opposed to non-responders (NR) having increased levels of terminally differentiated effector-memory (TEMRA) CD8+ and memory CD4+ T cells. TCR analysis revealed that, at baseline, R were significantly more clonally expanded, compared with NR. On treatment, R displayed an influx of newly emerging clonotypes of low expansion, as well as expansion of the precursor CD8 cluster.

Collectively, our data suggest that baseline clonal expansion could be a potential predictor of response to chemoimmunotherapy and that both clonal reinvigoration of pre-existing tumor-reactive T cells and clonal replacement on treatment are key drivers of a protective response to chemoimmunotherapy.

OR008

CANCER SCULPTS GRANULOPOIESIS TO GENERATE TUMOR-SUPPORTIVE NEUTROPHILS.

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Neutrophils dominate the immunological landscape of multiple solid tumors and strongly associate with cancer progression. Because neutrophils are short-lived cells, granulopoiesis is increased to meet the demands of cancers not only in cell number but also by rewiring the neutrophils' functional properties to be produced. Indeed, tumoral neutrophils favor the generation of immunosuppressive and angiogenic environments that allow tumors to thrive. Whether these functions are acquired in the tumor microenvironment or are imprinted early during their development in the bone marrow remains unknown.

To define how cancer rewires the production and properties of neutrophils during cancer progression, we assessed the epigenetic, transcriptomic, and proteomic landscape of neutrophils at single-cell resolution. We built a comprehensive atlas based on the single-cell transcriptomes of mouse neutrophils in health and disease, which we refer to as NeuMap. The NeuMap encompasses over 50 different physiological and pathological conditions, including multiple cancer-associated neutrophils from pancreatic, breast, and lung cancer mouse models, aging models, and acute and chronic diseases in both female and male mice, and at different stages of organismal development.

This comprehensive map allowed us to identify three unique developmental trajectories of neutrophils from the bone marrow into tissues in health, acute inflammation, and in cancer. In addition to promoting granulopoiesis, we observed that cancer skews the developmental trajectory to favor the generation of immune-suppressive and angiogenic cells. Neutrophils from the bone marrow of tumor-bearing mice express early immunosuppression and angiogenesis markers and notably blocked interferon and antigen presentation programs. Consistently, multiparametric flow cytometry of tumor-associated neutrophils uncovered a subset of neutrophils expressing immunosuppressive markers already in the bone marrow of cancer-bearing mice. Functional assays using immature neutrophils from the bone marrow of these mice showed that exposure to tumor-microenvironment factors drove a stronger immunosuppressive profile than in neutrophils coming from healthy mice. Combined inspection of chromatin accessibility and gene expression at the single cell level (DOGMA-seq) of neutrophils from naïve, cancer-bearing, and endotoxin-exposed (inflamed) mice revealed that, compared with inflammatory neutrophils which display type I Interferon (IFN) signaling, those associated with cancer had programs driven by the JunB and Bhlhe40 transcription factors. To functionally validate these observations, we generated mice with depleted expression of *Ifnar*, *JunB* or *Bhlhe40* in neutrophils using the MRP8CRE driver (IFNARdN, JunBdN, and Bhlhe40dN mice, respectively). In vitro exposure to multiple cytokines and conditioned media revealed that Bhlhe40 is needed for full activation of the immunosuppressive program. Similarly, in vivo assays in matrigel implants and ex vivo T-cell suppression assays revealed that JunBdN drives angiogenetic and immunosuppressive properties of neutrophils. In contrast, IFNAR repressed these immunosuppressive properties, and consequently, IFNARdN mice showed accelerated growth of lung tumors.

These results, based on a holistic analysis of the granulocytic compartment, suggest that the generation of tumor-associated neutrophils occurs through systemic alteration of early granulopoietic trajectories in the bone marrow. It will be now important to assess the additional contributions of local re-programming at the tumor microenvironment. Identifying the drivers that favor cancer-associated immune trajectories should enable rational targeting of pro-tumoral granulopoiesis to curb tumor

onset or growth. This is especially relevant for neutrophil-rich types of cancers, such as pancreatic, breast, and lung cancer, in which other immunotherapeutic strategies have proved ineffective.

OR009

IMPROPER PRIMING OF TUMOR ANTIGEN-SPECIFIC HUMAN CD4+ T CELLS LEADS TO A DISTINCT DYSFUNCTIONAL STATE IN IMMUNOTHERAPY-TREATED CANCER PATIENTS

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Failure of tumor control is attributed in part to hyporesponsive T cell states, including exhaustion or peripheral tolerance. Dysfunctional CD4+ T cell states to self-shared tumor antigens or even neoantigens can stem from incorrect priming and lack of costimulatory signals in the tumor microenvironment, and contribute to progression by creating an immune-permissive niche for tumor growth. However, studying human priming and peripheral tolerance of tumor-specific CD4+ T cells, as orchestrators of adaptive immune responses, is challenging to do in vivo. To model such mechanisms preventing effective priming of anti-tumor immunity in humans, we took advantage of two tumor-antigen vaccine clinical trials where patients were immunized with tumor antigen in the absence or presence of immunological adjuvant. Inadequate priming of antigen-specific CD4+ T cell responses in the absence of adjuvant leads to long-term peripheral tolerance. After sorting poorly in vivo primed antigen-specific CD4+ T cells, we characterize their transcriptomic and epigenetic landscape and compare them to baseline precursors and conventionally primed responses to understand hyporesponsiveness.

Twelve patients with ovarian or non-small cell lung cancer were selected from clinical trials LUD2006-001 and LUD99-010, vaccinated respectively with NY-ESO-1 and MAGE-A3, using antigen alone (no adjuvant, emulating a hyporesponsive phenotype and tolerization over time) or with TLR-containing immunological adjuvants. From PBMC at baseline, after 1 and 2 vaccines, we expanded antigen-specific CD4+ T cells using CD40L-guided sorting and performed bulk and single cell RNAseq, paired bulk and single cell TCRseq, and bulk RRBS methylation assays. Mixed linear effect models were used for differential expression and abundance analysis. Significance was stringently defined as false discovery rate adjusted p-values < 0.05.

We show that repeated administration of recombinant tumor antigen vaccines without immunological adjuvant emulates an hyporesponsive phenotype that cannot be rescued even by adjuvanted vaccines in vivo or repeated in vitro expansions. Differential expression analyses of antigen stimulation, adjuvant presence, and temporal changes revealed that hyporesponsive CD4+ T cells upregulated Golgi protein retention, chromatin condensation, and cytoskeleton regulation while downregulating proteasome, RNA transport, and ribosomal activity. Expected helper and effector T cell activation genes (STATs, IFNs and ISGs) were only weakly detectable in the no-adjuvant group compared to properly immunized CD4+ T cells. Following unadjuvanted vaccines, most gene clusters reverted to precursor baseline levels. In contrast, genes from CD4+ T cells post adjuvanted vaccines remained different from baseline. Gene pathways related to oxidative stress and glycolysis were de-enriched in tolerized-like cells while they peaked in properly immunized cells, even at steady-state in the absence of antigen restimulation in vitro. Surprisingly, promoter methylation changes were not associated with differential regulatory CD4+ states. Overall, a downregulation of protein and sugar metabolism was representative of hyporesponsive CD4+ T cells, specifically by suppression of transcription-related zinc finger families, galactosidase metabolism, and protein transport. Finally, TCRseq analysis showed that hyporesponsive CD4+ T cells from unadjuvanted vaccines had fewer dynamic clonal changes compared to greater clonal expansion seen after adjuvanted vaccines.

We characterized the transcriptional and epigenetic signature of a hyporesponsive tumor antigen-specific CD4+ T cell phenotype distinct from exhausted profiles, which may simulate poor priming conditions encountered in the tumor microenvironment of nascent tumors. This study provides

relevant avenues to potentially reverse improperly primed CD4+ T cells by identifying genes, pathways, and cellular states from a human in vivo experimental setting.

OR010

PRIMING OF SYNNOTCH CAR T CELLS VIA CNS-SPECIFIC ANTIGEN ALLOWS SPATIAL AND TEMPORAL REGULATION OF CAR EXPRESSION, PERSISTENCE OF T CELLS IN THE CNS, RESULTING IN THE COMPLETE AND SUSTAINED ERADICATION OF AGGRESSIVE GLIOBLASTOMA

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Glioblastoma (GBM), the most common malignant primary brain tumor, exhibits marked inter- and intra-tumoral antigen heterogeneity. Chimeric Antigen Receptor (CAR) therapy targeting single antigens, such as the tumor-specific neoantigen epidermal growth factor receptor variant III (EGFRvIII), has failed to show clinical benefit in patients with GBM due to the tumor escape following the loss of the CAR antigen. In contrast, more homogeneously expressed GBM antigens (EphA2 and IL13Ra2) are non-ideal because the expression in other normal organs can result in cross-reactive toxicity. Multi-antigen recognition circuits built using synthetic Notch receptor (synNotch) enable engineered T cells to integrate multiple imperfect but complementary antigens.

Notably, our recent work has shown that the sequential prime-and-kill strategy using EGFRvIII-sensing synNotch receptor paired with a tandem CAR can achieve precise killing of tumor cells expressing imperfect target antigens. However, less than 20% of adult GBM cases express EGFRvIII, limiting the eligible patient population. Furthermore, EGFRvIII expression can diminish over time, even after its detection in the primary GBM. To overcome the challenge of tumor antigen loss and bypass the need for tumor-specific antigens, the synNotch receptor can be designed to take antigen cues from the tissue environment for inducing the expression of a CAR against tumor-associated antigens.

We evaluated several brain-tissue-specific extracellular proteins as the priming signals for the synNotch receptor. The brain tissue-specific priming of the synNotch receptor allows the expression and cytolytic function of the CAR to be restricted at the tumor site. Here, we present our results with the synNotch receptor designed to recognize Brevican (BCAN), an extracellular matrix protein in the brain.

When mice bearing intracerebral GBM6 patient-derived xenograft (PDX) tumors received a single IV infusion of T cells engineered with the α -BCAN synNotch--> α -EphA2/IL-13R α 2 CAR (B-SYNC) circuit, all mice (22/22) demonstrated complete and durable regression of the tumor. Furthermore, these B-SYNC T cells were significantly more efficacious than constitutively expressed EphA2/IL-13R α 2 (tandem) CAR T cells. The brain-specific priming ensured that cytolytic activity of the tandem CAR is localized to the tumor-bearing brain but absent in the flank tumor that lacks the expression of the priming antigen BCAN. The synNotch-controlled CAR expression maintains a higher fraction of the T cells in the naïve-like state, associated with superior persistence and less exhausted phenotype in vivo. B-SYNC T cells persist in the brain, protecting mice against tumor rechallenge, even several months after infusion.

Thus, the B-SYNC approach represents a conceptual novelty of CNS tissue-specific CAR activation, which has not been previously evaluated. This also enhances the translational significance of synNotch-CAR T cells by widely extending the eligibility to EGFRvIII-negative glioma patients. We will develop and conduct a phase I study to evaluate our hypothesis.

OR011

IDENTIFICATION OF POTENT EFFECTOR CD8 T CELLS IN TUMORS

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T cells integrate their encounters with antigens over their lifetime, which decisively influence their differentiation state and responsiveness to future encounters. Within the broadly immunosuppressive tumor microenvironment, pockets of rare reactive immunity exist, such as conventional type 1 dendritic cells that support T cells through antigen presentation. Although the path to T cell exhaustion is increasingly well understood, a converse and reactive T cell population has remained largely undefined. To identify such exceptional effector CD8 T cells, one must separate cells that have had encounters and history leading to exhaustion and integrate the current protein expression that highlights successful ongoing activation. While upregulation of surface levels of a protein like CD69 is sometimes associated with ongoing or recent T cell stimulation, analysis of a series of datasets showed that a history of repeated stimulation is associated with decreasing transcription of the Cd69 gene.

We leveraged this by generating mice in which DNA encoding the teal fluorescent protein (TFP) was inserted at the 5' end of the Cd69 locus to record its transcriptional activity, thus giving us a simultaneous readout of transcript and protein to demarcate activation states. Through a series of experiments on Cd69-TFP reporter CD8 T cells, we established this mouse as a novel genetic tool to report T cell stimulation history in vivo, with naïve cells being largely TFPhiCD69- (Q1) and progressively acquiring a TFPlCD69- (Q3) resting phenotype with repeated stimulation. As opposed to TFPlCD69+ (Q4), TFPhiCD69+ (Q2) cells in this reporter system emerge as ones that are both recently activated yet have not been subject to chronic and exhaustive stimulation.

Demarcating intratumoral T cells in this way, Q2 cells were found to be transiently prominent and quickly give way to dysfunctional Q4 cells in growing tumors, while during tumor control, Q2 cells remain the dominant activation phenotype. Combining single cell RNA-Seq with this TFP-mediated demarcation of activated T cells revealed functionally superior T*Eff T cells, at the intersection of intermediate TEff cells and the Q2 activation state. These cells, marked by CD81 and XCL1 expression in this tumor model, are rare cells in growing tumors which nevertheless retain the ability to produce functional proteins like GzmB, TNF-alpha and IFN-gamma and are expanded with robust tumor killing capacity in regressing tumors. A similar T*Eff signature is derived by similar RNA/protein gating on human cancer patient CITE-Seq and RNA-Seq data, associated with favorable patient outcomes.

In summary, we have defined the existence of a rare T*Eff population, which is difficult to isolate from transcriptional states alone but is delineated by a judicious juxtaposition of protein and RNA expression. The systematic use of Cd69 transcription, along with its surface protein expression to delineate potent activation states, as described here, is imminently applicable in tumors and other important contexts including vaccination, resident memory formation, autoimmunity not only in CD8 T cells but potentially in CD4 T cells, NK cells and B cells alike.

OR012

IDENTIFYING MECHANISMS OF TUMOR IMMUNE COMPOSITION CONTROL IN PANCREATIC CANCER SUBTYPES

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Immunotherapies have shown good response rates across a range of human cancers, but not pancreatic ductal adenocarcinoma (PDAC) so far, indicating that PDAC employs additional mechanisms to evade immune control. The tumor microenvironment (TME) of PDAC is known to be immunosuppressive. However, how PDACs orchestrate this cancer protective TME is not established. Therefore, identifying the genes responsible for these phenotypes is of central importance and their targeting could be beneficial to enhance immunity against this disease.

Here, based on a molecularly defined cohort of mouse genetically engineered PDAC cell cultures, we generated syngeneic in vivo models recapitulating key aspects of clinically relevant human PDAC subtypes. To examine the impact of the candidate subtype-specific drivers of immune escape on growth and TME composition, we are employing a spatial functional genomics platform called Perturb-map, which enables dozens of different CRISPR-edited tumors to be resolved by multiplex imaging and spatial transcriptomics. Thereby, we are able to assess the extracellular function of cancer cell-derived genes (e.g., cytokines) in pancreatic tumors of diverse histopathological and molecular origin, with and without adaptive immune selective pressure.

Focusing initially on the classical PDAC subtype, we observed that knock-out of a selected set of 35 candidate genes has no impact on tumor cell fitness in vitro. In contrast, in vivo we identified two negatively selected genes, *Serpib2* and *Pthlh*, whose loss specifically altered tumor fitness and TME composition. *Pthlh* deleted tumor cells tended to be lost both in immunocompetent and *Rag2* deficient mice, suggesting tumor clearance independent of adaptive immunity, but not cell intrinsic since there was no fitness change in *Pthlh* knockouts in vitro. Instead importantly, the *Serpib2* knock-out induced tumor progression only in *Rag2*^{-/-} mice, suggesting that *Serpib2* protects PDAC via control of adaptive anti-tumor immunity. Validation experiments confirmed that loss of *Serpib2* led to reduced PDAC growth in immunocompetent animals and was associated with remodeling of the TME and an increase of intratumoral CD8⁺ T cells. This suggests that *Serpib2* promotes PDAC progression via extracellular control of the immunological state of the PDAC microenvironment.

We identified drivers of tumor growth and immune escape in the classical PDAC subtype, which might provide novel opportunities for immune centered therapeutic interventions. We expect that the spatial functional genomics platform of the distinct PDAC subtypes we are currently investigating will ultimately reveal the distinct modes that they utilize to escape immunity. In conclusion, our studies will establish the function of candidate drivers of immune-escape across PDAC subtypes - how they affect growth and immunity - and importantly, will be at the basis of the identification of novel actionable therapeutic targets for enhancing PDAC immunity.

OR013

IL-23 STABILIZES AN EFFECTOR TREG PROGRAM IN THE TUMOR MICROENVIRONMENT

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Regulatory T cells (Tregs) are crucial for the maintenance of immune tolerance and restrict immunopathology during inflammation, yet their presence in the tumor microenvironment (TME) can dampen anti-tumor immune responses. Effector Tregs (eTregs) are thought to be the cellular subset equipped with the strongest immunosuppressive and cancer promoting properties, but the mediators of eTreg induction in the TME remain largely elusive. The pro-inflammatory cytokine IL-23, primarily produced by myeloid cells, paradoxically promotes tumor growth in preclinical models and correlates with poor clinical outcomes. However, the specific mechanisms underlying the tumor-promoting effects of IL-23 remain unclear.

We used multiple preclinical cancer models, along with a novel reporter mouse strain and single cell analysis, to comprehensively map the cellular responders and sources of IL-23 in the TME. Additionally, we created conditional knockout mice to investigate the impact of IL23R signaling in Tregs within the TME in several preclinical tumor models. We performed high-dimensional flow cytometry with algorithm-guided analysis, single cell RNA sequencing, and functional assays, to understand the influence of IL23R on Treg stability and suppressive function in both the murine and human TME.

Our study identified tumor-associated macrophages as the primary producers of IL-23 in the TME. Surprisingly, among the cells sensing IL-23, we discovered a subset of highly suppressive tumor-infiltrating Tregs. The specific ablation of IL23r in Tregs resulted in significantly reduced tumor growth in various preclinical models. Notably, the tumor growth reduction observed in the Treg-specific ablation mirrored the kinetics seen in full IL23r-deficient mice, suggesting that Tregs are the relevant target of IL-23 in the TME. Upon loss of IL23R in Tregs we observed a higher infiltration of T cells displaying a more activated and cytotoxic phenotype. In addition, the analysis of the myeloid compartment revealed a shift from an immunosuppressive phenotype towards enhanced costimulation and antigen presentation. These findings suggest that IL-23 sensing of Tregs supports the formation of an immunosuppressive TME. Mechanistically, IL23R signaling promoted an effector Treg (eTreg) program characterized by increased expression of the master transcription factor Foxp3 and key downstream targets involved in suppressive function. IL23r-deficient Tregs exhibited reduced suppressive capacity and displayed signs of Treg destabilization such as production of IFN γ and altered cellular metabolism. Single cell analysis at the protein and transcriptome level of tumor infiltrating Tregs, where IL23r deficient and competent Tregs are present within the same TME revealed that IL23R signaling is crucial for the transition of Tregs from an activated to an eTreg differentiation program. Importantly, these findings were validated in the context of human cancer across various solid tumor types.

Our findings underscore the significance of IL23R signaling in promoting an eTreg program in the TME, leading to enhanced suppressive function of Tregs and thus limiting efficient anti-tumor immunity. This study not only provides new insights into the tumor-promoting functions of myeloid-derived IL-23 through Tregs, but also sets the sound base for selective targeting of Tregs through IL-23 or IL23R blockade to expand the therapeutic armamentarium of cancer immunotherapy.

OR014

NACL PRIMES CD8+ T CELLS FOR ENHANCED EFFECTOR FUNCTION IN CANCER IMMUNOTHERAPY

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CD8+ T cells play a central role in anti-tumor immunity but inevitably become dysfunctional or “exhausted” in the tumor microenvironment. Ionic metabolism is emerging as a novel regulator of CD8+ T cell function, but its role in anti-tumor immunity remains ill-defined.

We used multi-omic interrogation of the tumor immune infiltrate, responding or not to immunotherapy, to identify novel regulators of potent CD8+ T cell effector functions. Transcriptomics, metabolomics and adoptive T cell transfer models in vivo were used to validate our findings.

Here we show that sodium chloride (NaCl) counteracts T cell exhaustion to promote cancer regression. NaCl supplementation during CD8+ T cell culture induced potent effector differentiation, IFN-g production and cytotoxicity while maintaining gene networks responsible for stem-like plasticity. Accordingly, adoptive transfer of tumor-specific T cells resulted in enhanced persistence and superior anti-tumor immunity in humanized models. In mice, high salt diet (HSD) administration reduced growth of experimental murine tumors in a CD8+ T cell-dependent manner, inhibited the generation of Toxhigh Eomeshigh terminally exhausted CD8+ T cells, and promoted highly activated CD8+ T cells simultaneously showing potent effector functions and stem-like characteristics when compared to mice fed with regular diet. Mechanistically, NaCl supplementation enhanced glutamine consumption that was indeed critical for cellular reprogramming. We additionally found that a transcriptomic signature of HSD response in vivo was highly enriched in human and mouse CD8+ T cells reinvigorated by anti-PD-1/PD-L1 immunotherapy. In line with these data, cancer patients with increased baseline levels of NaCl were associated with improved progression-free survival following treatment with checkpoint blockade immunotherapies, suggesting promotion of anti-tumor immunity.

Collectively, we identify NaCl metabolism as a major regulator of CD8+ T cell effector function. We also propose that supplementing NaCl during the production of anti-tumor T cells potentially enhances their function upon subsequent adoptive transfer.

OR015

KEOPS COMPLEX LINKS TRNA MODIFICATION TO T CELL ACTIVATION IN MELANOMA

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The stimulation of the immune system in order to direct an anti-tumoral response is now holding significant promises in the treatment of cancer patients. Melanoma is the most aggressive form of skin cancer whose incidence and mortality constantly increased over the last 40 years. Recent discoveries changed the therapeutic intervention landscape of melanoma patients. In our lab, we discovered the key role of anticodon tRNA modifications and specific mRNA translation reprogramming in therapy resistance in melanoma (Rapino et al, Nature 2018). In this study, we aim to uncover the importance of tRNA modifications in the regulation of the melanoma immune response.

We assessed the propensity of >50 tRNA modifying enzymes to regulate proteome homeostasis using aggregation marker and flow cytometry to systematically links tRNA modifying enzymes to proteome homeostasis. In vivo validation using immune-deficient NOD-SCID mice and immune competent C53/BL6 mice highlighted the importance of tRNA modifying enzymes playing a role in the antitumor immune response. Flow cytometry was used to fully understand the immune population of the tumor. To better characterize our candidate, transcriptomic and proteomic analyses were performed, along small RNA sequencing such as tRNA, tsRNA. In order to understand the impact of the identified enzyme on translation, Ribosome sequencing was achieved.

Our in vitro and in vivo screens reveal the importance of OSGEP, the catalytic subunit of the KEOPS complex, in the trigger of an anti-tumor immune response. As part of the KEOPS complex, OSGEP is responsible for the addition of the t6A modification at position 37 of tRNAs, adjacent to the anticodon. We discovered that the loss of OSGEP in melanoma tumors reduced tumor growth in mice in a T-cell-dependent manner. We showed that of OSGEP knockdown affects mRNA translation and correlated with the accumulation of protein aggregates. Strikingly, cytokine array analyses revealed that the loss of OSGEP correlates with the production of pro-inflammatory cytokines, including type I interferon. We discovered that RIG-I was specifically activated upon OSGEP depletion and is essential for subsequent T cell response and control of tumor growth. Key experiments are ongoing to further pinpoint the mechanisms linking t6A tRNA modification to RIG-I activation and type I interferon response.

Together, our work uncovered the importance of OSGEP and t6A tRNA modification in the regulation of the tumoral immune response in melanoma. By triggering RIG-I activation and subsequent inflammation, we here demonstrate that specific modulation of t6A tRNA modification promotes T cell attraction and activation, in a context of poorly immunogenic melanoma tumors.

OR016

THE INCREASING COMPLEXITY OF THE MELANOMA TRANSCRIPTOMIC LANDSCAPE AND ITS CHANGES UNDER IMMUNOTHERAPY

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Immune checkpoint blockade (ICB) targeting the programmed cell death protein-1 (PD1) has become the standard of care in the treatment of advanced melanoma. While ICB is particularly successful in some patients, more than half will not obtain a durable response. It can be due to primary resistance, but can also be acquired during the treatment. Biomarkers that reliably discriminate between ICB responders and non-responders and therapeutic strategies that overcome therapy (cross) resistance remain high unmet clinical needs.

Most of the studies elucidating predictors or mechanisms of the resistance to this ICB were based on samples collected either before treatment or late after treatment. We hypothesized that treatment-induced changes in the tumor microenvironment can already be observed early after the first administration of ICB. Therefore, we conducted a prospective longitudinal clinical study in which matching drug-naïve and on-treatment melanoma biopsies from patients on ICB therapy were subjected to single-cell (including spatial) multi-omics profiling. All on-treatment samples were collected after only one cycle of treatment, reasoning that patient stratification at such an early time point may be possible and clinically useful. The scRNA-seq data (10X Genomics) was generated from 46 human metastatic lesions (11 responders, 11 non-responders according to RECIST1.1) stage III, and IV melanoma patients. More than 55K cells were identified and analyzed using standard Seurat pipelines. To overcome the challenge of identification of malignant cells clusters on the integrated data across patients posed by the high degree of inter-patient genetic variability, this analysis was further guided by deep scRNAseq from Tyr::NRasQ61K^o;Ink4a^{-/-} murine melanoma lesions, which showed high transcriptomic, but little genetic, ITH. Moreover, to spatially map the identified cell types/states we leveraged spatial transcriptomic approaches: Visium (10X Genomics) and Molecular Cartography (Resolve Biosciences).

We first established a comprehensive view of the cellular architecture of the treatment-naïve melanoma ecosystem and defined 7 distinct melanoma transcriptional metaprograms. Consistent with a non-random geographic distribution of melanoma cell states, we demonstrated that this complex transcriptomic heterogeneity is, at least partly, driven by the tumour microenvironment. Importantly, two of the melanoma transcriptional metaprograms were associated with divergent clinical responses to ICB. The Antigen Presentation cell population was more abundant in tumours from patients who exhibited a clinical response and were observed in the immune cell rich niches. Mesenchymal-like melanoma (MES) cells, which are known to drive resistance to targeted therapy, were significantly enriched in early on-treatment biopsies from non-responders to ICB, and their presence significantly predicted lack of response. Importantly, we validated these results in scRNAseq data from a breast cancer cohort within strongly similar study. Finally, we identified TCF4 (E2-2) as a master regulator of the MES program and suppressor of both melanocytic and Antigen Presentation programs. Targeting TCF4 expression in MES cells either genetically or pharmacologically using a bromodomain inhibitor increased immunogenicity and sensitivity to ICB.

In summary, our study described an increasingly complex melanoma transcriptional landscape and its rapid evolution under ICB. It also identified a putative biomarker of early response to ICB and an epigenetic therapeutic strategy that increases immunogenicity of ICB-refractory melanoma.

OR017

LINOLEIC ACID POTENTIATES CD8+ T CELL METABOLIC FITNESS AND ANTI-TUMOR IMMUNITY

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Adoptive T Cell Therapy (ACT) aims to re-educate the immune system against cancer, enabling unprecedented clinical responses. Yet, efficacy of ACT in solid tumors is still marginal. The metabolic state represents a major hurdle limiting ACT efficacy. Indeed, specific lipids can harm CD8+ T cell (CTL) mitochondrial integrity, leading to defective anti-tumour responses. However, the extent to which lipids can affect CTL functions and fate remains unexplored.

We screened different lipids and assess their ability to shape CTL differentiation and activity using multi-parametric flow cytometry, functional assays, together with a complete transcriptomic and epigenomic profiling. Metabolic profile of CTL cells was examined by bioenergetic flux measurements paired with metabolomic and lipidomic analysis. Finally, the anti-tumour responses of metabolically-edited CTL was evaluated in a pre-clinical mouse model, known to poorly respond to immunotherapy, as well as in the clinical setting of human CAR-redirectioned T cells.

We identified Linoleic Acid (LA) as a major positive regulator of CTL activity by improving metabolic fitness, preventing exhaustion and stimulating a memory-like phenotype with superior effector functions. Mechanistically, LA treatment enhances the formation of ER-mitochondria contacts (MERC), which in turn promotes calcium (Ca²⁺) signaling, mitochondrial energetics, and CTL effector functions. As a direct consequence, the anti-tumour potency of LA-instructed CD8 T cells is superior towards different type tumors, both in vitro and in vivo following adoptive transfer into tumour bearing mice.

In conclusion, our study demonstrates that LA could be considered as a molecular switch to fine-tune memory T cell formation and metabolic fitness maintenance, linking lipid metabolism to anti-tumour surveillance. This paved the way for a new generation of adoptive T cell-based therapies, where LA can be used during ex vivo CAR- and TCR- T cell manufacturing to achieve metabolic reprogramming and long-term functionality, broadening the therapeutic efficacy of ACT to a wide range of malignancies. Thereby, we propose here a novel strategy to boost ACT efficacy by implementing CTL long-term functionality, metabolic fitness and preventing exhaustion through lipid-induced mitochondrial rewiring (PCT/EP2022/081824).

OR018

FERROPTOSIS-TARGETING AGENTS POTENTLY MODIFY IMMUNOTHERAPY-INDUCED ANTIMELANOMA IMMUNE RESPONSES

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Identifying methods to enhance existing immunotherapy regimens and overcome resistance remains critical for improving outcomes in melanoma and other cancers, and ferroptosis has received significant attention as a cell death modality to target for this purpose. However, important questions remain regarding the relevance of ferroptosis to endogenous immune responses and the impact of ferroptosis inducers on the broader tumor microenvironment and immunotherapy responses. In this study, we aimed to investigate the role of ferroptosis in immunotherapy-induced immune responses to melanoma and assess the therapeutic potential of ferroptosis inducers alone and in combination with immune checkpoint inhibitors.

To begin, we performed single-cell RNA sequencing (scRNA-seq) on murine melanoma tumors to identify a ferroptosis signature associated with successful immune cell-mediated tumor clearance. We validated our findings through functional studies assessing for evidence of intratumoral ferroptosis in a preclinical mouse melanoma model treated with multiple immunotherapies. We then evaluated the effects of multiple ferroptosis inhibitors and inducers alone and in combination with immune checkpoint blockade. To determine the relevance of these findings to human melanoma patients, we analyzed melanoma patient tumors by scRNA-seq before and after immunotherapy treatment as well as a published dataset of treatment naïve and immunotherapy resistant human melanoma tumors.

Pathway analysis identified ferroptosis as key feature of murine melanoma tumors undergoing successful immune cell-mediated clearance. Deeper investigation revealed diverse ferroptosis-related genes with functions pertaining primarily to glutathione synthesis, redox biology, lipid production and storage, iron regulation, metabolism, transcriptional regulation, and immune signaling. Lipid peroxides were increased in tumors treated with multiple forms of immunotherapy, and ferroptosis inhibitors completely abrogated the effects of these immunotherapies. Conversely, multiple ferroptosis inducers stimulated potent antitumor immune responses and enhanced responses to immune checkpoint blockade. Mirroring our murine model, analysis of tumors from human melanoma patients responding to immunotherapy identified a prominent ferroptosis signature. Additional analyses revealed depletion of this signature in a distinct dataset of immunotherapy resistant human melanomas.

This study links single cell transcriptomic data from murine and human melanoma tumors with functional studies in our preclinical mouse melanoma model to demonstrate that ferroptosis is a vital component of immunotherapy-induced tumor clearance and ferroptosis inducers synergistically enhance responses to immune checkpoint inhibitors. These results offer crucial insight into the role of ferroptosis in antitumor immunity and highlight the promise of ferroptosis-targeting agents as immunotherapy adjuvants.

OR019

OVERCOMING THE INHIBITORY FCYRIIB BARRIER UNLOCKS FC-DEPENDENT ACTIVITY OF HUMAN ANTI-CTLA-4 ANTIBODIES

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While antibodies targeting CTLA-4 were developed to block inhibitory signals in T cells, studies in mouse models demonstrated that Fc-dependent mechanisms, including depletion of tumor-infiltrating regulatory T cells (Tregs), are required for complete antitumor activity. However, the two clinically available antibodies Ipilimumab and Tremelimumab do not show substantial Fc-dependent activity in cancer patients, which may limit their therapeutic efficacy.

Here, we investigate the mechanisms restraining the Fc-dependent activity of human anti-CTLA-4 antibodies in a novel humanized mouse model expressing both human CTLA-4 and human Fcγ receptors (FcγRs).

As observed in cancer patients, Ipilimumab and Tremelimumab failed to deplete tumor-infiltrating Tregs in humanized mice, indicative of poor Fc activity. We found that FcγRIIB, the inhibitory FcγR, was highly expressed in mouse and human tumors, acting as a potential barrier to Fc-dependent responses. Antibody-based blocking of FcγRIIB unlocked the Treg-depleting capacity of Ipilimumab and unleashed its antitumor activity. Alternatively, Fc-engineering of Ipilimumab to enhance binding to activating FcγRs while limiting binding to FcγRIIB significantly increased Treg depletion and antitumor activity. We obtained similar results with a Fc-optimized antibody targeting CCR8, a chemokine receptor selectively expressed on tumor-infiltrating Tregs. Finally, we found that Fc-optimized Ipilimumab increased MECA-79+ tumor-associated high endothelial venules, suggesting a potential for therapeutic induction of lymphoid-like structures in tumors.

Our results define FcγRIIB as a major barrier limiting Fc-dependent activity of human anti-CTLA-4 antibodies in the tumor microenvironment and support the development of Fc-optimized antibodies for improved cancer immunotherapy in patients.

OR020

PD-1/CDC1 AXIS REGULATES STEM-LIKE CD8+ T CELL DIFFERENTIATION IN THE TUMOR DRAINING LYMPH NODE

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Stem-like CD8+ T cells constitute a subset of activated cytotoxic CD8+ T cells that retain high proliferative capacity and self-renewal potential, enabling them to continuously give rise to terminally differentiated effector cells that promote clearance of infected and transformed cells. Importantly, stem-like CD8+ T cells – characterized by their high expression level of the transcription factor TCF-1 – have been identified as the major responding T cell subset during cancer treatment with checkpoint blockade. It is increasingly appreciated that tumor draining LNs (tdLN) serve as reservoirs from which stem-like CD8+ T cells in the tumor sites can be replenished. However, how activated CD8+ T cells interact with their surrounding microenvironment within tdLN during differentiation into stem-like cells, and how TCR-dependent signaling during this process promotes differentiation into TCF-1+ stem-like cells in a highly antigenic and inflammatory environment remains unresolved.

To address these questions, we employed multiplexed 3D tissue microscopy to analyze the immune compartments of optically cleared tdLNs that drain intradermally transplanted OVA-expressing mouse Kras/p53 (KP) tumors, focusing on late T cell differentiation phase (days 4-9 after initiation of tumor antigen-specific responses).

Well after the early phase (days 1-4 post tumor initiation) of T cell activation and proliferation, we identified distinct cellular niches within the T cell zone of tdLN predominantly occupied by clustering antigen-specific TCF-1+ PD-1+ stem-like CD8+ T cells that are closely associated with dense networks of antigen cross-presenting XCR1+ conventional dendritic cells (cDC1). Antigen-specific signaling can be detected in situ among these TCF-1+ stem-like cells, suggesting that these immune cellular niches serve as sites of ongoing antigen presentation. They are indispensable to the expansion of TCF-1+ stem-like CD8+ T cells, as in vivo ablation of XCR1+ cDC1 during this late differentiation period led to significant reduction of stem-like T cell formation. Interestingly, clustering TCF-1+ stem-like cells in tdLN expressed elevated levels of PD-1, PD-L1, and SLAMF6 surface proteins, and high-resolution tissue imaging revealed polarization of these inhibitory molecules towards the membrane interface of not only XCR1+ cDC1 but also neighboring T cells. Thus, we have uncovered a previously undefined late antigen presentation phase that persists days after initial CD8+ T cell priming and through which engagements of TCF-1+ stem-like cells with both antigen-presenting cDC1 as well as surrounding stem-like T cells appear to be involved in the regulation of optimal stem-like cell formation.

Intriguingly, PD-1 expression among polyclonal antigen-specific TCF-1+ stem-like cells is positively correlated with tetramer binding, suggesting that during effector differentiation, the PD-1/cDC1 axis within the cluster formation functions to promote selective enrichment and expansion of high affinity TCF-1+ stem-like cells. In vivo blockade of PD-1/PD-L1 signaling through checkpoint therapy eventually led to the loss of high affinity stem-like clones in the tdLN, along with a phenotypic shift towards terminally differentiated TCF-1- effector cells.

Our findings suggest that a PD-1/cDC1 axis formed during late differentiation phase is an integral component involved in sustaining optimal expansion of TCF-1+ stem-like CD8+ T cells. These results also raise questions about whether prolonged use of PD-1 checkpoint therapy in cancer patients interferes with maintenance of a key precursor population necessary for the robust effector generation needed to achieve full tumor regression.

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OR0021

CYTOTOXIC PD-L1/PD-L2 DUAL-SPECIFIC ANTIBODIES EFFECTIVELY TREAT BOTH IMMUNE “HOT” AND “COLD” CANCERS

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Antibody blockade of either the Programmed Death 1 (PD-1) immune checkpoint receptor on T cells or its cognate ligand PD-L1 on tumor cells and stroma has revolutionized oncology. Despite this transformational efficacy in immune-infiltrated “hot” cancers, these drugs fail to regress “cold” tumors lacking in substantial intra-tumoral T cell density. We leveraged the iterative selection capacity of yeast-displayed fully human antibody selection to identify dual-specific antibodies that bind to unique epitopes in both PD-L1 and PD-L2 blocking their engagement of PD-1. Affinities for each ligand, as well as the capacity to block PD-L1 to B7-1 cis binding, were enriched to therapeutically desirable levels through subsequent rounds of maturation and selection. Leads were selected with higher PD-L2 affinity compared to PD-L1, as human PD-L2 binds PD-1 with a 3-fold higher affinity than does PD-L1 and is significantly more selective in its expression in cancers versus in normal tissues. Recognizing the suppressive, T cell exclusionary stroma as a major obstacle to immune checkpoint blockade (ICB) responses in “cold” tumors, we Fc engineered these dual-specific antibodies to mediate efficient antibody-dependent cellular cytotoxicity (ADCC) and phagocytosis (ADCP) against PD-L1/L2 expressing cells.

Antibody binding to PD-L1 and PD-L2 of murine, cynomolgus, and human origin was measured on an Octet system (ForteBio). The Promega PD-1 assay system was used to measure EC50s for antibody reversal of PD-1 driven T cell inhibition following PD-L1 and/or PD-L2 engagement. Retrogenix cell microarray technology was used to determine on-target versus off-target binding. Both surrogate (Promega ADCC / ADCP Assay Kits) and direct (NK cell co-culture and cytotoxicity assays) assays were used to measure ADCC and ADCP efficiency against PD-L1/PD-L2+ target cells. Murine in vivo tumor models either unmodified (e.g., CT26 parental) or engineered to express PD-L2 (e.g., B16-PD-L2, CT26-PD-L2) to replicate human cancer PD-L2 expression were studied for the capacity of PD-1 blockade versus PD-L1/PD-L2 dual-specific antibodies to mediate tumor regression. High parameter flow cytometry was used to characterize changes in the tumor immune microenvironment in response to each agent. Also, human tumor xenografts were implanted into immune deficient mice to study the ADCC/ADCP based depletion pattern and therapeutic potential of these antibodies separate from their checkpoint blockade activity.

We generated Fc-enhanced PD-L1/PD-L2 dual-specific antibodies which efficiently mediated ADCC and ADCP based killing of human PD-L1+ and/or PD-L2+ cells in vitro using both NK and myeloid effector cells. In vivo studies of murine syngeneic tumor models revealed that these PD-L1/L2 antibodies significantly outperform PD-1 blockade across both “hot” and “cold” cancers. A final round of rational mutagenesis augmented PD-L1/L2 affinity and removed off-target binding potential discovered in the Retrogenix cell microarray test yielding 38002 (IMGS-001) a dual-specific antibody capable of driving tumor rejection of 50% of “cold” B16-PD-L2 tumors as a monotherapy. Flow cytometry showed that depletion of multiple subsets of tumor myeloid stroma correlates with T cell and dendritic cell expansion and therapeutic response in syngeneic cancer models. Lastly, treatment of nu/nu mice bearing a human triple negative breast cancer line (MDA-MB-231) with these cytotoxic dual PD-L1/L2 antibodies significantly inhibited tumor growth and reduced infiltration of CD11b+ cells.

This effector enhanced PD-L1/PD-L2 dual-specific antibody, 38002, represents a second generation of PD-1 pathway therapeutic which couples superior PD-1 pathway blockade with direct depletion of both immunosuppressive and T-cell exclusionary stroma and tumor cells themselves. An ongoing Phase I trial will establish whether these mechanisms are capable of conferring the same advantages to “cold” tumor patients.

OR022

LACK OF TREM2 DURING ANTI-PD1 THERAPY REPROGRAMS INTESTINAL MACROPHAGES AND MICROBIOTA TO ENHANCE TUMOR REJECTION

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Immune checkpoint therapy (ICT) is successfully used to activate anti-tumor T cell responses in the treatment of several types of cancer. However, while many patients respond durably to ICT, a significant number remain unresponsive, prompting the investigation of complementary therapeutic avenues to improve ICT. Reprogramming TAM by either blocking or deleting the macrophage receptor TREM2 attenuates tumor growth and promotes ICT. Another strategy to improve checkpoint therapy relies on the intestinal microbiota. Here we found that the synergistic effect of TREM2 deficiency with anti-PD1 is dependent on the microbiota.

We analyzed the anti-PD1 response of the MCA/1956 cell line injected subcutaneously. To address the contribution of the microbiota in wild-type and TREM2-deficient mice, we analyzed tumor growth in different housing conditions, we performed antibiotic treatments, cecal microbial transplant (CMT) experiments, 16S sequencing, and we treated mice with isolated microbial species. To characterize the immune response in the tumor and in the gut in our experimental settings, we performed flow cytometry and single cell RNAseq analyses. We took advantage of photoconvertible mice to track cell migration from the gut to the tumor bed.

We found that anti-PD1 combined with TREM2 deficiency induces proinflammatory programs in intestinal macrophages and a concomitant expansion of *Ruminococcus gnavus* (*R. gnavus*) in the gut microbiota. Colonization of wild-type mice with *R. gnavus* recapitulated enhancement of anti-PD1-mediated tumor elimination promulgated by the absence of TREM2. The intestinal proinflammatory environment coincided with expansion, increased circulation and migration of TNF-producing CD4⁺ T cells to the tumor bed.

TREM2 remotely controls anti-PD1 checkpoint blockade through modulation of the intestinal immune environment and microbiota; *R. gnavus* is a potential probiotic agent for overcoming resistance to anti-PD1.

OR023

ENGINEERED PROBIOTICS DIRECT THE ANTIGEN-SPECIFICITY OF CAR-T CELLS IN SITU FOR BROAD TUMOR TARGETING

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Antigen-targeting therapies such as chimeric antigen receptor (CAR)-T cells have achieved unparalleled success in the treatment of hematological malignancies. However, broad success has remained bottlenecked by the lack of targets that are specifically, and uniformly, expressed on heterogeneous solid tumors. In contrast, certain strains of bacteria are gaining recognition as a new class of antigen-agnostic cell therapies due to their selective growth within the immuno-suppressive niche of the solid tumor microenvironment (TME). Bridging these approaches, we developed a platform of probiotic-guided CAR-T cells (ProCARs) – in which tumor-colonizing probiotics release synthetic targets that efficiently tag tumor tissue for CAR-mediated lysis.

Here, we engineered a well-characterized strain of probiotic bacteria, *E. coli* Nissle 1917, to sustain the intratumoral production of genetically encoded CAR targets and T cell attracting chemokines in multiple xenograft and syngeneic models of human and murine tumors. We design orthogonal CAR targets (Tags) as modified dimers of GFP that broadly anchor to collagens, fibronectins, and heparin sulfates found in high abundance within the solid TME to achieve antigen-agnostic tumor targeting.

We demonstrate that Tags robustly coat the surface of cancer cell lines through interaction with cell surface matrix proteins, thus leading to the activation of GFP CAR-T cells and Tag-dependent killing across a panel of genetically distinct target cells. We additionally show that injected probiotics selectively grow within the tumor core and maintain the intratumoral production of Tags and human chemokines – leading to therapeutic efficacy across multiple subcutaneous and orthotopic tumor models. Moreover, we demonstrate a systemic antitumor benefit in immune-competent hosts, whereby unilateral treatment of primary tumors leads to a significant reduction in the growth rate of distal, untreated tumors. Finally, we show that intratumoral bacteria provide natural TLR agonists that trigger substantial activation of human and murine ProCAR-T cells.

Our findings highlight the potential of the ProCAR platform to address the critical roadblock of identifying suitable CAR targets by providing an antigen in situ that is orthogonal to both healthy tissue and tumor genetics. Altogether, the use of a bacterial delivery platform in the ProCAR system offers a partner organism that naturally enhances CAR-T cell effector function and broadens the scope of CAR-T cell therapy to include previously difficult to target tumors.

OR024

GENERATION OF NOVEL ANTI-TUMOR CHIMERIC ANTIGEN RECEPTORS INCORPORATING T CELL SIGNALING PROTEINS

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Chimeric antigen receptors (CARs) are molecules composed of an antibody fragment specific for a tumor antigen, fused to a transmembrane domain and a T-cell-signaling moiety. CAR T cells have been successfully used to treat lymphoma, leukemia, and multiple myeloma but their efficacy in solid tumors has been more challenging. CAR-T cell exhaustion, cytokine-mediated toxicity, and disease relapse in situations where there is low density of target antigen have posed challenges to CAR-T immunotherapy. Although CARs have been designed to harness T Cell Receptor (TCR) signaling, they are much less sensitive than TCRs. Thus, there may be multiple benefits to improving current CAR-T design. While many ongoing efforts are focused on the identification of cell surface target antigens, we have taken advantage of our recent super-resolution microscopy studies of TCR-mediated activation to modify CAR signaling domains. Visualization of T cell activation highlighted signaling clusters of adapter molecules that are distinct from the TCR complex, leading us to hypothesize that direct activation of adapter proteins may be sufficient to mediate T cell activation. Furthermore, by direct triggering of the downstream signaling cascade, our novel Chimeric Adapter Proteins (CAPs) may bypass several proofreading steps required for crossing the T cell activation threshold as well as inhibitory regulation of upstream molecules, possibly leading to a more sensitive and potent activation of T cells.

We have designed and generated novel CAPs that bypass the TCR zeta domains used in current FDA-approved CAR designs. These CAPs fuse an extracellular targeting domain to intracellular domains derived from downstream signaling proteins that we have identified in distinct signaling clusters. CAPs harboring an scFv binding CD19 (FMC63) and fused to LAT or SLP76 adapter moieties in tandem with the ZAP-70 kinase domain, were generated. CAPs resulting in cell surface expression following lentiviral transduction of primary T cells were then screened in vitro.

CAP-Ts expressing adapter moieties promoted high levels of basal cytokine secretion in an antigen-independent manner, a feature that has been correlated with enhanced cytotoxicity in vivo and were not further assessed. In contrast, CAPs that exclusively contained ZAP70 domains demonstrated low basal activation and high antigen-specific cytokine production and cytotoxicity and were further evaluated in vivo. We named them CAR-Tyrosine Kinases (CAR-TKs). First generation CAR-TKs containing ZAP70 domains, and second-generation CAR-TKs, containing ZAP70 and CD28 costimulatory domains, were evaluated for their ability to eliminate CD19+ leukemia in an NSG xenograft model. Second generation CAR-TKs exhibited high anti-tumor efficacy, and significantly enhanced and persistent in vivo tumor clearance in leukemia-bearing NSG mice as compared with conventional CD19-28zeta CAR-T. Mechanistically, CAPs were activated in an Lck-independent manner and displayed slower phosphorylation kinetics and a longer duration of signaling compared with 28zeta-CARs.

The unique signaling properties of CAR-TKs may therefore be harnessed to improve the in vivo efficacy of T cells engineered to express an anti-tumor chimeric receptor.

P001

A TETRAVALENT NANOVACCINE THAT INHIBITS HEAD AND NECK CARCINOMA VIA DENDRITIC AND T CELL ACTIVATION

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Abstract

Background: The prevalence of head and neck carcinoma (HNC) caused by the human papillomavirus (HPV) is constantly rising. HPV's oncogenic proteins E6 and E7 are co-responsible for malignant cell proliferation, and are therefore appropriate therapeutic targets.

Methods: The current study aimed at developing therapeutic vaccination against HNC. We produced a tetravalent vaccine consisting of virus-like nanoparticles (Q \square) loaded with a potent TLR9 ligand and displaying four elongated HPV peptides (Q \square -HPVag). Peptide epitopes from E6 and E7 were selected based on published data, according to their in vivo antitumor efficacy, immunodominance and binding affinity to MHC-I molecules. The antitumor efficacy of the Q \square -HPVag vaccine was evaluated in the murine mEERL95 HNC model. Tumor-infiltrating CD8+ and CD4+ T cells and DC subsets (cDC1, cDC2, DC3 and migratory DCs) were determined.

Results: Therapeutic vaccination with the tetravalent Q \square -HPVag could significantly hinder tumor progression and enhance the infiltration of HPV-specific CD4+ and CD8+ T cells which readily produced interferon gamma (IFN- γ) and tumor-necrosis factor α (TNF- α). The resulting antitumor efficacy was associated with favorable immune-repolarization of the tumor microenvironment through expansion of activated DCs and their different subsets, including CCR7+ migratory DCs, cDC1 and cDC2. Furthermore, Q \square -HPVag decreased post-surgical tumor recurrence and prolonged the survival.

Conclusion: Monotherapy with the tetravalent Q \square -HPVag vaccine shows promising antitumor efficacy in an HNC murine model. Future clinical application using this strategy is readily feasible and practical, as click chemistry coupling of elongated synthetic HPV E6/E7-derived peptides to nanoparticles can be done at the bedside directly before injection.

P002

CYTOKINE-ARMORED DENDRITIC CELL PROGENITORS FOR ANTIGEN-AGNOSTIC CANCER IMMUNOTHERAPY

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Dendritic cells (DCs) are specialized myeloid cells with the ability to uptake, process, and present antigens to T lymphocytes; they also generate cytokine and chemokine gradients that regulate immune cell trafficking, activation, and function. Monocyte-derived DCs (moDCs) pulsed with tumor antigens have been used as a platform for therapeutic vaccination in cancer. However, in spite of significant development and testing, antigen-loaded moDCs have delivered mixed clinical results.

Here we present a DC therapy that uses a population of mouse or human DC progenitors (DCPs) engineered to produce two immunostimulatory cytokines, IL-12 and FLT3L.

In the absence of antigen loading, cytokine-armoured DCPs efficiently differentiated into conventional type I DCs (cDC1) and inhibited tumor growth in melanoma and autochthonous liver cancer models. Tumor response to DCP therapy involved synergy between IL-12 and FLT3L and was associated with massive T and NK cell infiltration and activation, robust M1-like macrophage programming, and ischemic tumor necrosis. Mechanistically, anti-tumor immunity was dependent on endogenous cDC1 expansion and interferon- γ (IFN γ) production and signaling, but did not require CD8+ T cell cytotoxicity. In one application, cytokine-armoured DCPs synergized with antigen-specific CAR-T cells to eradicate intracranial gliomas in mice

Engineered DC secreting pro inflammatory cytokine IL12 act synergistically with cell surface presented targets in hard to treat tumor models including glioblastoma.

P003

THE ATF4 / LCN2 AXIS CONTROLS IMMUNE EVASION IN KRAS-DRIVEN LUNG CANCER

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The tumor microenvironment (TME) and the fitness and activation of immune cells play a critical role in determining patient response to immunotherapy and clinical outcomes. However, the biological mechanisms underlying immune evasion remain largely elusive. Solid tumors, such as lung cancer, are characterized by a metabolically stressed TME, with common features such as nutrient deprivation, toxic waste accumulation, and oxidative stress significantly impacting cells in the TME. Recent research has highlighted the important role that metabolic stress and other tumor characteristics play in the development and level of anti-tumor immunity.

Multiple stress-related pathways, including the integrated stress response (ISR), play a role in tumorigenesis, with the ISR being activated by metabolic stressors and leading to the selective induction of mRNA, including activating transcription factor 4 (ATF4). ATF4 acts as a master regulator of cellular adaptive functions and survival and has been associated with cancer type and therapy, although its impact on the TME and immune composition of different tumors remains to be determined. The involvement of ATF4 in pro- and anti-tumor pathways makes its role in cancer progression unclear.

GEMM mice of *Kras*LSL-G12D/+; *Trp53*fl/fl *Rosa26*LSL-Cas9-P2A-GFP/LSL-Cas9-P2A-GFP background aged 6 – 10 weeks were randomly selected to begin tumor initiation studies with the USEC lentivirus. *Kras*/P53 primary LUAD cell lines were obtained from Tyler Jacks laboratory. CRISPR/Cas9 screen with pUSEPR lentiviral plasmid composed of 3240 sgRNAs targeting 470 ATF4 regulated genes and 278 CTR sgRNAs was used in vitro and in vivo in C57Bl6J and NSG mice. Single cell RNA seq/ExCITE seq of approximately 12000 Lung immune cells from each condition (3 mice per condition) were sorted as live+/dead- CD45-circulating- CD45+. For ExCITE-seq tumor cells were sorted as live+/dead- CD45-circulating- CD45- GFP+ and added to immune cells prior to multiplexing. Then samples were multiplexed using cell hashing antibodies. Cells from each sample were pooled and loaded into 10X Chromium. Gene expression together with Hashtag oligo (HTO) libraries were processed using Cell Ranger. Additional material and methods available upon request.

Using unbiased genetic screens, we identified lipocalin-2 (LCN2) as the ATF4-dependent protein that robustly suppresses anti-tumor immune responses. Genetic and pharmacological inhibition of the ATF4/LCN2 axis delays tumor growth and prolongs survival in mice transplanted with primary KP cells. Furthermore, ATF4/LCN2 KO in the GEMM of LUAD results in delayed tumor progression. CD4 and CD8 depletion in vivo experiments lead to recovery in ATF4/LCN2-deficient tumor growth pointing to adaptive immunity involvement as critical component in observed phenotype. Pharmacological (ISRIB) and genetic (CRISPR, shRNA) disruption of the ATF4/LCN2 axis resulted in increased CD4, CD8 TILs and decreased specifically FoxP3+ CD4+ T cells. We developed a neutralizing aLCN2 antibody that showed potent antitumor efficacy in vivo, with delayed tumor growth and prolonged survival. Our results suggest that targeting LCN2 is an effective strategy against LUAD with significant impact on immune TME remodeling.

Cancer immunotherapy has been a promising approach to cancer treatment, but its success has been limited by various factors. We found that disrupting the tumor intrinsic ATF4/LCN2 axis reinvigorates anti-cancer immunity, revealing a novel therapeutic approach for cancer immunotherapy. Our team then developed an antibody against LCN2 with potent in vivo efficacy,

providing mechanistic proof of concept. We are now studying aLCN2 antibody-based combination strategies with ICB to evaluate its clinical potential. This new approach has significant implications for cancer treatment, and we look forward to seeing its impact in the clinic.

P004

ERADICATION OF TUMORS WITH PRE-EXISTING ANTIGENIC HETEROGENEITY BY VACCINE-MEDIATED CO-ENGAGEMENT OF CAR T AND ENDOGENOUS T-CELLS

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Chimeric antigen receptor (CAR) T-cell therapy in solid tumors have been largely disappointing. A key challenge is the loss of the antigen targeted by the CAR due to tumor heterogeneity. Even in B-ALL patients initially responding to CD19 CAR T therapy, loss or downregulation of the CD19 antigen has often resulted in disease relapse. These observations highlight the urgent need to develop alternative strategies to prevent antigen loss-induced tumor escape. Antigen spreading (AS) is the induction and amplification of immune responses to secondary antigens distinct from the original therapeutic target. However, to date there is limited clinical evidence of CAR T therapy itself inducing therapeutically meaningful AS, and mechanisms by which AS could be promoted during CAR T therapy remain poorly understood.

We used a novel amphiphilic vaccine (amph-vax) to specifically stimulate and enhance CAR T therapy directly through the CAR. The amph-vax molecules were generated by linking a CAR-ligand to albumin-binding phospholipid-polymers. These lipid-polymer conjugates preferably accumulate in the lymph nodes (LNs) and insert in the antigen-presenting cell (APC) membranes on arrival in LN allowing APCs to stimulate CAR T cells in tandem with natural costimulation and cytokine support, enhancing CAR T expansion and functionality. We used two sets of CAR T, vaccine and tumor models: 1) anti-EGFRvIII CAR T, amph-pepvIII vaccine and EGFRvIII +/- CT-2A murine glioblastoma cells. 2) anti-FITC/TRP1 bispecific CAR T, amph-FITC vaccine and TRP1+ B16F10 murine melanoma cells.

In both CT-2A and B16F10 tumor models, amph-vax boosted CAR T therapy (termed "CAR T-vax therapy"), but not CAR T therapy alone, elicited pronounced AS in both CD4+ and CD8+ T-cell compartments. Single-cell RNA-seq of 21,835 tumor infiltrating-endogenous T cells confirmed significant increase of cytotoxic CD8 T cells and induction of Th1 CD4 T cells. Transcriptomic analysis demonstrated that vaccine boosting significantly enhanced CAR T cell metabolism, including oxidative phosphorylation (OXPHOS). AS was reduced by ~50% using PGC-1 α -deficient CAR T-vax therapy. IFN γ blockade using an anti-IFN γ antibody or IFN γ -knockout CAR T abolished AS. Blockade of IL12 pathway using anti-IL12 (p75) antibody or IL12rb2 knockout mice phenocopied IFN γ blockade, resulting in negligible AS. We further observed significant reduction of AS in BatF3-deficient mice or mice with deficient IFN γ signaling in CD11c+ DCs. Finally, we showed that IFNGR1- or IL12rb2-deficient CAR T-vax therapy failed to induce AS. Using a heterogeneous tumor model with EGFRvIII+ and EGFRvIII- CT-2A cells mixed at pre-defined ratios, we found that CAR-T vax therapy cured ~50% animals bearing tumors with up to 20% EGFRvIII- cells, and further elevating CAR T-intrinsic IFN γ expression increased the cure rate to ~80%. And the therapeutic response was completely lost in Rag1^{-/-} mice.

We demonstrated vaccine boosting of CAR T therapy as a clinically-translatable strategy to promote control of antigenically heterogeneous tumors via antigen spreading. Mechanistically, we showed that IFN- γ production by CAR T-cells and sensing of IFN- γ by both host cells and the CAR T-cells themselves are critical to enable AS. CAR T-derived IFN- γ were propagated via a positive feedback regulation through crosstalk with DC-derived IL-12. Engineering CAR T cells to forcibly express IFN- γ in response to antigen receptor stimulation enhances AS, thereby enhancing control of antigenically heterogeneous tumors. As few solid tumors express target antigens on >90% of tumor cells, the findings described here provide guidance for engineering CAR T therapies to more effectively treat solid tumors with pre-existing antigenic heterogeneity. As vaccines for CAR T-cells

are already being explored clinically, these findings can inform future clinical trials aiming to enhance CAR T treatment of solid tumors.

P005

IL-18 SECRETION ENABLES CAR T CELLS TO OVERCOME LOW ANTIGEN EXPRESSION IN MULTIPLE MYELOMA

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Multiple myeloma is a disease of transformed plasma cells that is thought to develop in the bone marrow. While therapeutic advancements in the last two decades have extended the survival of myeloma patients significantly, the disease is still considered incurable. Chimeric antigen receptor (CAR) T cells targeting B cell maturation antigen (BCMA) were recently FDA approved for relapsed and refractory multiple myeloma due to their impressive responses in patients. Clinical data indicate that BCMA can be weakly expressed at baseline, downmodulate, or undergo genetic loss following BCMA-directed treatment, resulting in disease relapse. Therefore, additional cellular engineering strategies are required to overcome low antigen expression in multiple myeloma.

To overcome insufficient BCMA expression, we engineered a CAR based on a proliferation inducing ligand (APRIL), which is the natural ligand for two myeloma-associated antigens: (1) BCMA and (2) Transmembrane activator and CAML interactor (TACI). We also engineered these CAR T cells with expression of the pro-inflammatory cytokine IL-18 to boost anti-myeloma activity in vivo against antigen-high and antigen-low disease. This project utilizes MOPC315.BM, a BALB/c mineral oil plasmacytoma that was sequentially passaged in vivo to more closely mimic clinical features of myeloma. Using CRISPR-Cas9 we created lines of MOPC315.BM with low antigen expression to recapitulate what is observed in some patients. This syngeneic and immunocompetent mouse model enables us to interrogate how these CAR T cells interact with other cell types in the myeloma microenvironment.

Our results demonstrate clearance of both antigen-high and antigen-low disease with optimal costimulation and engineered cytokine secretion. In vivo, 4-1BB costimulation is required for APRIL CAR T cell activity against antigen-high myeloma. These efficacy data correlate with greater T cell abundance and persistence as measured with in vivo bioluminescent imaging and peripheral blood analysis. Constitutive expression of IL-18 also allows APRIL-CD28 CAR T cells to gain anti-tumor activity in vivo while further enhancing the efficacy of 4-1BB containing APRIL CAR T cells. In a low antigen expression setting, constitutive IL-18 expression rescues the activity of both BCMA scFv-based and APRIL CAR T cells. As expected, T cells with optimal 4-1BB costimulation, constitutive IL-18 secretion, or both undergo increased NF- κ B signaling when exposed to myeloma in vitro. Flow cytometry and single-cell sequencing reveal that IL-18 secreting CAR T cells increase activation of T cells and antigen presentation by myeloid cells in the bone marrow. Peripheral blood analysis shows elevated interferon-gamma (IFN-g) in the serum of IL-18 CAR T cell treated mice, which correlates with transcriptomic upregulation of interferon response pathways in myeloid cells. Co-culture of IL-18 expressing CAR T cells with macrophages also results in enhanced myeloma killing. These co-culture data, along with the pro-inflammatory myeloid phenotype observed in vivo, indicate that macrophages contribute to the anti-myeloma activity of IL-18 expressing CAR T cells.

APRIL CAR T cells show activity against antigen-high myeloma and co-expression of IL-18 is required for activity against antigen-low myeloma. IL-18 increases T cell activation, antigen-presentation, and IFN-g levels in vivo. This increase in IFN-g may activate bystander antigen presenting cells in vivo and enable them to license myeloma clearance. IL-18 secreting CAR T cells can be an attractive strategy to combat low antigen expression.

P006

A SURGICALLY OPTIMISED INTRAOPERATIVE POLY(I:C) RELEASING HYDROGEL PREVENTS CANCER RECURRENCE

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Surgery remains the main treatment option for most solid cancers, including soft tissue sarcoma. However, recurrences are common and associated with a poor prognosis. Adjuvant systemic immunotherapy with checkpoint inhibitors has provided improvements in survival, but only in selected cancers and in a proportion of patients.

We developed a surgically optimised hyaluronic acid-based hydrogel for sustained local delivery of innate immune agonists and mapped its in-vivo degradation kinetics. We assessed the safety, dose, and scheduling of low dose, local innate immune agonists and demonstrated the efficacy of hydrogel-loaded immunotherapy using mouse models of incomplete tumour resection. The underlying immunological mechanisms were characterised using flow cytometry, RNA sequencing and cytokine blocking studies. Finally, we assessed the safety and feasibility of the hydrogel in a veterinary clinical trial in canine patients undergoing surgical removal of soft tissue sarcoma.

The surgically optimised hydrogel could be easily applied in the wound bed after cancer surgery. We identified TLR3 agonist poly(I:C) as the optimal local immunotherapy, resulting in local and systemic anti-tumour immunity, when degraded over 2-3 weeks by the hydrogel, and improving surgical wound healing. The poly(I:C) hydrogel prevented tumour recurrence in multiple mouse models. Mechanistically, poly(I:C) induced a transient IFN α response that reshaped the tumour microenvironment (TME), attracting inflammatory monocytes and depleting Tregs from the TME. In addition, RNAseq analysis showed that a pre-existing IFN gene signature predicted response to the hydrogel, and we demonstrated that the hydrogel sensitised tumours to anti-PD-1 or anti-CTLA4. Finally, the hydrogel proved safe in canine cancer patients and was easy to use for the surgeon. Using KLH as a systemically tractable biomarker, we found the hydrogel induced a measurable antigen-specific systemic immune response.

The poly(I:C) hydrogel provides a safe and effective approach to prevent recurrence of solid tumours following surgery.

P007

INTEGRATED SINGLE-CELL PROFILING DISSECTS CELL-STATE-SPECIFIC ENHANCER LANDSCAPES OF HUMAN CD8+ TUMOR-INFILTRATING T CELLS AND PRIORITIZES GENES FOR MANIPULATION IN CAR T CELLS

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Despite extensive studies on the chromatin landscape of exhausted T cells, the transcriptional wiring underlying functional and dysfunctional states of human tumor-infiltrating lymphocytes (TILs) is incompletely understood. However, T cell exhaustion limits tumor control by T cells and related cell therapeutics including chimeric antigen receptor (CAR) T cells.

We use an optimized plate-based single-cell chromatin profiling method to map the chromatin accessibility landscape of human CD8+ tumor infiltrating lymphocytes covering four cancer entities. We further include control CD8+ populations isolated from PBMCs of healthy donors or from tumor-adjacent material. This data is integrated with publicly available single-cell RNA-seq data for downstream analyses. We further employ CRISPR interference and activation to target accessible chromatin sites to study their impact on gene-regulation. CRISPR/Cas9 gene editing is used to delete putative dysfunction drivers from CAR T cells to explore possibilities to improve their efficacy.

We identify tissue-specific and general gene-regulatory landscapes in a wide breadth of CD8+ TIL functional and dysfunctional states. Our results reveal a human core chromatin trajectory to TIL dysfunction and identify enhancers, transcriptional regulators, and deregulated target genes involved in this process. By integrating single-cell chromatin accessibility with single-cell RNA-seq data from tumor entity-matching samples we map enhancer-promoter interactions in human TILs and prioritize key state-specific genes by super-enhancer analysis. We started to investigate if CRISPR/Cas9-based knockouts of prioritized putative T cell dysfunction drivers can improve the function of CAR T cells. We further validate enhancer regulation at loci encoding immunotherapy-relevant genes by targeting non-coding regulatory elements with CRISPR activators and repressors.

In summary, our study advances the understanding of molecular regulation of human TIL (dys-)function. Our work provides a framework for modulating relevant genes for therapeutic purposes and to study mechanisms of gene regulation in human T cells.

P008

GENETIC AND PHARMACOLOGICAL TARGETING OF CHROMATIN EFFECTOR PYGO2 PROMOTES CYTOTOXIC T CELL RESPONSES AND OVERCOMES IMMUNOTHERAPY RESISTANCE

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Advanced prostate cancer (PCa) shows overwhelming de novo resistance to immune checkpoint blockade (ICB). We recently identified Pygo2 (Pygo2) as the driver for the amplicon 1q21.3 in PCa. However, it remains unclear whether Pygo2's role in PCa involves immune regulation.

To determine Pygo2 function during autochthonous PCa development, we crossed Pygo2 conditional null allele to the PB-Cre/Pten/Smad4 (DKO) mice and generated PB-Cre/Pten/Smad4/Pygo2 (TKO) PCa mouse model. Various immune-phenotyping techniques (single cell RNA-seq, CyTOF, flow cytometry and immunostaining) were performed on spontaneous and syngeneic DKO and TKO tumors, as well as the syngeneic PCa models. Transcriptomic and epigenetic profiling followed by gain and loss of function studies were conducted to decipher the underlying mechanisms. Pygo2-selective inhibitors were synthesized and used for monotherapy and combination immunotherapy. Both in silico database and clinical samples were used for clinical correlation validations.

We found that Pygo2 deletion decelerated tumor progression, diminished metastases, and extended survival. Pygo2 loss augmented the infiltration of cytotoxic T lymphocytes (CTLs) and sensitized tumor cells to T cell killing. Mechanistically, Pygo2 orchestrated a p53/Sp1/Kit/Ido1 signaling network to foster a microenvironment hostile to CTLs. Genetic or pharmacological inhibition of Pygo2 enhanced the anti-tumor efficacy of immunotherapies using ICB, adoptive cell transfer, or myeloid-derived suppressor cell inhibitors. In human prostate cancer samples, PYGO2 expression was inversely correlated with CD8+ T cell infiltration. Analysis of the ICB clinical database showed association between high PYGO2 level and worse outcome. Ongoing AI-based virtual screening followed by experimental screening have identified more drug-like Pygo2 inhibitors with promise to sensitize cold tumors to immunotherapy.

Our results highlight a promising path to improving immunotherapy with PYGO2-targeted therapy for lethal prostate cancer and other cold solid tumors.

P009

WHY HIGH-TMB BIOMARKER IS ONLY SUCCESSFUL IN CERTAIN CANCERS?

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The FDA recently approved the use of a high tumor mutational burden (TMB-high) biomarker, defined by ≥ 10 mutations/Mb, for treating solid tumors with pembrolizumab, an immune checkpoint inhibitor (ICI) targeting PD1. Despite this advancement, recent studies have reported that TMB-high biomarkers only stratify ICI responders in specific cancer types, and the mechanisms responsible for this observation remain unclear. The tumor immune microenvironment (TME) of a cancer type might modulate the ability of TMB to predict ICI response in that particular cancer type (termed TMB power). This study aims to systematically investigate this hypothesis by analyzing the TME of different cancer types in The Cancer Genome Atlas and identifying key immune factors that determine TMB power across various cancer types.

To explore our hypothesis, we inferred the levels of 31 immune-related factors characteristic of the TME of different cancer types using data from The Cancer Genome Atlas. We integrated this information with TMB and response data from 2,277 patients treated with anti-PD1 at MSKCC. Using this integrated dataset, we aimed to identify key immune factors that influence TMB power across 14 different cancer types.

Our analysis revealed that high levels of M1 macrophages and low resting dendritic cells in the TME characterized cancer types with high TMB power. We developed a model based on these two immune factors, which strongly predicted TMB power in a given cancer type during cross-validation and testing (Pearson Rho = 0.76). Using this model, we predicted the TMB power in nine additional cancer types, including rare cancers, for which TMB and ICI response data are not publicly available.

This study uncovers immune-related factors that may modulate the relationship between high tumor mutational burden and ICI response. Our findings suggest that TMB-high may be highly predictive of ICI response in cancer types with high levels of M1 macrophages & low levels of resting dendritic cells. This analysis also revealed cervical squamous cell carcinoma as a top cancer type candidate for which ICB clinical trials should be prioritized. By understanding the immune factors that influence the stratification power of TMB, we can better predict ICI response in various cancer types, ultimately helping to prioritize cancer types for clinical trials and improve treatment strategies.

P010

FASTING RESHAPES TISSUE-SPECIFIC NICHES TO IMPROVE THE INNATE IMMUNE RESPONSE TO CANCER.

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Fasting has long been associated with the improved elimination of cancer cells. However, the distinctive role of specific immune subsets mediating these anti-tumor properties remain elusive.

Here, we implemented a cyclic fasting diet (CFD) after tumor initiation to assess how cyclic fasting impacts the natural killer (NK) response to cancer.

We observed that adherence to a CFD improves anti-tumor immunity against both solid and metastatic tumors in a NK cell-dependent manner. During a CFD, NK cells undergo tissue redistribution and experience tissue-specific remodeling through cytokine-driven priming and metabolic rewiring that endow them with enhanced anti-tumor effector functions. NK cells from the spleen of CFD mice are exposed to elevated concentrations of fatty acids and glucocorticoids, leading to increased fatty acid metabolism via expression of CPT1A, which is essential for their survival and anti-tumor functions. In parallel, NK cells that are redistributed to the bone marrow during CFD in a S1PR5- and CXCR4-dependent manner are primed by an increased pool of IL-12-expressing cells.

Together, these data uncover a novel dietary strategy to improve tumor clearance and identifies a previously unknown mechanistic link between dietary restriction and optimized innate immune responses.

P011

THE TEMPORAL PROGRESSION OF IMMUNE REMODELING DURING METASTASIS

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Cancer metastasis is the cause of death for 50-90% of patients with solid tumors in part because clinically-approved therapies which target metastasis do not exist. Prior work has shown that tumor-mediated immune remodeling is necessary for metastasis. As a result, targeting metastatic disease by reversing or reorienting immune dysregulation in the metastatic niche of distant organ sites represents an appealing yet unexplored strategy. However, since most preclinical studies of tumor-immune interactions have relied upon cross-sectional analyses of immune cells in primary tumors and unaffected tissues, our understanding of how immune populations are dynamically remodeled in the metastatic niche remains incomplete.

Longitudinal single-cell genomics analyses of immune remodeling in distant organ sites during metastasis has the potential to both refine our understanding of metastatic immune remodeling and identify candidate pathways for anti-metastatic immunotherapy development. To this end, we constructed a longitudinal single-cell RNA-sequencing (scRNA-seq) cell atlas of lung immune cells in the PyMT mouse model of spontaneous metastatic breast cancer. Our atlas systematically chronicles how the fully-intact metastatic niche immune compartment responds during primary tumorigenesis, through pre-metastatic niche formation, to the final stages of metastatic outgrowth in the lung. Analyses of these data expand on known models of pro-metastatic immune remodeling, identify novel metastasis-associated immune signatures, and discover candidate anti-metastatic immunotherapy pathways.

To construct our longitudinal scRNA-seq atlas of lung immune cells during metastasis, we used MULTI-seq to multiplex lungs isolated weekly from PyMT+ female mice between 6-15 weeks of age. MULTI-seq enabled 35 lungs to be processed in a pooled format in order to reduce costs and avoid technical confounders such as batch effects and cell-cell doublets. After next-generation sequencing and scRNA-seq data quality-control, we analyzed a final dataset representing 104,314 immune cells across all sampled time points.

In-depth computational analysis of our longitudinal scRNA-seq atlas yielded three key insights.

First, we observed that lung myeloid cells specifically in the pre-metastatic niche engage in TLR inflammatory signaling through the NFkB pathway. While 'sterile' TLR-NFkB inflammation plays a known role in metastatic progression, our analyses identify cell types that engage in TLR-NFkB inflammation which were not previously known. Moreover, our data suggests that primary tumor-derived cues act at a distance to reprogram lung myeloid cells to participate in this process via induction of CD14 expression.

Second, we observed that cytotoxic NK cell proportions increased in the lung over time. This finding is at odds with the known anti-metastatic effects of cytotoxic NK cells and documented NK cell phenotypes in the PyMT primary tumor microenvironment. Thus, this finding leads to secondary hypotheses regarding how metastatic tumors evade NK cell cytotoxic responses.

Third, cell-cell signaling inference analysis predicted metastasis-associated interactions which may organize the metastatic niche. Specifically, we observed IGF1-IGF1R signaling between interstitial macrophages and neutrophils that is suppressed over time, as well as reciprocal regulation of Ccl6 expression in alveolar macrophages and neutrophils. While perturbing IGF1 and CCL6 signaling

inhibits metastatic progression in other contexts, our data provide new mechanistic details into these observations and position these pathways as anti-metastatic immunotherapy candidates.

Our study identifies changes in immune cell gene expression profiles, population structure, and cell-cell signaling interactions which correlate with metastatic progression. We anticipate that the longitudinal scRNA-seq atlas presented here will serve as a foundational resource for the cancer immunology community.

P012

BILE ACIDS SYNTHESIS PRESENTS A NOVEL METABOLIC CHECKPOINT TO ANTI-TUMOR IMMUNITY IN LIVER TUMORS

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The success and failure of immune-checkpoint blockade depends heavily on generation of effector CD8⁺ T cell responses and their recognition of target tumor cells. Despite recent developments in the identification of novel immune checkpoints, majority of the tumors and/or patients fail to respond to or only partial respond to immune checkpoint blockade. Hepatocellular carcinoma (HCC) is one such tumor despite being infiltrated by CD8⁺ T cells, fail to respond to immune checkpoint blockade as well as adoptive T cell transfer therapies. Often liver cancers are presented with elevated levels of bile acids (BAs) which can directly or indirectly contribute to tumor progression. However, the contribution of these BAs in blunting tumor-specific T cell responses even in the presence of immune checkpoint blockade has been poorly investigated. We have identified that the metabolic state of liver cancer cells enables them to evade immune recognition by regulating bile acids levels within tumor microenvironment.

To test the role of BAs in liver cancer we used mouse models of liver cancer and mouse derived liver organoids. We used AAV8 based CRISPR-CAS9 to knockdown various enzymes involved in BA synthesis pathway. We also used in vitro activated CD8⁺ T cell culture system to delineate the mechanism by which bile acids affected T cells.

We observed that in mouse models of liver tumors and HCC patients displayed increased levels of conjugated BAs. Interestingly, these BAs are specifically enriched within tumor regions where tumor-specific T cells are abundant. Tumor specific knockdown of major steps in BA synthesis pathway led to the observation that the rate-limiting enzyme of BA synthesis pathway - CYP7A1 and BA conjugating enzyme BAAT are essential for regulating BA levels and T cell responses within tumors. Thus, tumor-specific knockdown of CYP7A1 or BAAT resulted in significant reduction in conjugated BAs and altered levels of secondary BAs which is associated with increased tumor-specific T cell responses (both survival and function). More importantly, combining BAAT knockdown with anti-PD-1 resulted in complete elimination of tumors, whereas anti-PD-1 alone did not influence T cell responses or tumor burden. The therapeutic benefit of modulating BA synthesis pathway was dependent on the direct effects of BAs on T cells as tumor specific-T cells within liver accumulated BAs. Additionally, culturing in vitro activated CD8⁺ T cells with various BAs indicated that while primary conjugated BA (mainly TCDCA) dose-dependently affected T cell survival, secondary BA (especially LCA) inhibited T cell effector functions. Intriguingly, TCDCA induced mitochondrial oxidative stress in T cells leading to inhibition mitochondrial respiration, and Reactive Oxygen Species (ROS) induced cell death. Hence, supplementing CD8⁺ T cells with N-Acetyl Cysteine (antioxidant) or over-expression of catalase inhibited TCDCA induced T cell death. Subsequently, adoptive transfer of Catalase over-expressing T cells improved their fitness within liver tumors. Furthermore, immunohistological analysis of liver samples from patients with HCC displayed higher expression of BAAT compared to the patients without HCC. Interestingly, BAAT expression negatively correlated with CD8⁺T cell infiltration indicating that BAAT mediated conjugated bile acids can negatively regulate T cell abundance and may further determine responses to immunotherapy.

Taken together, we have identified bile acid synthesis pathway as a novel metabolic checkpoint mechanism by which tumors derived BAs impede tumor-specific T cell responses and thwart the efficacy of immune checkpoint blockade.

P013

ADVANCING SOLID TUMOR IMMUNOTHERAPY: 3D BIOMATERIAL-DRIVEN IN SITU IMMUNO-ENGINEERING OF T-CELLS

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T-cell immunotherapy has demonstrated remarkable efficacy in the treatment of hematological malignancies. However, its application to solid tumors has been challenging due to their localized nature, protection from circulating T-cells, and inherent heterogeneity. While localized delivery of tumor-specific T-cells using biomaterials has shown promise, the complex and costly procedures involved in generating a sufficient number of tumor-specific T-cells present significant obstacles. To address these limitations, we propose an innovative strategy to engineer T-cells in situ, utilizing a 3D biomaterial-based scaffold. This approach eliminates the need for large-scale in vitro generation and culture of tumor-specific T-cells and facilitates the use of host cells as opposed to allogeneic cell products, thus overcoming a major hurdle.

We developed polyethylene glycol (PEG) based 3D scaffolds as biomaterial platforms for T-cell engineering. Using carbamide chemistry, we conjugated positively charged PLL to the scaffold, enabling efficient loading of lentiviruses carrying tumor-specific T-cell receptors (TCRs). We characterized the scaffold's physical and biological properties to ensure suitability for physiological application. In vivo gene delivery efficiency was evaluated using luciferase-expressing lentiviruses, quantifying gene expression via non-invasive imaging. Implanting the scaffold with OVA-TCR lentiviruses in a B16-OVA tumor model, we assessed tumor reduction, presence of transduced T-cells, and overall anti-tumor responses, including reductions in immunosuppression markers and systemic cytokine responses.

The PEG-based 3D scaffolds functionalized with PLL showed high cell attachment and enhanced retention of lentiviral particles through electrostatic interactions. The scaffolds had an interconnected and macroporous structure which provided large surface area for lentivirus immobilization. They also exhibited excellent biocompatibility with splenic T-cells and hemocompatibility. In vitro studies demonstrated efficient delivery of OVA-TCR gene into primary T-cells via lentiviruses. The engineered T-cells exhibited potent cytotoxicity against B16-OVA tumor cells during co-culture. Implantation of scaffold in C57BL/6 mice promoted immune cell infiltration without causing any toxicity. Further, implantation of luciferase-expressing lentivirus-loaded scaffolds showed higher transduction efficiency compared to bolus lentivirus delivery. When scaffolds carrying OVA-TCR expressing lentiviruses were implanted into C57BL/6 mice with B16-OVA tumors, it resulted in significant tumor reduction compared to the no treatment control group. Immune cell profiling revealed a significantly high percentage of transduced T-cells and a notable reduction in suppressor immune cells within the tumors of mice implanted with scaffold.

To summarize, our strategy to engineer T-cells in vivo using a 3D biomaterial-based scaffold offers a promising solution for the treatment of solid tumors. By circumventing the challenges associated with large-scale in vitro production of tumor-specific T-cells and leveraging the host cells' potential, we can enhance the effectiveness and feasibility of T-cell immunotherapy in the context of solid tumors. This approach holds great potential for improving patient outcomes and expanding the application of T-cell immunotherapy to a wider range of cancer types.

P014

A MULTIPLEXED SPATIAL PROFILING OF THE TUMOR MICROENVIRONMENT OF EXTENSIVE-STAGE SMALL CELL LUNG CANCER TO FIND PREDICTIVE BIOMARKERS OF BENEFIT FROM CARBOPLATIN-ETOPOSIDE PLUS ATEZOLIZUMAB FIRST-LINE TREATMENT

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First-line systemic treatment with carboplatin-etoposide plus atezolizumab (CEA) is a new standard of care for extensive-stage small cell lung cancer (ES-SCLC). No predictive biomarkers for patient selection have been identified so far.

This is a single-center retrospective/prospective translational study on ES-SCLC patients receiving first-line CEA, investigating the predictive value of tissue biomarkers. Gene expression profiling (GEP) was performed analyzing 770 immune/cancer-related genes, while two 9-color multiplex immunofluorescence (mIF) panels were used to assess immune checkpoint expression, tumor-infiltrating immune cell populations and their spatial relationships. Moreover, the expression of ASCL1, NeuroD1 and POU2F3 transcription factors was investigated to define ES-SCLC molecular subtypes. The level of each biomarker was correlated with clinical endpoints.

Forty-two patients were included; median follow-up was 7.7 months. Overall response rate was 66%, median time to treatment failure (TTF), progression free survival (PFS), duration of response (DoR) and overall survival (OS) were 5.3 (95%CI 4.8-5.7), 5.3 (95%CI 3.9-6.6), 3.6 (95%CI 1.5-5.8) and 7.8 months (95%CI 2.9-12.6), respectively. Responding patients and those with a longer duration of response had a higher cell proliferation, DNA damage repair and epigenetic regulation scores. In the vast majority of patients, more than 80% of tumor cells were negative for the HLA-class I expression, which in turn reflects an immune-cold tumor microenvironment. Non-responding patients had a higher density of intratumoral CD163+ M2-polarized tumor-associated macrophages (TAMs) as compared to responders. Moreover, a higher ratio between TAMs and CD8+ T cells was associated with a shorter DoR, TTF and OS, as well as a higher percentage of CD8+ cells in close proximity to CD163+ macrophages. Patients with higher density of CD4+ T cells, CD8+ cytotoxic T lymphocytes (CTL) and B lymphocytes had a better outcome, as well as those with a higher percentage of CTL close to HLA-I+ tumor cells, CD4+ T cells and B lymphocytes. Furthermore, higher expression of exhaustion CD8 markers, and higher density of PD-1+ or PD-L1+ cells were associated with a better prognosis, as well as a higher percentage of PD-1+ CTL in close proximity to PD-L1+ tumor cells or macrophages. According to the expression of ASCL1, NeuroD1 and POU2F3 transcription factors, each ES-SCLC patient was assigned to the SCLC-A (ASCL1+, 35.7%), SCLC-N (NeuroD1+, 19%), SCLC-P (POU2F3+, 12%) and SCLC-I (triple-negative, 33.3%) subtypes. NeuroD1+ tumors had different molecular and phenotypical characteristics as compared to the other subtypes, associated to a more aggressive phenotype and worst patient outcome.

We identified predictive and prognostic immune signatures, immune cell populations and cell-to-cell interactions in ES-SCLC patients receiving chemo-immunotherapy through a multiplexed spatial profiling. These results highlight the importance of the tumor microenvironment and spatial interactions in tumor response and survival.

P015

FUNCTIONAL ROLE OF TUMOR-SPECIFIC B-CELL RESPONSES IN PRECLINICAL MODELS OF IMMUNE CHECKPOINT THERAPY

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Immune checkpoint inhibition (CKI) demonstrated breakthrough therapeutic efficacy in a variety of cancer types. Enhancement or de-novo induction of tumor specific T-cell responses is assumed as major mode of action, but other lymphatic subpopulations also contribute to the efficacy of CKI. We have shown that B cells mediate immune response in gastrointestinal cancer and recent data suggest an important role of B cells for susceptibility of cancer patients to CKI.

To advance our understanding of B cell specific mechanisms in the setting of CKI, we used three different mouse models. In the subcutaneous PancO2-OVA tumor model, mice received anti-PD-1 treatment at onset of tumor appearance. For optimal mimicry of the major types of gastric adenocarcinomas, we used two recently developed orthotopic transgenic mouse models, which express a tamoxifen-inducible Cre-recombinase in the stomach-specific *Anxa10* locus. The genomically stable (GS) cancer model carries the alterations *Cdh1fl/fl*, *KrasG12D/+* and *Smad4fl/fl* resulting in a slow progressing tumor growth as observed by magnetic resonance imaging (MRI). The chromosomally instable (CIN) model contains mutations of *KrasG12D/+*, *Tp53R172H/+* and *Smad4fl/f* and show a more rapid, intestinal tumor growth. Both mouse strains were thoroughly characterized with regard to their immune phenotype and received single or combination checkpoint therapy with anti-PD-1 and anti-4-1BB at early and midterm stages of cancer development.

CKI in the PancO2-OVA model resulted in a significant increase of OVA-specific B cells and secretion of OVA-specific antibodies. This was accompanied by an increase of T follicular helper cells and activated CD86+ B cells. In the orthotopic gastrointestinal cancer models, dysplasia of stomach tissue occurred two weeks after tamoxifen induction, which was apparent by a thickening of the stomach wall in MRI. Metastases of liver, lymph nodes and lungs were observed at later stages. We detected B-cell clusters in the tumor microenvironment indicating the presence of tertiary-lymphoid structures, which is usually not the case in mouse models of cancer. In the GS model, anti-PD1 treatment did not result in a significant reduction of tumor growth. However, we observed increased percentages of memory B cells, germinal center B cells and activated CD86+ B cells.

Our results underline the role of B cells in CKI.

P016

NOVEL GRP78 CAR-NK IMMUNOTHERAPY FOR DRUG-RESISTANT LIVER CANCER TREATMENT

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Currently, most cancers are treatable through early detection and precision medicine strategies, leading to long-term remission. However, drug resistance makes liver and pancreatic cancers difficult to treat. Drug resistance emerges when tumors have genetic changes leading to tumor evolution. The mutations in the cancer genome cause drug-resistant, making these cancers difficult to treat, which explains why chemotherapies and immune checkpoint blockade are ineffective for the long-term treatment of most liver cancer. Natural Killer (NK) cells are the first-line soldiers that survey the body killing tumor cells, dysfunctional cells, or virally infected cells. NK Cells alone cannot kill tumors because tumors can silence immune cells, also known as immune evasion. Therefore, we can engineer NK cells using tools like chimeric antigen receptors (CARs) that detect tumors more precisely while activating NK cells. CAR-NK therapies successfully treat blood cancers like lymphomas with minimal side effects to patients. However, major challenges remain in treating solid tumors (e.g., liver and pancreatic cancers), leading to poor outcomes.

Our lab has recently developed novel Chimeric Antigen Receptor- Natural Killer (CAR-NK) therapies to target drug-resistant liver cancers by targeting Glucose Receptor Protein 78 (GRP78). GRP78 is a heat-shock protein that resides in the endoplasmic reticulum normally. However, the GRP78 protein will migrate to the cell's surface, whereby the cell undergoes stress. The high-throughput assays for evaluating GRP78-CAR-NK killing of drug-resistant liver cancers in vitro and in vivo have been conducted in this study.

In our study we show surface expression of GRP78 across various solid tumors. We show improved survival in mice treated with the GRP78 CAR-NK compared to control groups.

This study proposes a unique approach to use specific CAR-NK immunotherapy to target the survival of solid tumors. GRP78 assists in protein homeostasis by being involved in stabilizing oncogenic misfolded proteins. GRP78 supports tumor cell plasticity, immune evasion and chemoresistance. GRP78-CAR therapy has not been tested in a CAR-NK model for treating solid tumors. We hypothesize that we can target GRP78 with CAR-NK therapy to control the growth of solid tumors. The study's long-term goal is to understand the efficacy of targeting surface heat shock proteins to treat drug-resistant solid tumors.

P017

UTILISING MOSQUITO SALIVA PEPTIDE TO AUGMENT ONCOLYTIC THERAPY IN SOLID TUMOURS

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The global health burden of solid tumours, such as Hepatocellular carcinoma (HCC) and melanoma, is increasing annually. Due to the resistance of many tumours to conventional therapy, more efficient anti-tumour therapy is needed. Oncolytic therapy is a promising cancer treatment in which oncolytic viruses (OVs) are used to selectively infect and kill cancer cells, as well as indirectly promote anti-tumour immune responses and thereby enhance survival. However, there is an urgent need to increase their efficacy. Here, we suggest a radically different approach to enhance anti-tumour immunes elicited by OVs. Our group has identified a unique peptide, derived from mosquito saliva called sialokinin (SK), that induces rapid vascular permeability and influx of leukocytes. It is well established that this response acts to inadvertently enhance mosquito-borne virus infection, many of which have also been used as experimental OVs. Here, we hypothesise that intra-tumoural administration of SK will enhance; localisation of oncolytic virus to tumours; infection of tumour cells with virus; and the recruitment of leukocytes that aid both virus replication and activation of anti-tumour immunity.

Material and Methods: BNL 1ME A.7R.1 HCC or B16-ova melanoma tumour cells were subcutaneously injected into BALB/c mice or C57BL/6 mice respectively. Once tumours were established, mice were injected with treatments of oncolytic virus alone (i.t. or i.v.), saliva/SK peptide (i.t.) alone or combined with virus. AT 24 hours post treatment, tissues (tumour, spleen) and blood were collected to analyse virus titre, quantify leukocyte influx and immune gene expression (by flow cytometry, qPCR and plaque assay).

We report *in vivo* experiments that investigate whether sialokinin or abiotic mosquito saliva modulates extent of two prototypic OVs and activation of innate immunity, in HCC and melanoma. Sialokinin was able to induce vascular leakage and oedema in skin and lower doses of SK recruited leukocytes in a dose dependent manner. Interestingly, we found that although virus infection was not enhanced in tumours, induction of innate immune responses including CD8 T cell recruiting chemokines were enhanced by mosquito saliva.

Together, we show that there is potential in utilising immune modulating peptides from mosquitoes to enhance host immune response to OV therapy.

P018

ADAPTIVE IMMUNITY AGAINST RETROELEMENTS CONTROLS TISSUE PHYSIOLOGY

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A symbiotic dialogue between the microbiota and host is essential to promote protective immune responses in the skin. Integrated retroviral elements, or retrotransposons (RTNs), comprise up to 45% of the human genome. We previously found that immune responses to the skin microbiota are dependent on the reactivation and keratinocyte-intrinsic sensing of RTNs. Here, we explored the possibility that RTNs may also represent a source of antigens recognized by the skin adaptive immune system.

We first established a model of sterile injury, where topical application of a mild detergent reactivated RTNs, and recruited CD8 T cells to the skin in an RTN-dependent manner.

RNAseq analysis of RTN expression revealed Langerhans cells (LCs) have the most abundant RTN expression amongst skin dendritic cells. Our work demonstrated that CD8 T cell responses to mild detergent were entirely dependent on LCs, supporting the idea that LCs could present RTN antigens to CD8 T cells. To identify RTN-specific T cells, we selected peptides that were uniquely expressed in LCs, but not in thymic epithelial cells which mediate negative selection. Several peptides promoted cytokine production from injury-elicited CD8 T cells, and were then used to generate tetramers. Following contact with mild detergent, tetramer-positive cells were identified, confirming the presence of RTN-reactive CD8 T cells in the skin. RNA-seq analysis suggests RTN-specific CD8 T cells may contribute to wound repair and immunoregulation. Epithelial regrowth in response to punch biopsy wounding was significantly enhanced in mice which were pre-applied with mild detergent. In fact, 30% of mild detergent-applied mice had fully healed wounds.

Thus, mild stress, such as that induced by mild detergent, is sufficient to promote adaptive immunity to defined antigens derived from RTNs. Our work also proposes that homeostatic immunity to ancient retroviruses promotes a defined class of T cell responses aimed at maintaining barrier integrity in response to daily stressors encountered by the skin.

P019

HMGB2 REGULATES THE DIFFERENTIATION AND STEMNESS OF EXHAUSTED CD8+ T CELLS DURING CHRONIC VIRAL INFECTION AND CANCER

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Chronic infections and cancers evade the host immune system through mechanisms that induce T cell exhaustion. The heterogeneity within the exhausted CD8+ T cell pool has revealed the importance of stem-like progenitor (Tpex) and terminal (Tex) exhausted T cells, although the mechanisms underlying their development are not fully known.

Using TCR transgenic CD8+ T cells, acute and chronic lymphocytic choriomeningitis virus (LCMV) and melanoma tumor models, we found a key function for high mobility group box 2 (HMGB2) proteins in responding T cells.

Here we report HMGB2 expression is upregulated and sustained in exhausted CD8+ T cells, and HMGB2 expression is critical for their differentiation. Through epigenetic and transcriptional programming, we identify HMGB2 as a cell-intrinsic regulator of the differentiation and maintenance of Tpex cells during chronic viral infection and in tumors. Despite Hmgb2^{-/-} CD8+ T cells expressing TCF-1 and TOX, these master regulators were unable to sustain Tpex differentiation and long-term survival during persistent antigen. Furthermore, HMGB2 also had a cell-intrinsic function in the differentiation and function of memory CD8+ T cells after acute viral infection. We found decreased Hmgb2^{-/-} Tpex cells in melanoma tumors, and these T cells had decreased protective capacity than WT cells in the survival of melanoma tumor-bearing mice.

Our findings show that HMGB2 is a key regulator of CD8+ T cells and may be an important molecular target for future T cell-based immunotherapies.

P020

SNS-101, A CLINICAL-STAGE PH-DEPENDENT AND TME-SELECTIVE ANTI-VISTA ANTIBODY WITH FAVORABLE PHARMACOKINETIC AND SAFETY CHARACTERISTICS, PROMOTES ANTI-TUMORAL M1 MACROPHAGE POLARIZATION IN PD-1 REFRACTORY SETTINGS

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VISTA (V-domain Ig suppressor of T-cell activation) is an immune checkpoint expressed on abundant myeloid-lineage cells, including monocytes and neutrophils, suppressing T-cell activation when engaged with its target receptor PSGL-1. Importantly, VISTA is only active at low pH (~pH 6) such as in the tumor microenvironment (TME) due to protonation of surface exposed histidine residues. VISTA inhibition demonstrated excellent therapeutic combinability with CTLA-4 or PD-1/PD-L1 T-cell checkpoint inhibitors in preclinical studies. However, clinical development of anti-VISTA antibodies has been challenging due to (1) high clearance via target-mediated drug disposition (TMDD) by VISTA-positive neutrophils and monocytes at physiologic pH and (2) immune activation and cytokine release syndrome (CRS) at sub-therapeutic doses upon engagement of VISTA in the blood. Moreover, the molecular mechanism by which VISTA mediates immune suppression in the TME remains elusive. To prevent TMDD and mitigate potential CRS, we developed SNS-101, a human monoclonal IgG1 antibody specific for the protonated, active form of VISTA. We assessed the pharmacokinetic (PK) and toxicity profile of SNS-101 in cynomolgus monkeys. Additionally, we analyzed serum from MC38 tumor-bearing animals treated with either SNS-101 alone or in combination with anti-PD-1 to test the hypothesis that VISTA inhibition repolarizes myeloid cells to a proinflammatory/immune activating state.

A multi-dose GLP toxicology study in cynomolgus monkeys was conducted, dosing SNS-101 at 3, 10, and 100 mg/kg by intravenous infusion every 2 weeks (total of 3 doses), followed by a 6-week treatment-free recovery period. Blood samples for cytokine analysis were collected on days 1 and 29, and at 2 and 24 hours after the end of each infusion. Blood samples for leukocyte count determination and immunophenotyping were collected from animals in all groups at days 14, 31 and 71. Anti-tumor efficacy was assessed in VISTA-KI mice implanted with syngeneic MC38 tumors, and serum of SNS-101-treated animals was analyzed using a 49-plex cytokine/chemokine panel.

The PK profile of SNS-101 shows linear elimination kinetics in target-bearing cynomolgus monkeys, demonstrating absence of any target-mediated clearance. There were no SNS-101-related peripheral blood immunophenotyping changes or changes in plasma IFN-gamma, TNF-alpha, or IL-6 concentrations at any time point or dose level compared with the control group. Moreover, recursive feature selection by machine learning identified significant changes in key cytokines/chemokines related to tumor growth inhibition. Levels of CXCL-12, CCL-24 and IL-3 inversely correlated, and CCL-2, -7, -12 and CXCL-10 directly correlated with tumor volume reduction, respectively.

Our data demonstrate that SNS-101's exquisite selectivity for active, protonated VISTA significantly reduced CRS risk. Furthermore, no SNS-101-related adverse effects were observed up to doses of 100 mg/kg/dose. Previously observed rapid clearance with non-pH-sensitive VISTA antibodies was eliminated. As VISTA engagement may enforce a myeloid immunosuppressive program, our cytokine/chemokine data supports a model in which SNS-101 targets suppressive signaling in the myeloid compartment, and in combination with anti-PD-1, helps shift the balance of macrophage polarization toward an anti-tumor M1 phenotype. SNS-101 has recently received IND-clearance and

entered clinical trials either as monotherapy or in combination therapy with a PD-1 inhibitor in patients with advanced solid tumors (NCT05864144).

P021

LIGAND-INDEPENDENT TREM-1 BLOCKERS AS INNATE IMMUNE CHECKPOINT INHIBITORS TO OVERCOME CANCER IMMUNOTHERAPY RESISTANCE

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Since the first immune checkpoint inhibitor (ICI) was introduced in 2011, cancer immunotherapy has revolutionized the treatment of many types of advanced solid tumors. However, most patients still do not derive benefit. Notable examples are patients with pancreatic ductal adenocarcinoma (PDAC). These patients show no or poor response to standard anti-PD-1/PD-L1 and anti-CTLA-4 immunotherapies. A growing line of evidence suggests that overexpression of triggering receptor expressed on myeloid cells 1 (TREM-1) in tumors correlates closely with infiltration of immune-suppressive cells, immune regulation, and poor clinical outcome, highlighting TREM-1 as a novel innate immunity target for cancer immunotherapy. To block TREM-1, we developed a TREM-1 inhibitory peptide sequence GF9 that employs a novel, ligand-independent mechanism of action, addressing the problem of the unknown TREM-1 ligand(s). Here, we demonstrated that well-tolerable GF9 sequence-based TREM-1 inhibitors reduce inflammation and tumor-associated macrophage (TAM) content, inhibit tumor growth, and overcome resistance to anti-PD-L1 immunotherapy in mouse models of PDAC.

Free GF9 peptide and macrophage-targeted lipopeptide complexes of GF9 sequence-based TREM-1 inhibitory peptides were synthesized and characterized in biophysical, biochemical, and cell-based assays. Mouse studies were performed using subcutaneous human AsPC-1, BxPC-3, CAPAN-1, MIA PaCa-2, and PANC-1 xenograft athymic nude mouse models as well as a fully immunocompetent mouse model of PDAC generated by inoculation of the primary bioluminescent (a firefly luciferase, Luc, expressing) Kras (G12D)/Trp53 null/Pdx1-cre (KPC) mouse tumor chunk into the subcapsular region of the pancreas of wild-type C57BL6 mice. In the xenograft models, TREM-1 inhibitors were intraperitoneally (i.p.) injected at various doses once a day for 5 days per week for 30 days on average, and tumors were measured twice weekly until sacrifice. TAM content in xenografts was measured by F4/80 immunostaining and serum cytokines were analyzed by commercially available ELISA kits. In the syngeneic orthotopic model, TREM-1 inhibitors were i.p. administered at various doses once a day for 21 days either alone or in combination with an anti-mouse PD-L1 antibody i.p. administered at a 10 mg/kg dose twice a week for 3 consecutive weeks, and tumor progression was monitored using IVIS® bioluminescent imaging on days 4, 8, 11, 15, 18, and 22.

Ligand-independent TREM-1 blockers alone inhibited tumor growth and extended survival in xenograft and syngeneic models of PDAC. In the xenograft models used in these studies, TREM-1 blockade substantially suppressed the release of serum proinflammatory cytokines (interleukin-1alpha, IL-1a, and IL-6) and macrophage colony-stimulating factor (M-CSF or CSF-1), and reduced the intratumoral macrophage content. Furthermore, the higher TAM content in xenografts, the higher was the efficacy of the TREM-1 inhibitory formulations tested. In the syngeneic orthotopic mouse model of PDAC, ligand-independent blockade of the TREM-1 signaling pathway significantly inhibited tumor progression and substantially improved the therapeutic efficacy of PD-L1 blockade compared with TREM-1 inhibitor and anti-PD-L1 treatments alone.

These data further confirm that TREM-1 plays an important role in the pancreatic cancer pathogenesis and suggest that ligand-independent TREM-1 inhibition represents a novel strategy to overcome immunotherapy resistance in patients with PDAC. In line with several recent independent studies that demonstrated that TREM-1 inhibitory peptide GF9 sensitizes hepatocellular carcinoma to immunotherapy in different syngeneic mouse models, this suggests that ligand-independent

TREM-1 blockers can be developed into a new class of innate ICIs aimed to overcome resistance of poorly sensitive tumors to current cancer immunotherapies.

P022

LPP FORMULATED PERSONALIZED CANCER RNA VACCINES ENCODING NEOANTIGENS ELICITED STRONG IMMUNE RESPONSES IN CANCER PATIENTS

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Neoantigen cancer vaccines motivating cancer specific-T cell responses have achieved promising anti-tumor efficacy in recent preclinical models and clinical trials. Optimization of delivery platforms and vaccine formulations could promote tumor regression. Here, we showed that a novel therapeutic personalized cancer vaccine (PCV) composed of a lipopolyplex shell and tandem neoantigens mRNA sequence core was highly potent in conferring neoantigen immunogenicity. CT26, MC38 and B16F10 syngeneic tumors were effectively controlled with the efficiently prime and expansion of antigen-specific cytotoxic effector CD8+ and CD4+ T cells. We further observed that PCV completely inhibits tumor growth in a prophylactic immunization setup. Memory T cells persisted in vivo for long and protected from tumor rechallenge. Additionally, PCV immunization combined with immune checkpoint inhibition provided a superior tumor suppression and reversed the tumor immunosuppressive microenvironment. More importantly, we evaluated the safety and efficacy of LPP PCV in investigator-initiated trials. LPP formulated PCV elicited strong immune responses in patient with late-stage solid tumor. The specificity of T cell immune responses were validated by tetramer staining and TCR sequencing. In conclusion, our data offers a novel lipopolyplex-based personalized mRNA cancer vaccine for personalized immunotherapy.

Methods include flow cytometry, ELISpot, tetramer staining, TCR sequencing etc.

To be presented.

To be provided.

P023

CHARACTERIZATION OF AN 125I-ANTI-MARCO ANTIBODY AS A POTENTIAL TARGETING AGENT FOR CANCER THERAPY

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Tumor microenvironment has a critical role in tumor cell survival and progression. MARCO is a scavenger receptor expressing on tumour-associated macrophages (TAMs). TAMs can have pro-tumor and tumor-suppressive effects. It has been demonstrated that targeting the MARCO receptor could reduce tumor growth. Accordingly, targeting TAMs with anti-MARCO antibodies is a potential therapy and revive immunotherapy. In this study, a novel MARCO-targeting radio-immunoconjugate was created in order to facilitate kinetic and biodistribution assessments.

Iodination of anti-MARCO and negative-control (NC) antibodies was optimized. The receptor-antibody specific binding was assessed in MARCO-transfected L929 cells by specificity assays and LigandTracer real-time measurements. Purified 125I-antibodies were intravenously administered to non-tumor-bearing and tumor-bearing mice (B16 and EO771). SPECT/CT imaging visualized 125I-antibody uptake 2, 6 and 24 h post-injection. Organs were collected at 6 and 24 h post-injection and activity was measured in a gamma counter.

Antibodies were reproducibly radiolabeled and purified. Receptor-specific binding was demonstrated for the 125I-anti-MARCO antibodies on MARCO-transfected L929 cells. Biodistribution in non-tumor bearing and B16 tumor models indicated a high accumulation of 125I-anti-MARCO antibody in the spleen, in line with SPECT/CT imaging at assessed time points. In the EO771 tumor model the biodistribution differed, with no marked differences in spleen and lymph nodes uptake between 125I-anti-MARCO and 125I-NC antibodies. This signifies that the EO771 tumor may stimulate MARCO+ macrophages to relocate. Tumor-to-blood ratios didn't indicate any significantly higher tumor uptake. However, localizing MARCO+ macrophages in tumor site isn't necessarily required to initiate anti-tumor immune response.

In conclusion, anti-MARCO antibody was successfully radioiodinated with no adverse effects from labeling. Modified biodistribution of MARCO+ macrophages in the EO771 model may induce more anti-tumor effects. Further studies should be performed to assess whether other immune cells are involved in anti-tumor responses after anti-MARCO antibody treatment, clarify their roles and evaluate the potential therapeutic approaches.

P024

OBESITY REPROGRAMS BOTH COLORECTAL CANCER CELL AND THE TUMOR MICROENVIRONMENT TO PROMOTE METASTASIS

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Liver metastasis is the most common site of metastases for colorectal cancer (CRC) and is the critical cause of CRC-related death. Epidemiological studies increasingly point to the role of modifiable factors such as diet and obesity in tumor initiation and cancer risk, but dietary impact on tumor metastasis, distinct from tumor initiation or cancer risk, remains unexplored. The precise mechanisms by which pro-obesity high fat diets (HFD) can impact cancer metastasis remains an essential question to optimize dietary interventions for cancer patients. A key challenge in the field had been the lack of physiologically relevant in-vivo models of CRC metastasis, which are required to study systemic changes such as diet.

To this purpose we have a) generated a series of fluorescently (tdTomato+) labelled murine CRC organoids (CRCO) comprising many genotypic combinations observed in human CRC including APC, P53, KRAS and PTEN; and b) developed a high-fidelity model where CRCOs are transplanted via colonoscopy into recipient host colons, forming tumors that later metastasize to the liver. Our overarching objective is to leverage these physiologically relevant CRC models to determine how CRC with different genetic mutations metastasize to the liver microenvironment in pro-obesity HFD. C57BL/6J mice fed a Control (CD) or HFD with 60 kcal% fat to induce obesity, were transplanted with tdTomato+ CRCOs carrying APC-/-, KRASG12D, P53-/- and PTEN-/- . Transplanted mice developed primary tumors in the colon, and assessment for fluorescent tumor cells in the liver showed distant metastasis.

HFD significantly reduced survival of mice with tumors, primarily due to colon tumor burden and obstruction of the colon. HFD also significantly increased frequency (~2-fold) of liver metastasis. Sensitivity of PTEN-/- CRC to HFD indicates mechanisms independent of insulin/PI3K metabolic reprogramming to be responsible for the observed increase in metastasis. Interestingly, immunocompromised mice with Rag2 deficiency, lacking mature lymphocytes, transplanted with murine CRCOs also showed significantly decreased survival and significantly increased liver metastasis in HFD, ruling out lymphocyte activity as a mechanism for HFD induced metastasis. We further generated organoids derived from colon and liver metastatic tumors from CD and HFD mice and serially transplanted them into CD mice to assess the long-term impact of diet on metastatic potential. HFD-derived organoids showed increased liver metastasis (~4-fold) as compared to CD-derived CRCOs when transplanted into CD mice.

To characterize dynamic changes in tumors and their microenvironment, we performed single cell RNA-sequencing on both colon and liver metastatic tumors. We found that HFD induced tumor-specific stemness for tumor cells and increased phenotypes of several pro-tumor populations including cancer associated fibroblasts and macrophage populations. Differentially expressed candidate metastasis modulators identified in our screening will be further interrogated in functional and genetic assays. We anticipate that exploration of these drivers of CRC metastasis and their modulation in obesity will provide recommendations for dietary interventions and mechanistic insights for metastasis-specific therapies.

P025

BACTERIAL INTERACTIONS WITH HUMAN HOST CELLS IN EPITHELIAL CANCERS

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The tumor microenvironment (TME) is composed of a heterogeneous cell population including stromal and immune cells that provides a favorable niche for cancer cells to survive. Pan-cancer molecular studies have revealed the presence of bacterial communities within tumor tissue from the 33 most common cancer types. Further, animal and cellular models have supported the role of specific members of the intratumoral microbiota in the initiation and progression of these cancers. Imaging techniques have revealed that intratumoral bacteria can be located intracellularly, within host immune and epithelial cell types; yet functional impact of intracellular bacteria on infected cell types within the TME was unexplored. In this work by adapting spatially resolved techniques we have characterized the microniches that contain bacteria in altering the tumor immunity and development of epithelial cancers.

This work aims to develop new technologies to assess the spatial distribution of the bacterial communities across the tumor tissue and the host cells that they interact with. By implementing spatially resolved techniques such as the 10x visium spatial transcriptomics we have mapped in an untargeted and unbiased manner the bacterial communities that reside in the tumor tissue. By using the GeoMx Digital Spatial Profiling we have characterized the microniches that contained bacteria by measuring the expression of 77 target proteins that are involved in modulating the tumor immunity and cancer progression. By adapting 10x genomics 5' scRNAseq for simultaneous analysis of host-bacterial transcripts (INVADEseq), we revealed the identity of intracellular bacteria and the host cells they infect, along with the transcriptional impact on bacteria on infected cells. By implementing functional assays based on CRC-spheroids embedded in collagen matrices we have described the biological behaviors that are altered in neutrophils and cancer epithelial cells when they are exposed with a cancer strain of *Fusobacterium nucleatum* isolated from a CRC sample.

Our spatially resolved techniques have demonstrated that the bacteria communities populate microniches that are largely immunosuppressive, less vascularized and coincided with cancer epithelial cells that express lower levels of wild type p53. Our single-cell RNAseq data has revealed that intratumoral bacteria interacted mostly with myeloid cells that exhibited a gene expression profile that were compatible with pro-inflammatory macrophages and cancer cells that displayed more aggressive gene signatures involved in metastasis and chemoresistance. The functional assays have demonstrated that neutrophils were recruited at the infection site of CRC spheroids where they could form cell clusters. Additionally, infected cancer cells started to invade the surrounding environment as single cells following cell detachment from the body mass of the spheroid, in contrast uninfected cancer invaded the adjacent area as a collective maintaining the epithelial-to-epithelial cell junctions.

Our data has demonstrated that the intratumoral microbiota is not distributed uniformly along the tumor tissue, instead they populated specific microniches that are characterized to be immunosuppression and contain transformed cells with metastatic and chemoresistance potential. Our single-cell RNAseq data has revealed that the intratumoral bacteria can trigger a myeloid response possibly hampering the T cell activity against tumors. Moreover, intratumoral bacteria can be also found in transformed epithelial cells with chromosome instability that exhibit a gene expression profile that is involved in metastasis and cell dormancy. Functional studies have revealed that intratumoral bacteria can modify the biological behavior of neutrophils and cancer cells promoting cell heterogeneity in the tumor microenvironment of human cancers.

P026

TUMOR PROMOTER CYSTEINYL LEUKOTRIENE RECEPTOR 1 REGULATES PD-L1 EXPRESSION IN COLON CANCER

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Immunotherapy targeting programmed death-ligand 1 (PD-L1) or PD-1 in solid tumors has been shown to be clinically beneficial. However, in colorectal cancer (CRC), only a subset of patients benefit from PD-1/PD-L1 treatment. Previously, we showed that high Cysteinyl leukotriene receptor 1 (CysLT1R) levels are associated with poor prognosis in CRC patients. Recently, we have revealed the role of the tumor promoter CysLT1R in drug resistance and stemness in colon cancer (CC) cells. Here we intend to understand the role of colon cancer promoter Cysteinyl leukotriene receptor 1 in PD-L1 regulation in order to improve the sensitivity of colon cancer patients towards immunotherapy.

We have used colon cancer cell lines, SCID mice models and colorectal cancer patient cohort along with public datasets of colorectal cancer patients to test and validate the hypothesis.

Here, we show the role of the CysLT1R/Wnt/ β -catenin signaling axis in the regulation of PD-L1 using both in vitro and in vivo preclinical model systems. Interestingly, we found that both endogenous and IFN γ -induced PD-L1 expression in CC cells is mediated through the upregulation of CysLT1R, which enhances Wnt/ β -catenin signaling. Therapeutic targeting of CysLT1R with its antagonist montelukast (Mo), as well as CRISPR/Cas9-mediated or doxycycline-inducible functional absence of CysLT1R, negatively regulated PD-L1 expression in CC cells. Interestingly, an anti-PD-L1 neutralizing antibody exhibited stronger effects together with the CysLT1R antagonist in cells (Apcmut or CTNNB1mut) with either endogenous or IFN γ -induced PD-L1 expression. Additionally, mice treated with Mo showed depletion of PD-L1 mRNA and protein. Moreover, in CC cells with combined treatment of a Wnt inhibitor and an anti-PD-L1 antibody was effective only in β -catenin-dependent (APCmut) context.

These results elucidate a previously underappreciated CysLT1R/Wnt/ β -catenin signaling pathway in the context of PD-L1 inhibition in CC, which might be considered for improving the efficacy of anti-PD-L1 therapy in CC patients.

P027

MICROBIAL-DERIVED BILE ACIDS INFLUENCE LIVER IMMUNITY DURING VIRAL INFECTION AND CANCER

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Host-microbiome interactions are crucial for maintaining the overall health and well-being of the host. One significant factor derived from microbes, secondary bile acids, are implicated in various processes ranging from aging and mood behavior to diseases like autoimmunity and cancer. Besides intestines, liver is first organ that is influenced by the microbiome and their derived factors due to the anatomical and physiological connection to each other. Consequently, the liver is exposed to bile acids synthesized both by itself and by the gut microbiome. Despite ongoing efforts to understand the role of microbial-derived secondary bile acids in cancer, their specific effects on the immune system, particularly CD8⁺ T cell responses, which are crucial for combating intracellular pathogens and cancer, remain poorly understood.

To investigate this, we utilized a mouse model of liver cancer induced by SV40 and acute infection LCMV-Arm.

Our findings confirmed that the tumor microenvironment is enriched in secondary bile acids, specifically LCA and its derivatives, while levels of UDCA were reduced. Furthermore, culturing in vitro activated CD8⁺ T cells with various secondary bile acids, revealed that LCA and its derivatives inhibited CD8⁺ T cell responses in a dose-dependent manner, without affecting cell survival. Conversely, UDCA promoted T cell function. Notably, we discovered that UDCA could rescue the suppressive effects of LCA on T cell function. To further examine the impact of the LCA/UDCA ratio, we fed mice with LCA, resulting in an increased LCA/UDCA ratio, which led to reduced anti-viral and anti-tumor CD8⁺ T cell responses. This, in turn, caused an increase in circulating viral load during LCMV-Arm infection and an elevated tumor burden in SV40-induced liver cancer. Conversely, feeding liver tumor-bearing mice with UDCA, which reduced the LCA/UDCA ratio, significantly decreased liver tumor burden and enhanced CD8⁺ T cell responses. These observations suggest that the LCA/UDCA ratio within the host significantly affects T cell responses in the context of infection and cancer. Mechanistically, LCA and its derivatives induced endoplasmic reticulum (ER) stress, leading to the expression of Nr4a1 (NUR77), an orphan nuclear receptor highly expressed in tumor-infiltrating and tissue-resident memory T cells. Notably, knockdown of NUR77 effectively rescued T cell suppression in vitro and in vivo in the presence of LCA.

In summary, our data indicate that microbial-derived secondary bile acids regulate CD8⁺ T cell responses to infection and cancer by modulating ER stress-driven Nur77 expression.

P028

TIME COURSE TRANSCRIPTOMIC ANALYSIS DURING IMMUNE CHECKPOINT THERAPY REVEALS TRANSIENT ACTIVATION OF IFN β DETERMINES RESPONSE

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Immune checkpoint therapy (ICT) results in complete regression of tumours in some patients, but no response in others. The biological determinants of the response to ICT remain incompletely understood. Little is known about dynamic biological events that underpin therapeutic efficacy due to the inability to frequently sample tumours in patients.

We developed bilateral tumour mouse models where ICT either leads to a response or a failure to respond in both tumours, allowing us to take one tumour during ICT while determining the response from the remaining tumour. We mapped the gene expression of 144 responding and non-responding tumours from two mouse models at four time-points during ICT using bulk and single-cell RNAseq.

We found that responding tumours displayed a dynamic on/off pattern of type I interferon (IFN) signaling, while in non-responsive tumours IFN was slowly and persistently activated. By mimicking the on/off IFN signal using time-dependent sequential dosing of recombinant IFNs and neutralizing antibodies we markedly improved ICB efficacy, but only when IFN β or its receptor was targeted, not IFN α . We identified Ly6C+/CD11b+ inflammatory monocytes as the primary source of IFN β and found that active type I IFN signaling in tumour-infiltrating inflammatory monocytes was associated with T cell expansion in patients treated with ICB.

Our results suggest that on/off activation of IFN β signaling is critical to the therapeutic response to ICT, which can be exploited to drive clinical outcomes towards response. This is a key example of time-dependent modulation of a drug target being required to achieve optimal anti-cancer activity.

P029

DYNAMIC COORDINATION OF INNATE IMMUNE STIMULI BY KINETICALLY ACTIVATING NANOADJUVANT REDUCES IMMUNE CELL EXHAUSTION AND ENHANCES THE SENSITIVITY OF TUMORS TO IMMUNOTHERAPY

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Despite the contributions of various immunotherapy approaches to immune activation, the clinical benefits of cancer immunotherapy are still constrained by the induction of immunosuppression and immune cell exhaustion. Engineering-based strategies, such as the 'pathogen-mimicking' approach, have utilized key features of pathogen size, shape, and surface-molecule organization to regulate innate immunity and augment adaptive immunity. However, their contribution to immune activation ultimately leads to exhausted APCs and T cells, compromising the overall effectiveness of therapeutic efficacy. Taken cues from pathogen immune stimulation, our research highlights that the maturation of APCs is influenced not only by the type of immune stimuli but also by the duration, combination, and timing of immune stimuli. These factors collectively impact the subsequent T cell responses and play a role in preventing immune exhaustion. To address this, we have developed a kinetically activating nanoadjuvant (K-nanoadjuvant) that dynamically integrates two waves of innate immune stimuli in terms of the order, duration, and time window between the spatiotemporally activating Toll-like receptor 7/8 agonist and other Toll-like receptor agonists.

Our study aimed to examine the impact of K-nanoadjuvant on immune responses in both the tumor microenvironment and the tumor-draining lymph nodes. Specifically, we wanted to determine if K-nanoadjuvant could enhance T-cell and NK-cell-mediated antitumor immunity in two different mouse models: B16OVA melanoma and TC-1 tumor. In order to assess the effects of various factors such as combination, duration, and time window, we compared mice treated with K-nanoadjuvant to those treated with a combination of R848 and poly(I:C). Additionally, we sought to understand the key factor responsible for the enhanced antitumor immunity observed with K-nanoadjuvant by studying the effects of neutralizing IL-12 after treatment.

The K-nanoadjuvant promotes an optimal immune response, specifically, it extends the duration of dendritic cells activation and induces sustained secretion of interleukin-12, resulting in the generation of effector and non-exhausted CD8⁺ T cells within both tumor draining lymph node and tumor microenvironment. In experimental models using mice, treatment with K-nanoadjuvant as a monotherapy or in combination with anti-PD-L1 or liposomes (doxorubicin) demonstrates potent antitumor immunity while exhibiting minimal systemic toxicity.

These findings provide a promising strategy for synchronously and dynamically tailoring innate immunity to enhance cancer immunotherapy.

P030

NFAT5 INDUCTION BY THE TUMOR MICROENVIRONMENT ENFORCES CD8 T CELL EXHAUSTION

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Persistent exposure to antigen during chronic infection or cancer renders T cells dysfunctional. The molecular mechanisms regulating this state of exhaustion are thought to be common in infection and cancer, despite obvious differences in their microenvironments.

We studied by transcriptomics the overexpression and knock-out of NFAT5 in cancer and chronic infection. We generated a new mouse model to compare the expression of NFAT5 in the two pathologies and generated new tools to measure NFAT5 activity in vivo.

We discovered that NFAT5, an NFAT family member lacking an AP-1 docking site, is highly expressed in exhausted T cells from murine and human tumors and is a central player selectively in tumor-induced exhaustion. While NFAT5 overexpression reduced tumor control, NFAT5 deletion improved tumor control by promoting the accumulation of tumor-specific CD8 T cells expressing less TOX and PD-1 and producing more cytokines among precursor exhausted cells. Conversely, NFAT5 did not promote T cell exhaustion during chronic infection. While NFAT5 expression was induced by TCR triggering, its transcriptional activity was specific to the tumor microenvironment and not chronic infection and required hyperosmolarity.

NFAT5 thus promotes CD8 T cell exhaustion in a tumor-selective fashion.

P031

TARGETING GLIOBLASTOMA WITH A TCR SPECIFIC TO THE HLA-A*02-RESTRICTED, GLIOBLASTOMA-ASSOCIATED ANTIGEN PTPRZ1

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The development of effective T cell therapies for patients with glioblastoma (GBM) remains challenging. This is attributed, among other features, to a comparatively low mutational burden and resulting paucity of immunogenic, tumor-specific target antigens. To overcome these challenges, our study leveraged vaccine-induced T cell responses uncovered as part of the Glioma Actively Personalized Vaccine Consortium (GAPVAC) trial. In GAPVAC, human leukocyte antigen (HLA)-A*02+ GBM patients were vaccinated with peptides encompassing glioblastoma-associated antigens (GAA), including protein tyrosine phosphatase receptor type Z1 (PTPRZ1). PTPRZ1 is upregulated in GBM, particularly in GBM stem cells, with low expression across adult tissues. Therefore, we set out to target PTPRZ1 in GBM using T cell therapy.

From a GAPVAC trial patient who experienced a favorable outcome with clinical response to a GAA peptide cocktail including an immunogenic PTPRZ1 peptide, autologous PTPRZ1-reactive T cells were expanded in vitro. PTPRZ1-reactive T cells were sorted and subjected to single-cell VDJ sequencing. The dominant T cell receptor (TCR) clonotypes were cloned and evaluated using Jurkat reporter cell lines. Among the highly expanded clones, a PTPRZ1-reactive TCR was subsequently selected. Next, the TCR was transduced into primary human T cells, and the resulting TCR-T cells were co-cultured with HLA-A*02+ target cell lines expressing either the minimal PTPRZ1 epitopes or endogenously high levels of PTPRZ1. Flow cytometry and LDH release assay were employed to examine TCR activation and cytotoxicity against target cell lines. Lastly, the in vivo therapeutic efficacy of PTPRZ1-TCR-transgenic primary human T cells was evaluated in immunodeficient NSG mice that were inoculated with the target cell lines.

Of the sorted PTPRZ1-reactive T cells, the repertoire was oligoclonal. After cloning and testing the top ten expanded TCRs in Jurkat reporter cells, one TCR exhibited strong reactivity to tumor cell lines expressing the immunogenic PTPRZ1 epitopes. Subsequently, primary human TCR-T cells were generated and co-cultured with target cell lines, demonstrating significant antigen-specific activation and cytotoxicity. Although CD8+ T cells primarily serve as effectors, the assistance of CD4+ T cells maximized the cytotoxicity. To assess the therapeutic potential of the TCR-T cell product, tumor-bearing NSG mice received adoptive transfer of primary human T cells transduced with the PTPRZ1-reactive TCR. PTPRZ1-TCR T cell transfer resulted in regression of established tumors and prolonged survival. 30% of the mice presented complete tumor regression by the experimental endpoint.

We identified a patient-derived, vaccine-induced, TCR reactive against an HLA-A*02-restricted epitope of the GAA PTPRZ1, and confirmed its therapeutic efficacy in vivo. In light of these promising results, a phase 1 clinical trial, Intraventricular T cell receptor transgenic T cell therapy to treat glioblastoma (INVENT4GB), is in preparation and aims to assess the feasibility and safety of intravenous and intracerebroventricular PTPRZ1-TCR-transgenic T cells in patients with recurrent GBM.

P032

PRIMARY NASAL VIRAL INFECTION REWIRES THE TISSUE-SCALE MEMORY RESPONSE

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Recent studies of human nasopharynx have identified muted antiviral epithelial cell states and myeloid reprogramming to be associated with severe respiratory viral infection. However, it is difficult in human studies to control for prior exposure, align infection trajectories, and functionally test the contributions of protective and detrimental cell states to disease trajectory. Understanding whether and how primary infection shapes memory responses to secondary challenge in the nose is critical for informing the rational design of nasal-targeting therapeutics and vaccines, especially in immunocompromised individuals and those receiving chemo- and immunotherapy.

Using a murine influenza A virus (IAV) infection model, we tested the hypothesis that intranasal viral infection confers tissue-scale memory across innate and adaptive immune responses that can collectively respond upon rechallenge. We generated the largest single-cell transcriptional atlas of the murine nasal mucosa to date (n= 198,419 cells), sampling three distinct anatomical regions throughout acute IAV infection and during a memory response (n=60 samples).

Sampling host and viral RNA in conjunction with immunofluorescence imaging, we found infection was largely restricted to respiratory mucosa and induced stepwise changes in cell type, subset, and state composition over time. Interferon (IFN)-responsive neutrophils appeared 2 days post infection (dpi) and preceded transient IFN-responsive/cycling epithelial cell responses 5 dpi, which coincided with broader antiviral monocyte and NK cell accumulation. By 8 dpi, monocyte-derived macrophages expressing Cxcl9 and Cxcl16 arose alongside effector cytotoxic CD8 and Ifng-expressing CD4 T cells. Following viral clearance (14 dpi), rare, previously undescribed Meg3+MHC-II+ epithelial cells and Krt13+ nasal immune-interacting floor epithelial (KNIIFE) cells expressing multiple genes with immune communication potential increased concurrently with tissue-resident memory T (TRM)-like cells and IgG+/IgA+ plasma cells. Proportionality analysis coupled with cell-cell communication inference underscored the CXCL16–CXCR6 signaling axis in effector CD8 T cell and TRM cell formation in the nasal mucosa. Using heterologous IAV strains to avoid antibody neutralization, we show that mice lacking CXCR6 more slowly clear virus following challenge when compared with wild type mice, supporting the role for CXCR6 in maintaining effective TRM and CD4 T cell responses. Secondary influenza challenge administered 60 dpi induced an accelerated and coordinated myeloid and lymphoid response with reduced IFN-responsive epithelial activity, illustrating how tissue-scale memory to natural infection engages both myeloid and lymphoid cells without broad epithelial inflammation.

Together, this atlas serves as a reference for viral infection in the upper respiratory tract, highlights key epithelial and immune subsets contributing to host responses, and illustrates an optimal and coordinated memory response to secondary challenge. This reference atlas can be readily applied to new datasets generated from the nasal mucosa in diverse models to learn how memory is perturbed in distinct challenge settings.

P033

DEFECTS IN THE NECROPTOSIS MACHINERY ARE A CANCER RESISTANCE MECHANISM TO CHECKPOINT INHIBITOR IMMUNOTHERAPY

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Immunotherapy with checkpoint inhibitors targeting PD-1 or CTLA-4 (re-)invigorate T cell immune responses against tumor cells. In many patients these therapies fail, because the development of spontaneous immune responses is often compromised, as the tumor microenvironment (TME) lacks proinflammatory signals resulting in suboptimal activation of antigen-presenting cells (APC). Under certain circumstances, tumor cells can undergo a special form of inflammatory programmed cell death that favors recognition and elimination by the immune system. One form of such immunogenic cell death (ICD) is necroptosis, which has mainly been studied in the context of pathogenic infections. Activated by RIPK3, the central executioner protein of necroptosis MLKL has a pore-forming activity that results in the leakage of danger-associated molecular patterns (DAMPs) during programmed cell death. At this stage it is unclear whether these mechanisms play a role in tumor immunosurveillance or -therapy with checkpoint inhibitors.

With tumor cells lines that lack specific components of the cell death machinery (generated via CRISPR/Cas9-mediated mutagenesis) and murine models, we addressed the importance of necroptotic ICD for the efficacy of immune checkpoint inhibitors. These preclinical data were aligned with genome-wide transcriptional programs in melanoma patient tumor samples at diagnosis and during checkpoint inhibitor treatment for the activity of these pathways and possible association with treatment outcome.

Tumor cell-intrinsic genetic defects in critical components of the necroptosis machinery (MLKL or RIPK3) abrogated successful tumor immunosurveillance and -therapy. C57BL/6 mice bearing syngeneic, MLKL- or RIPK3-deficient B16 malignant melanomas failed to control tumor growth in response to anti-PD-1/anti-CTLA-4 immunotherapy, and showed poor overall survival. Similar results were observed in the murine CT26 colon carcinoma model, but not in Panc02 pancreatic adenocarcinoma. Mechanistically, defects in the necroptosis pathway resulted in reduced tumor antigen cross-presentation by type 1 conventional dendritic cells (cDC1) in tumor draining lymph nodes, and subsequently impaired immunotherapy-induced expansion of circulating tumor antigen-specific CD8⁺ T cells and their accumulation and activation in the TME. In vitro, co-culture of tumor cells undergoing necroptotic (induced by exposure to TNF-alpha, a SMAC-mimetic and the pan-caspase inhibitor zVAD) but not apoptotic programmed cell death resulted in increased uptake by phagocytic cells, associated with maturation and activation of dendritic cells. Treatment of tumor cells with the epigenetic modulator azacytidine or a type-I interferon induced enhanced intrinsic transcriptional activity of the necroptosis machinery, and hence their susceptibility to necroptotic ICD. In humans, transcriptome analysis of primary melanoma samples revealed a strong association between high expression of MLKL and prolonged overall survival. In melanoma patients treated with anti-PD-1 and/or anti-CTLA-4 checkpoint inhibitor, high MLKL transcriptional activity significantly associated with durable clinical response to immunotherapy.

Collectively, these data suggest that a functional necroptotic machinery is imperative for checkpoint inhibitor treatment efficacy. Necroptotic tumor cells act as an immunogenic stimulus, initiating anti-tumor T-cell immunity. Future experiments will focus on the molecular triggers of necroptosis in tumor cells, and how this process can be modulated to circumvent cancer resistance to immune checkpoint inhibitor therapy.

P034

HARNESSING CYTOMEGALOVIRUS IMMUNITY AGAINST PANCREATIC TUMORS FOR IMMUNOTHERAPY

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Immunotherapy has had very limited success in pancreatic cancer, due to its low mutational burden and immunosuppressive microenvironment. Our approach consists in redirecting pre-existing antiviral immunity against pancreatic tumors by delivering viral antigens using the tumor penetrating peptide iRGD. This peptide, targets the tumor vasculature through alpha-v integrins and neuropilin-1, delivering conjugated or co-administered cargo to tumors.

Here we used mouse cytomegalovirus (CMV) as an infection model. CMV is a β -herpesvirus that induces a strong T-cell response in mice and humans, comprising >10% of all circulating CD4 and CD8 T cells mostly with effector-memory phenotype. Importantly, human CMV infects over 60% of the world's population rendering it a suitable candidate for translation.

Mice latently infected with CMV were orthotopically implanted with KPC pancreatic tumor cells and treated with systemic injections of vehicle or iRGD plus CMV class I and II binding peptides. Another cohort of age matched uninfected mice was used as a control. Tumor growth and mouse weight were monitored twice a week and CMV specific immune responses were measured in spleen and tumor by flow cytometry, using tetramer staining and CMV specific peptide recall.

CMV infected mice receiving iRGD + CMV peptides responded to treatment as evidenced by delayed tumor growth associated with increased tumor necrosis and T cell infiltration. In addition, flow cytometry analysis of tumor and spleen showed a significant increase in CMV specific T cells that produced significant amounts of IFN- γ and TNF- α upon CMV peptide recall. Furthermore, tumor regulatory T cells (Treg) were significantly reduced and the CD8/T reg ratio was greatly increased. Survival studies showed a 68% increase in median survival in the treated and infected group.

Taken together, this data shows we can deliver antigens to pancreatic tumors via iRGD and elicit an anti-tumor immune response that results in delayed tumor growth and has a survival benefit. This is a mutation agnostic approach with high translational value, since more than 60% of the population has previous CMV immunity and iRGD in combination with chemotherapy is already in phase II clinical trials for advanced pancreatic cancer.

P035

EPIGENETIC REGULATION OF CELL STATE GOVERNS ANTI-TUMOR IMMUNITY IN HIGH-RISK NEUROBLASTOMA

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Childhood neuroblastoma with MYCN-amplification is classified as high-risk and often relapses after intensive treatment regimen. Immune checkpoint blockade therapy shows limited efficacy in neuroblastoma patients and the cancer intrinsic immune regulatory network is poorly understood.

We performed genome-wide CRISPR/Cas9 genetic screens in a human tumor-immune co-culture system (TICS) to reveal neuroblastoma intrinsic resistance genes to nivolumab. The gene of interest was deleted using CRISPR and cells were characterized using flow cytometry, ATACseq and proteomics. Response of control or KO tumors to PD1 blockade was tested in immunocompetent mice bearing the MYCN-driven 9464D tumors. Immunological changes were characterized using flow cytometry and a nanostring gene panel. Expression of the target gene was tested using published scRNAseq data from patients with neuroblastoma and the prognostic value was examined using multiple transcriptomics datasets of neuroblastoma tumors.

Here, we leveraged genome-wide CRISPR/Cas9 screens in a human tumor-immune co-culture system and identified H2AFY as a resistance gene to nivolumab. Analysis of single-cell RNA-sequencing datasets from neuroblastoma patients revealed that H2AFY mRNA was enriched in adrenergic tumor cells but was absent in cells with the malignant mesenchymal phenotype. Because neuroblastoma cells at the mesenchymal state are more susceptible to immune-mediated cytotoxicity, we hypothesized that H2AFY may serve as a master regulator for response to immunotherapy. Indeed, genetic deletion of H2afy in murine MYCN-driven 9464D neuroblastoma cells reverted in vivo resistance to PD-1 blockade by eliciting concurrent activation of the adaptive and innate immunity. Mapping of the epigenetic and translational landscape in MYCN-driven neuroblastoma cells using ATACseq and mass spectrometry demonstrated that H2afy deletion by CRISPR/Cas9 promoted cell transition towards a mesenchymal-like state. Genes associated with the mesenchymal cell state were more epigenetically active and translated at higher levels, while the epigenetic and translational levels of adrenergic genes were suppressed in H2AFY KO cells. In patients with neuroblastoma, high H2AFY mRNA was associated with worse overall survival. Using a multi-omics approach combining patient datasets, CRISPR screens and mRNA expression in mouse tumors, we uncovered H2AFY-associated genes that were functionally relevant and prognostic in patients.

Altogether, our study uncovers the previously unknown function of H2AFY as an epigenetic gatekeeper for cell state and proposes its therapeutic potential to improve response to immunotherapy in high-risk human neuroblastoma.

P036

DEVELOPMENT OF ANTI-CD73 ANTIBODY FOR CANCER IMMUNOTHERAPY

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The hypoxic tumor microenvironment may induce CD73 ectonucleotidase expression in cancer cells. Highly expressive CD73 promotes the onset and progression of tumors and determines poor prognosis of patients. CD73 plays a pivotal role in generating an immunosuppressive microenvironment to boost cancer progression. CD73 dephosphorylates extracellular adenosine monophosphate (AMP) and subsequently leads to a massive production of adenosine in the tumor microenvironment. The increased extracellular adenosine in the tumor microenvironment interferes proliferation, apoptosis and metastasis in cancer cells and causes dysregulation of immune cell infiltrates by interacting with specific G-protein-coupled receptors. Therefore, CD73 is regarded as a potential immunotherapeutic target. The anti-CD73 antibody can effectively inhibit the CD73 activity to reduce the production of adenosine and restore the anti-tumor activity of immune cells in the tumor microenvironment. In combination of the anti-CD73 antibody with current cancer regimens can further improve the limited response rate of the current regimens.

Herein, DCB identified a novel humanized anti-CD73 antibody. The immunomodulation activity of DCB's anti-CD73 antibody was validated using CFSE-based T cell proliferation assay. The tumor growth inhibition activity of DCB's anti-CD73 was demonstrated using an MDA-MB-231 xenograft mouse model.

DCB's novel humanized anti-CD73 antibody displayed better inhibition activity of membrane-bound CD73 enzyme and better reversing effects on the AMP-attenuated CD4+ T cell proliferation than did benchmark MEDI9447. It also suppressed intra-tumoral CD73 activity and delayed tumor growth in in vivo models. In addition, the results of toxicology evaluation showed that the anti-CD73 antibody developed by DCB was stable and safe. A Chinese hamster ovary stable cell line that demonstrated excellent productivity has also been developed.

Hence, the DCB's anti-CD73 antibody has great potential for clinical development and can provide a new, safe and effective therapeutic efficacy for cancer patients.

P037

FIGHTING FIRE WITH FIRE: ALLEVIATING IMMUNE SUPPRESSION IN THE TUMOR MICROENVIRONMENT BY TARGETING COMPLEMENT INHIBITION

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Since the discovery of immune checkpoint inhibitors, cancer immunotherapy took its rightful place as an important pillar in the battle against cancer. Most of the current immunotherapies focus on cytotoxic T-lymphocytes and natural killer cells as the main effector cells of anti-tumor immunity. However, the complement system, one of the best characterized pathways in several pathophysiological conditions, is often overlooked in the context of tumor immunology. Overall, there is no consensus about its role within the tumor microenvironment, since both tumor-promoting and tumor-suppressing properties have been described, depending on the tumor type, stage and complement factor(s) involved. To avoid overaction, and ultimately complement dependent cell lysis (CDC) via formation of the membrane attack complex (MAC), the complement system is tightly regulated at different stages by a series of complement regulatory proteins (CRPs). However, several reports have described that overexpression of CRPs, such as CD46, CD55 and CD59, on cancer cells, correlates with a worse prognosis. The effect of blocking or downregulating CRPs on tumor growth and survival has been investigated, for only a limited number of cancer cell lines of which the B-cell lymphoma cell line Raji is by far the most extensively studied. As far as we know, the functional role of CRPs has never been studied in the context of melanoma. Therefore, we wanted to evaluate the effect of knocking-out (KO) CRPs, in a first instance CD59, in melanoma cell lines, using the efficient CRISPR-Cas9 technology and evaluate its effect on complement mediated tumor cell death, followed by an assessment of anti-tumor immune responses.

The expression of CD46, CD55 and CD59 on melanoma cell lines (624-mel, 888-mel, 1087-mel, CHL 1 and MZ2) and the CD20-expressing Raji cell line was analyzed using flow cytometry. To assess complement-dependent cell lysis (CDC), Raji cells and 624-mel cells (both KO and wildtype (WT)) were treated with 5% and 2.5% normal human serum (NHS) as a source of complement factors. To initiate the complement cascade, we added rituximab, an IgG1 monoclonal antibody (mAb) targeting CD20. The effect on Raji cells was evaluated using flow cytometry after staining with Fixable viability stain780 and the effect on 624-mel was monitored using the IncuCyte Live Cell Analysis system upon the addition of Cytotox Red as a live/death discriminator. An anti-calreticulin (CRT) mAb is used to determine immunogenic cell death (ICD) by flow cytometry.

All the melanoma cell lines were 100% positive for the expression of CD46, CD55 and CD59, but displayed variable expression levels on a per cell basis (MFI). In literature, Raji cells are almost invariably used as the model in complement-related research and indeed, we confirmed that they are prone to CDC. Interestingly, we found that Raji cells were 100% positive for CD46, yet only about 50% for both CD55 and CD59. Raji cells were subjected to complement factors and cells lacking CD55 and CD59 succumbed to the treatment, while CD55 and CD59 expressing cells survived, showing their importance for controlling CDC. This was also associated with CRT exposure, indicating the induction of ICD. To investigate whether a similar mechanism is at play in melanoma cells, we successfully KO CD59 in the 624- and 1087 mel cell line, using CRISPR-Cas9 and showed that even low concentrations of NHS (5%) induce cell death.

Our observation that Raji cells only partially express CD55 and CD59 and that the CD55/59 expressing cells survived a challenge with NHS, while cells lacking these CRPs died, highlights the importance of CRPs in controlling tumor cell death via CDC. A similar effect was observed for melanoma cells where CD59 was KO. To confirm this result, we will quantify the level of cell death by flow cytometry analysis and eventually evaluate whether the observed cell death is of an immunogenic type, as we already established for Raji cells.

P038

KDM6B MEDIATED EPIGENETIC REPROGRAMMING OF INTRATUMORAL MYELOID CELLS IMPROVES RESPONSE TO IMMUNE CHECKPOINT THERAPY IN GLIOBLASTOMA

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Immune-suppressive myeloid cells play a critical role in conferring resistance to immune checkpoint therapy (ICT). Therefore, developing therapeutic strategies to target immune-suppressive myeloid cells is a critical approach to enhance response to cancer immunotherapy and has been the focus of intense research for many years. However, many of the strategies to target myeloid cells have not been successfully translated to the clinic partly due to the functional heterogeneity and redundancy of pathways in myeloid cell subsets. The significant plasticity of myeloid cells highlights the important role of epigenetic regulation of their cell state. However, the impact of epigenetic regulation of intratumoral myeloid cell plasticity on therapeutic resistance remains largely unexplored.

Glioblastoma (GBM) is an aggressive form of brain tumor, highly infiltrated with immune-suppressive myeloid cells and demonstrates resistance to ICT. We have previously shown the persistence of tumor-associated macrophages (TAMs) in the GBM tumor microenvironment even after treatment with anti-PD1 therapy. In this study, we aimed to identify epigenetic factors regulating the functional phenotype of intratumoral myeloid cell subsets in order to reprogram these cells to a pro-inflammatory state thus enhancing anti-tumor immunity and efficacy of anti-PD1 therapy.

We performed scRNA-seq, spatial transcriptomic (VISIUM) and multiplex IF analyses of human GBM samples to identify key epigenetic factors in intratumoral myeloid cell subsets. Next, we adopted a reverse translational strategy to elucidate the mechanistic underpinnings using murine models of GBM. We performed scRNAseq, scATAC seq, sequencing, CyTOF analysis of murine models of GBM. Additionally, we performed in-vitro macrophage polarization and phagocytosis assays to functionally validate our findings.

scRNA seq of GBM tumors resected from patients demonstrated high expression of histone 3 lysine 27 demethylase (KDM6B) in myeloid cell subsets including monocytes, macrophages and dendritic cells (DCs). Further, spatial transcriptomic analysis of human GBM tumors showed significant infiltration of KDM6B expressing immune-suppressive myeloid cells in the tumor microenvironment (TME) which were not co-localized with effector CD8 T cells. KDM6B is an epigenetic enzyme that demethylates the repressive trimethylation mark at histone 3 lysine 27 (H3K27me3) thereby promoting gene transcription. Importantly, in murine models of GBM, *LysMcreKDM6B^{fl/fl}* mice carrying *Kdm6b* deletion in myeloid cells had enhanced pro-inflammatory pathways and improved survival compared with their wild-type counterparts. ChIP sequencing demonstrated that KDM6B directly regulates H3K27me3 enrichment of genes including *Mafb*, *Socs3* and *Sirpa* which inhibit critical pro-inflammatory pathways such as cytokine production and phagocytosis in macrophages, providing mechanistic insight into enhanced pro-inflammatory pathways noted in the absence of *Kdm6b*. In-vitro macrophage polarization assays also demonstrated the inability to acquire an immune-suppressive phenotype following the loss of *Kdm6b*. Further, pharmacological inhibition of KDM6B could recapitulate the functional phenotype of *Kdm6b* deleted myeloid cells and improve sensitivity to anti-PD1 therapy in GBM. Together, these findings have provided critical insight into KDM6B-mediated epigenetic regulation of intratumoral myeloid cell functions. Thus, this study identified KDM6B as an epigenetic regulator of the functional phenotype of myeloid cell subsets and a potential therapeutic target to improve response to ICT.

The strategy of inhibiting KDM6B, proposed in this study, not only adds to the existing repertoire of myeloid cell targeting strategies, it proposes a new paradigm of regulating the epigenetic machinery

to target intratumoral myeloid cell plasticity thus reprogramming them to a pro-inflammatory phenotype and improve response to immune-based therapy.

P039

ESCRT-DEPENDENT STING DEGRADATION INHIBITS STEADY-STATE AND cGAMP-INDUCED SIGNALING

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Stimulator of interferon genes (STING) is an intracellular sensor of cyclic di-nucleotides driving innate immunity against dsDNA. Along with anti-pathogen immunity, activation of the cyclic GMP-AMP (cGAMP) synthase (cGAS)/STING pathway is essential for antitumor responses, immune checkpoint therapy, development of autoimmune diseases and induction of cellular senescence. STING trafficking is tightly linked to its function. Upon cGAMP ligation, STING traffics to the Golgi and the TGN to interact with TBK1 and activate a type I IFN response. STING signaling shutdown is achieved by degradation of the sensor in the lysosome. Mutations in genes regulating these processes have been found in humans to drive autoinflammation. Despite the centrality of STING trafficking to STING responses, the proteins governing these processes and the signals triggering activated STING elimination are not well understood.

We used a combination of unbiased approaches to identify regulators of STING trafficking and degradation. By fusing STING to TurboID we generated a time-resolved map of the STING interactome during its trafficking employing proximity-ligation proteomics with quantitative TMT labeling. To associate functions to the proteins in the STING interactome, we then generated a STING-mNeonGreen reporter line to monitor STING levels upon cGAMP activation. Using this line, we performed genome-wide and targeted CRISPR screens to identify genes regulating STING trafficking and degradation.

By using a combination of proteomics and CRISPR screens, we show that oligomerization drives STING ubiquitination and that ubiquitinated STING recruits ESCRT in the endosomal compartment to achieve STING degradation and signaling shutdown. Ubiquitinated STING in the endosomal compartment creates a platform for the recruitment of an endosome-specific VPS37A/UBAP1-containing ESCRT complex. Association with this complex drives degradation of the sensor via fusion of vesicles coated with oligomeric STING with the endolysosome. By focusing on genes mutated in human disease, we show that a pathogenic mutant of the ESCRT-I subunit UBAP1 blocks STING degradation and leads to accumulation of STING in the endolysosomal compartment at steady state, driving spontaneous activation of the sensor.

Based on these findings, we propose an updated model of STING trafficking and degradation. Tonic cGAMP production primes a basal flux of STING trafficking from the ER to the lysosome with consequent ESCRT dependent constant degradation. Inactivating mutations in genes controlling STING trafficking represent a generalized mechanism inducing constitutive STING-dependent responses that could lead to or exacerbate disease.

P040

A VASCULAR-RESTRICTED, TUMOR-INDUCED NEUTROPHIL POPULATION DRIVES VASCULAR OCCLUSION, PLEOMORPHIC NECROSIS, AND METASTASIS

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Tumor necrosis is associated with poor prognosis in cancer. It is generally considered a passive process, occurring when tumor growth outpaces nutrient supply. Here, we report that it can be actively driven by a Ly6G^{High} Ly6C^{Low} neutrophil population that arises in tumor-bearing mice as the hematopoietic system becomes heavily skewed towards granulopoiesis. Compared to Ly6C^{High}, Ly6C^{Low} neutrophils harbored unique transcriptional and phenotypical properties, with increased neutrophil extracellular trap (NET) formation and, intriguingly, the inability to extravasate in response to inflammatory challenges. We identified a transcriptomic signature for these “vascular-restricted” neutrophils and found it to be expressed in neutrophils from breast cancer patients. Using whole mount tissue clearing of tumors from multiple murine models, we identified tumors with vascular-restricted neutrophils in blood and a pleomorphic architecture of the necrotic tissue. Pleomorphic necrosis was also found in biopsies from triple-negative breast cancer patients. We hypothesized that the pleomorphic necrotic structures could be related to the unusual properties of vascular-restricted neutrophils. Indeed, in the models harboring vascular-restricted neutrophils and pleomorphic necrosis, we found that neutrophils formed intravascular aggregates in a process involving NETs. This intravascular aggregation caused vascular occlusion and blood flow interruption in the tumor vasculature, particularly in pericyte-poor vessels harboring luminal exposition of basal membrane components, as well as fibrin and platelet deposition. Furthermore, blocking NET formation genetically and pharmacologically reduced the extent of necrosis in the primary tumors and the lung metastatic burden in several cancer models. This reduction in metastasis correlated with a reduction in the area of peri-necrotic tissue, where we found —using spatial transcriptomics and immunofluorescence— that cancer cells undergo transcriptomic and protein-level changes related to enhanced metastatic potential. Necrosis correlates with worse prognosis but is considered passive and non-targetable. Here, we show that genetic or pharmacologic blockade of NET formation abrogates the formation of pleomorphic necrosis, critically demonstrating that 1) tumor necrosis is not necessarily a passive phenomenon arising as a byproduct of tumor growth and 2) it can be blocked to reduce metastatic spread.

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P041

TNF BLOCKADE ATTENUATES CYTOKINE RELEASE SYNDROME AND CAR T CELL EFFICACY IN A PRECLINICAL MODEL

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CAR T cell-induced Cytokine Release Syndrome (CRS) limits the application of CAR T cells beyond inpatient settings in specialized centers. Understanding the cellular and molecular mechanisms driving CRS is crucial to rationally design interventions that can abate or prevent CRS. TNF serum levels are elevated in patients suffering from CAR T cell-induced CRS, and anecdotal evidence has shown that TNF blockade can abate symptoms of CRS in patients. Consistently, we have observed a correlation between CRS severity and serum TNF levels in a previously established murine model for CAR T cell-induced CRS.

To assess the role of TNF in CRS pathophysiology, we treated a murine model of CRS with TNF-blocking agent etanercept prior and post CAR T cell administration. We monitored CRS-induced weight loss and lethality. We used flow cytometry to assess the accumulation of myeloid immune cells at the tumor site and single cell RNA sequencing to assess the activation state of these myeloid immune cells. Peripheral blood samples were analyzed to assess both CAR T cell and myeloid derived cytokine levels. The effect of etanercept pre-treatment on CAR T cell efficacy was assessed in a murine model for acute lymphoblastic leukemia.

We observed that post-CAR T cell etanercept administration can promote recovery from non-lethal CRS in our murine model. Pre-treatment with etanercept had a more profound effect and completely prevented CRS-induced weight loss and lethality, even in severe CRS. In addition, etanercept pre-treatment significantly reduced the accumulation of macrophages at the tumor site and significantly reduced the expression of Nos2 (encoding inducible nitric oxide synthase) in these macrophages. Reduced activation of the recipient's myeloid immune system was further corroborated by the detection of significantly lower IL-6, G-CSF and MCP-1 serum levels in etanercept pre-treated animals. We also observed that etanercept pre-treatment lowers the serum level of CAR T cell-derived cytokines, including IL-2, suggesting reduced CAR T cell activation. Consistently, we observed that etanercept pre-treatment decreases the efficacy of CAR T cell in a murine model for acute lymphoblastic leukemia.

These results show that TNF is a master regulator of CRS and that its blockade abates important processes in CRS pathophysiology, including macrophage accumulation, Nos2 expression and IL-6 secretion in a preclinical model. Furthermore, etanercept prevents CRS-induced weight loss and lethality in this model. Our efficacy studies show that general blockade of TNF reduces the anti-tumor efficacy of CAR T cells in mice, warranting caution when intervening with the TNF pathway driving CRS.

P042

TRIPLE CHECKPOINT BLOCKADE, BUT NOT ANTI-PD1 ALONE, ENHANCES THE EFFICACY OF ENGINEERED ADOPTIVE T CELL THERAPY IN ADVANCED OVARIAN CANCER

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Over 20,000 women are diagnosed with ovarian cancer annually, and more than half will die within 5 years. This rate has changed little in the last 30 years, highlighting the need for therapy innovation. Although immunotherapy has revolutionized cancer treatment and is now considered a pillar of cancer care, efforts to harness endogenous patient immune responses have yielded limited therapeutic activity and benefited only a small fraction of ovarian cancer patients. T cells engineered to express a T cell receptor (TCR) targeting proteins uniquely overexpressed in tumors have the potential to overcome limitations posed by the endogenous T cell repertoire and control tumor growth without toxicity. Mesothelin (Msln) is over-expressed in ovarian cancer, contributes to the malignant and invasive phenotype, and has limited expression in healthy cells, making it a candidate immunotherapy target in these tumors.

The ID8VEGF mouse cell line was used to evaluate if T cells engineered to express a mouse Msln-specific high-affinity T cell receptor (TCRMsln) can kill murine ovarian tumor cells in vitro and in vivo. Immunohistochemistry and flow cytometry were used to interrogate inhibitory signaling pathways active in TCRMsln T cells. Tumor-bearing mice were treated with TCRMsln T cells plus anti-PD-1, anti-Tim-3 or anti-Lag-3 checkpoint-blocking antibodies administered alone or in combination, ultimately targeting up to three inhibitory receptors simultaneously. Single-cell RNA-sequencing was used to profile the impact of combination checkpoint blockade on both the engineered T cells and the tumor microenvironment. Cytokine secretion and survival assays were used to evaluate TCRMsln T cell antitumor function in the presence or absence of checkpoint-blocking antibodies.

In a disseminated ID8 tumor model, adoptively transferred TCRMsln T cells preferentially accumulated within established tumors, delayed ovarian tumor growth, and significantly prolonged mouse survival. However, our data also revealed that elements in the tumor microenvironment (TME) limited engineered T cell persistence and cytolytic function. Triple checkpoint blockade, but not single- or double-agent treatment, dramatically increased antitumor function by intratumoral TCRMsln T cells. Single-cell RNA-sequencing of tumor-infiltrating cells revealed distinct transcriptome changes in engineered T cells, endogenous T cells and myeloid-derived cells. Engineered TCRMsln T cells increased expression of pathways associated effector and memory gene signatures, including proliferation and metabolic function, and reduced expression of genes associated with exhaustion when combined with triple checkpoint blockade. Moreover, combining adoptive immunotherapy with triple checkpoint blockade significantly prolonged survival in the cohort of treated tumor-bearing mice, relative to mice that received TCRMsln T cells alone or with anti-PD1 or double-agent treatments.

Inhibitory receptor/ligand interactions within the tumor microenvironment can dramatically reduce T cell function, suggesting tumor cells may upregulate the ligands for PD-1, Tim-3 and Lag-3 for protection from tumor-infiltrating lymphocytes. In a model of advanced ovarian cancer, triple checkpoint blockade significantly improved the function of transferred engineered T cells and improved outcomes in mice in a setting in which single checkpoint blockade had no significant activity. These results suggest that cell therapy with triple blockade, which can be more safely pursued in a cell intrinsic form through genetic engineering, may be necessary for improved efficacy in patients.

P043

ATR INHIBITION UPREGULATES PD-L1 AND POTENTIATES THE ANTITUMOR IMMUNE RESPONSE TO CHEMOIMMUNOTHERAPY IN SMALL-CELL LUNG CANCER.

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Small cell lung cancer (SCLC) is the most aggressive subtype of lung cancer, with a very poor prognosis and limited therapeutic options. Recent clinical trials of immune checkpoint blockade (ICB) combined with chemotherapy delivered only very modest benefit. Here, we identified that ataxia telangiectasia and rad3 related (ATR), the primary activator of the replication stress response, is highly enriched in SCLC and inhibition of ATR induced DNA damage and apoptosis in multiple SCLC models.

In this study, we performed genetic and pharmacological inhibition of ATR in a panel of human and murine SCLC models. Furthermore, we investigated the effect of ATR inhibition alone or in combination with PD-L1 blockade as a first-line or a second-line treatment in multiple immunocompetent mouse models of SCLC. The downstream effects of ATR inhibition were assessed by single-cell RNA sequencing, multicolor flow cytometry, western blot analysis, IHC, and genomic analysis.

In multiple immunocompetent SCLC mouse models, ATR inhibition (ATRi) remarkably enhanced the anti-tumor effect of PD-L1 blockade. We next tested the ATR inhibition either alone or in combination with PD-L1 in the second-line regimen for SCLC. We observed that ATR inhibition in combination with PD-L1 blockade significantly reduced tumor volume and prolonged survival of aggressive mice models when compared to PD-L1 alone. Targeting ATR enhanced the expression of PD-L1, activated the cGAS/STING pathway, induced the expression of Type I and II interferon pathways, and caused significant infiltration of cytotoxic and memory/effector T-cells into tumors. Interestingly, ATRi also led to significant induction of MHC class I in SCLC in vitro and in vivo models.

Analysis of pre- and post-treatment clinical samples from a proof-of-concept study of a first-in-class ATR inhibitor, M6620 (VX970, berzosertib), and TOP1 inhibitor topotecan, in patients with relapsed SCLCs validated the induction of MHC class I and interferon pathway genes, for the first time in this disease.

Our findings highlight ATRi as a potentially transformative vulnerability of SCLC, paving the way for combination clinical trials with anti-PD-L1. Given the increasing importance of immunotherapy for the management of SCLC and that ATR inhibitors are already in clinical trials, combining an ATR inhibitor with PD-L1 blockade may offer a particularly attractive strategy for the treatment of SCLC and contribute to the rapid translation of this combination into the clinic.

P044

WEE1 INHIBITION ENHANCES THE ANTITUMOR IMMUNE RESPONSE TO PD-L1 BLOCKADE BY THE CONCOMITANT ACTIVATION OF STING AND STAT1 PATHWAYS IN SMALL CELL LUNG CANCER

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Small cell lung cancer (SCLC) is a poorly immunogenic, high-grade neuroendocrine carcinoma arising in the lung. Immune checkpoint blockade (ICB) added to chemotherapy is now the standard upfront therapy for SCLC but leads to a modest increase in overall survival and progression-free survival. These modest benefits underscore the critical need to identify pathways and targets that can durably enhance the antitumor responses of ICB in SCLC. SCLC is characterized by a ubiquitous loss of TP53 which disrupts the G1-S cell cycle checkpoint and as a result, most SCLC is dependent on G2-M cell cycle checkpoint regulators including WEE1. We and others have shown that DNA damage response (DDR) components are overexpressed in SCLC and targeting DDR could be an effective therapeutic strategy in SCLC.

In this study, we have performed WEE1 inhibition either alone or in combination with PD-L1 blockade in a panel of SCLC models.

We demonstrate that inhibition of WEE1 induces G2/M cell cycle arrest, DNA damage, and cytosolic DNA accumulation in SCLC models. We further show that WEE1 targeting activates the STING-TBK1-IRF3 pathway which increases type I interferons (IFN- α and IFN- β) and pro-inflammatory chemokines (CXCL10 and CCL5), facilitating an immune response via CD8+ cytotoxic T-cell infiltration. We further show that WEE1 inhibition concomitantly activates the STAT1 pathway, increasing IFN- γ and PD-L1 expression. Consistent with these findings, combined WEE1 inhibition (AZD1775) and PD-L1 blockade caused remarkable tumor regression, activation of type I and II interferon pathways, and infiltration of cytotoxic T-cells in multiple immunocompetent SCLC genetically engineered mouse models, including an aggressive model with stabilized MYC.

Our study demonstrates the cell-autonomous and immune-stimulating activity of WEE1 inhibition in SCLC. Given the increasing importance of immunotherapy for the management of SCLC and that WEE1 inhibitors are already in clinical trials, combining a WEE1 inhibitor with PD-L1 blockade may offer a particularly attractive strategy for the treatment of SCLC and contribute to the rapid translation of this combination into the clinic.

P045

UNVEILING THE IMPACT OF MRNA MODIFICATION ON MRNA VACCINE-INDUCED IMMUNE RESPONSES VIA SINGLE-CELL TRANSCRIPTOME PROFILING

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mRNA vaccines have emerged as a revolutionary approach offering a precise and personalized strategy to engage the immune system against many diseases. In particular, mRNA vaccines administered intravenously in the form of mRNA complexed to cationic lipids (mRNA-lipoplex) were shown to effectively induce tumor antigen-specific T cell responses. In addition, mRNA-lipoplexes activate innate immune signaling pathways through pattern recognition receptor mediated sensing of unmodified, uridine-containing mRNA which can be blocked by m1Y uridine modification. In this study, we investigated how such modification of mRNA affects the magnitude and phenotype of antigen-specific T cells as well as induction of innate signaling.

C57/BL6 mice were immunized with mRNA-lipoplexes containing unmodified or m1Y-modified Reps1 (neo-antigen) encoding RNA. Splenocytes and spleen-sorted antigen-specific CD8+ T cells from immunized mice were subjected to 10X Genomics platform's 3' single-cell RNA sequencing 3 hours and 19 days after immunization, respectively. Acquired raw data was processed with the 10X Genomics Cell Ranger single-cell pipeline and Seurat toolkit was employed for data integration and clustering analysis.

We could identify more than 10 different cell types in splenocytes and 7 phenotypes in the antigen-specific CD8+ T cells. Our data showed a notable increase in the expression of activation-related and interferon response-related genes, specifically in DCs, macrophages, and T cells in the unmodified mRNA group compared to its modified counterpart. Moreover, unmodified mRNA containing mRNA-lipoplexes resulted in the generation of a higher proportion of cytotoxic CD8+ T cells, known for their ability to directly eliminate target cells, while the frequency of central memory T cells was reduced in this group.

The findings of this study contribute to our understanding of the nature of immune responses elicited by antigen-specific mRNA vaccines and highlight the distinct effects of mRNA modification on phenotype of immune cells including vaccine-induced antigen-specific CD8+ T cells.

P046

BRCA1 AND BRCA2 MUTANT OVARIAN TUMORS CREATE DISTINCT TUMOR IMMUNE MICROENVIRONMENT AND DIFFERENTIAL SIALYLATION STATUS

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BRCA1 and BRCA2 both function in the Homologous Recombination DNA Repair Pathway and mutations in either gene increase the risk for ovarian cancer. Clinically however, BRCA2 mutated (BRCA2mt) ovarian tumors have consistently presented with a more indolent clinical course and patients harboring BRCA2 mutation showed better survival compared to patients with wild-type BRCA or BRCA1 mutated (BRCA1mt) tumors.

Host anti-tumor immune response is the first-line of defense against tumor initiation and progression. Ovarian tumors are known to create an immunosuppressive microenvironment characterized by significant infiltration of MDSC, which consequently prevent successful CD8 T cell cytotoxic response. The success with PD-1/PD-L1 therapies observed in other cancers is unfortunately, not seen in ovarian cancer.

Sialylation is the addition of sialic acid to terminal ends of glycoproteins or glycolipids and is upregulated in cancers. Sialic acid is a ligand for Siglec receptors expressed in various immune cells and serve as self-associated molecular patterns that promote immune suppressive mechanisms.

We show in this study that BRCA1mt and BRCA2mt ovarian tumors differentially affect host immunosurveillance mechanisms and that this is correlated with differential levels of cancer cell sialylation.

Isogenic ID8 mouse ovarian cancer cell lines ID8Trp53^{-/-};Brca1^{-/-} and ID8Trp53^{-/-};Brca2^{-/-} with complete knock-out of p53 and BRCA1 or BRCA2 were used to establish intraperitoneal (i.p.) tumors in C57BL/6 immunocompetent mice (n=8). Immunophenotype in peritoneal lavage was characterized by immunostaining and flow cytometry analysis. Levels of sialylation was quantified using anti-peanut agglutinin antibody (PNA). RNA sequencing data from high-grade serous ovarian cancer patients with somatic loss of function mutations in BRCA1 (BRCA1mt, n=16) or BRCA2 (BRCA2mt, n=15) were used to identify differentially expressed genes (p<0.05 and fold-change >1.6) and differentially regulated Biological Processes by Gene Ontology Analysis.

ID8Trp53^{-/-};Brca2^{-/-} ovarian tumors grew slower (p<0.0001) and produced less i.p. tumor burden than ID8Trp53^{-/-};Brca1^{-/-} tumors (p=0.0008). As such, mice bearing ID8Trp53^{-/-};Brca2^{-/-} tumors survived significantly longer (mean survival of 56 days vs 41 days for ID8Trp53^{-/-};Brca1^{-/-}; p=0.029). We observed an early increase in CD11b⁺/MHC Class II⁺ M1 anti-tumor macrophages in the peritoneal lavage of mice bearing ID8Trp53^{-/-};Brca2^{-/-} compared to mice bearing ID8Trp53^{-/-};Brca1^{-/-} tumors (p=0.0234). In contrast, a significant increase in immunosuppressive CD11b⁺/Ly6C⁺/Ly6G⁺ granulocytic MDSC was observed in ID8Trp53^{-/-};Brca1^{-/-} tumors (p<0.0001). More importantly, we observed significantly higher levels of CD8⁺ cytotoxic T cells in ID8Trp53^{-/-};Brca2^{-/-} tumors (p<0.0001) and further analysis showed that these cells express the activation markers IFN γ and STING. These immune differences correlated with the level of sialylation, which is significantly lower in ID8Trp53^{-/-};Brca2^{-/-} ovarian cancer cells (p=0.011). The immune and sialylation differences were also found in patients and transcriptomic analysis comparing BRCA2mt vs BRCA1mt patients showed immune-related biological processes to be differentially regulated such as antigen processing and presentation (0.0373), CD8⁺ T cell lineage commitment (0.0472), and common myeloid progenitor cell proliferation (0.0032). Finally, levels of sialyltransferases ST6GALNAC1 and ST6GALNAC4 was significantly downregulated in BRCA2mt ovarian tumors (6.9 fold and 2.7 fold, respectively compared to BRCA1mt tumors).

BRCA1mt and BRCA2mt ovarian tumors differentially regulate the tumor immune microenvironment and BRCA2mt tumors are more successful in maintaining immunesurveillance. The observed difference in the level of sialylation suggest the possible value of targeting the sialic acid/Siglec axis to improve anti-tumor immune response in ovarian cancer.

P047

SHORT-COURSE NEOADJUVANT INTRATUMORAL MBTA IMMUNOTHERAPY PREVENTS DISTANT METASTASES OF MURINE PHEOCHROMOCYTOMA

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Pheochromocytomas and paragangliomas (PHEO/PGL), neuroendocrine catecholamines-secreting tumors derived from neural crest cells, are considered as immunologically cold tumors due to their low amounts of neoantigens, somatic mutations, and lack of leukocyte infiltration. Our previous studies have demonstrated the efficacy of an intratumoral vaccination strategy, named MBTA therapy, in murine pheochromocytoma models. This therapy combines a phagocytosis ligand Mannan-BAM, three adjuvant TLR ligands, and immunostimulatory anti-CD40 antibody, resulting in complete tumor elimination in 63% of mice with subcutaneous PHEO, reduced distal metastases, and prolonged survival in metastatic PHEO models (PMID31083581, PMID34439097). However, such results have been accomplished by a treatment setup consisting of four pulses with three intratumoral injections every five days, which may be limiting in future clinical setting. Surgery remains an important treatment method for many solid tumors, including PHEO/PGL. Thus, we tested whether intratumoral MBTA therapy optimized for neoadjuvant setting and surgical resection could generate immunologic memory capable of preventing recurrence or distant metastases.

Mice bearing subcutaneous pheochromocytoma MTT tumors were treated with neoadjuvant intratumoral MBTA therapy or PBS with three to four pulses consisting of only one injection every five days and surgically resected two days after the last injection. Development of distal metastases from primary tumors was monitored.

Two independent experiments showed that neoadjuvant MBTA and surgical resection resulted in 100% protection of mice (n=25) against subsequent development of metastases from primary tumor even in three times bigger tumors (120.18 mm³) at the beginning of therapy compared to the PBS group (n=24, 40.50mm³) where 60% of mice developed distant metastases preferentially in livers, peritoneum, and lymph nodes. We also compared neoadjuvant PBS-injected and non-injected control and showed that intratumoral injection itself does not affect metastatic activity of murine subcutaneous PHEO. The retrospective comparison showed that metastatic spread was related to the size of tumors on at the day of surgery. Detailed analysis of re-transplantation plug consisting of MTT cell and Matrigel in MBTA and control mice, remaining free of metastasis after 120 days from surgery, showed that MBTA mice had increased and faster infiltration of T cells compared to the control group, confirming robust immune memory even after short-course MBTA therapy.

Collectively, neoadjuvant MBTA therapy followed by surgery generates immunologic memory capable of preventing distant metastases from primary subcutaneous PHEO tumors. These preclinical data support further investigation regarding the use of neoadjuvant MBTA therapy in patients with PHEO, and perhaps in other types of tumors, where immunotherapy is still limiting or distal recurrence after surgery is possible. This work has been done at the NICHD/NIH under grant number ZIAHD008735.

P048

THE LOCAL ADMINISTRATION OF POLY (I:C) AND RESIQUIMOD TRIGGER AN EFFECTIVE ANTITUMORAL RESPONSE IN MULTIPLE MODELS OF PANCREATIC CANCER

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Cancer immunotherapy has deeply improved the life expectancy of oncological patients and immune checkpoint blockade (ICB) is now a well-established approach in a wide range of malignancies. Nevertheless, ICB is poorly or not effective in some “difficult-to-treat” malignancies such as pancreatic ductal adenocarcinoma (PDAC) and novel compounds need to be implemented in the clinical practice to face ICB limitations. In a previous publication of our lab, we showed that poly (I:C) (pIC) and resiquimod (R848) are two immune modulators able to boost cancer killing when injected intratumorally in preclinical models of fibrosarcoma and lung cancer, acting mainly on Toll-like receptors (TLRs) expressed by tumor-associated macrophages (TAMs). The possibility to use this local intratumoral approach could be appealing also in PDAC, as this is characterized by a poorly immunogenic tumor microenvironment (TME), with an abundance of TAMs and fibrous tissue that dampens the arrival of medical compounds. Consequently, the goal of this project is to exploit the therapeutic potential of the local administration of pIC/R848 in preclinical models of PDAC and to characterize the immune modulation elicited by these drugs.

We generated two heterotopic (subcutaneous) models of pancreatic cancer by implanting in the flank of syngeneic wild-type mice either K8484 or Panc02 cell lines (hereby named K8484 s.c. and Panc02 s.c.), the first obtained from KPC mice and with a more epithelial phenotype while the second one with sarcomatous histology. We then generated an orthotopic model of PDAC by implanting K8484 luciferase-positive cells in the head of the pancreas of syngeneic mice. All these models were challenged with pIC/R848 either intratumorally (s.c. models) or intraperitoneally (orthotopic) and tumor growth was assessed over time.

In K8484 s.c. mice we observed a curative response following the intratumoral administration of 5 doses of pIC/R848 and a protection against the rechallenge with the same cell line twice, months after the first implant. On the other hand, only a partial response was observed in Panc02 s.c. using 7 doses of the drugs, suggesting a more aggressive behavior of this line. The use of pIC/R848 intraperitoneally in the orthotopic model of PDAC led to a potent reduction of tumor growth in all treated mice that was stable for more than 100 days in 90% of the animals. Mechanistically, the immune infiltrate of K8484 s.c. and Panc02 s.c. pIC/R848 treated mice highlighted a decrease of macrophages in the tumor, as well as an increase in mature CD8⁺ T cells and NK cells, suggesting a clear involvement of adaptive immunity. Indeed, depletion experiments in K8484 s.c. demonstrated that pIC/R848 lose their efficacy if CD4⁺ and CD8⁺ T cells are depleted. As CD4⁺ T cells are the main sources of IFN-gamma and as type I interferons lead to this cytokine's secretion in the TME, we implanted K8484 s.c. both in wild-type and Ifng KO mice, unable to produce IFN-gamma, in the presence or absence of an antibody blocking IFNAR-1, receptor for type I interferons. The interference with both types of IFNs results in little or no efficacy of the treatments, with a worse outcome when both types of IFNs are affected.

Taken together, the results of our study suggest that the intratumoral administration of pIC/R848 can be a valuable approach in PDAC. These two compounds elicit a type I and II IFN-driven antitumoral response and further investigations are ongoing to dissect the role that each cell type plays in producing and responding to IFNs in the TME.

P049

IN VITRO AND IN VIVO ANALYSIS OF MONOCYTE-DERIVED DCS DIFFERENTIATED IN THE PRESENCE OF LXR ANTAGONISTS FOR ANTITUMOR PURPOSES.

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Perturbation of cholesterol and lipid metabolism in solid tumors affects the antitumor immune response. Activation of the Liver X Receptors (LXR) by oxygenated derivatives of cholesterol (oxysterols), impacts on tumor infiltrating immune cells, dampening tumor immunity and immunotherapeutic strategies. We recently reported that the pharmacological manipulation of the oxysterol metabolism with a newly developed antagonist of the LXRs, named PFM037, delays tumor growth, promotes intratumoral monocyte-derived dendritic cell (mono-DC) differentiation, and synergizes with anti-PD-1 immunotherapy and adoptive T cell therapy. Preliminary data also suggested that PFM037 induces metabolic alterations in vivo. This behaviour discloses its potential antitumor role in human cancers.

Monocytes were purified from the bone marrow of C57BL/6 mice and cultured in vitro with M-CSF, IL-4 and TNF α , in the presence or in the absence of PFM037. After 48h, early mono-DC differentiation was evaluated by flow cytometry prior to perform gene expression analysis by RNA-seq and lipidomic analysis by liquid chromatography tandem mass spectrometry (LC-MS/MS). CyQUANT XTT Cell Viability assay was used to assess cell proliferation in vitro. For in vivo studies, C57BL/6 mice were intraperitoneally injected for 3 days with PFM037 and 5-Bromo-2' Deoxy uridine (BrDU). Some metabolic parameters (intracellular lipid content (BODIPY 493), lipid uptake (BODIPY 500 and CD36 expression) and peroxidation (BODIPY 581/591) were evaluated by flow cytometry. For antigen presentation assay, naïve CD8+ T cells, isolated from the spleen of Pmel-1 mice, were labelled with the carboxyfluorescein succinimidyl ester (CFSE) and then co-cultured with mono-DCs or cDCs loaded with the gp100 melanoma differentiation antigen. After 72h of coculture, CD8+ T-cell proliferation and differentiation were analysed by flow cytometry.

We investigated the effect of mono-DC differentiation in the presence of PFM037 and we observed in vitro proliferation of mono-DCs cultured in the presence of PFM037 as compared to vehicle. Moreover, when we treated mice for three consecutive days with PFM037, we observed the increased frequency of CD11b+CD11c+MHC-II+ cells co-expressing EdU in the bone marrow. Transcriptomic studies revealed the alteration of specific metabolic pathways, i.e., regulation of lipid localization, transport and storage, which were down-regulated in PFM037-differentiated cells compared to control. Gene expression analysis also highlighted *Abca1*, *Abcg1*, *Scd1* and *Scd2* among the top LXR-target genes down-modulated by PFM037 treatment during mono-DC differentiation.

Analysis of metabolic parameters showed that PFM037-treated mono-DCs down-regulated lipid uptake, a function paralleling the decreased expression of the CD36 marker. Then, we performed lipidomics and we observed the decrease of membrane lipids (phosphatidylethanolamines) and the increase of storage lipids (triacylglycerols and cholesterol esters) and messenger lipids, such as the lysophosphatidylethanolamines (LPE), in PFM037-treated mono-DCs. The above-reported transcriptomics and lipidomic modifications led us to undertake functional studies to evaluate the

antigen-presenting ability of PFM037-treated mono-DCs. Mono-DCs differentiated in the presence of PFM037 and loaded with gp100 peptide, stimulated the proliferation of CFSE-labelled Pmel-1 transgenic CD8⁺ T cells and promoted the differentiation of stem cell memory (CD8⁺CD44^{low}CD62L⁺) and central memory (CD8⁺CD44⁺CD62L⁺) antigen-specific T cells.

PFM037 induces mono-DC proliferation and affects their cholesterol and lipid composition. Functionally, the changes induced by PFM037 impact the immunomodulatory properties of these antigen-presenting cells during T cell activation and differentiation. These data support the need to further investigate the biologic properties of PFM037 to translate this compound in the clinical setting.

P050

MULTIDIMENSIONAL ANALYSIS OF PANCREATIC ADENOCARCINOMA TISSUE AND PANCREATIC JUICE REVEALS CLINICALLY RELEVANT SUBGROUPS

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Metabolic reprogramming is a hallmark feature of cancer characterizing pancreatic ductal adenocarcinoma (PDAC) molecular subtypes and capable to influence the tumor microenvironment (TME). A pancreatic juice (PJ) metabolic signature has been reported to be prognostic of exceptional survival for PDAC, as well as discriminating between PDAC and other pancreatic pathologies. Given the opportunity to use PJ as a source of biomarkers, the further characterization of such a signature by tumor transcriptome would help in identifying PDACs with peculiar tumor vulnerabilities.

We performed transcriptomic analysis of 26 PDAC samples previously grouped into 3 metabolic clusters (M_CL), according to PJ metabolic profile. We classified samples by molecular subtype and analyzed their transcriptional differences. Finally, we tested enrichment of immune signatures as predictive of outcome. Furthermore, we analyzed metabolic profile using untargeted metabolomic approach in an additional cohort (N=70) including PDAC and non-PDAC patients.

PJ metabolic profiling was associated with PDAC transcriptomic molecular subtypes ($p=0.004$). Enrichment analysis evidenced the upregulation of immune genes and pathways in PJ cluster M_CL1, which identified patients with long survival, compared to M_CL2, which was associated with a worse prognosis. This was consistent with differences observed between M_CLs, in terms of immune infiltrate assessed by multiple immunofluorescence and spatial analysis performed by digital pathology on tumors slides. Finally, the enrichment analysis of 39 immune signatures by xCell confirmed decreased immune signatures in M_CL2 compared to M_CL1. Stratification of patients according to the xCell enrichment score was associated with longer survival. Moreover metabolic profiles from PDAC and non-PDAC patients highlighted a list of molecules discriminating between these two categories.

PJ metabolic fingerprints reflect tumor molecular subtype as well as TME immune characteristics. Moreover PJ shows a strong diagnostic power in discriminating between pancreatic tumor and other pancreatic pathologies. Accordingly with these results PJ has a promising potential role as a source of biomarkers for personalized therapy.

P051

HOMEOSTATIC ACTIVATION OF ARYL HYDROCARBON RECEPTOR BY FOOD-DERIVED LIGANDS IS ESSENTIAL FOR THE EFFICACY OF IMMUNE CHECKPOINT BLOCKADE THERAPY

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Cancer immuno-surveillance and response to checkpoint blockade therapy can be affected by multiple environmental factors, including nutrition. However, the direct effects of individual nutrients on anti-tumoral immune responses remain poorly understood. Here we addressed the impact of dietary ligands of Aryl Hydrocarbon receptor (AhR), a transcription factor activated by indoles and tryptophan catabolites generated through food digestion and microbiota metabolism.

To this aim, we analyzed pre-clinical tumor models in mice fed on a diet naturally poor on AhR ligands, or the same diet enriched for Indole-3-carbinol, a phytonutrient cleaved by stomach enzymes into AhR agonists.

We found that diet-derived AhR ligands were essential for the efficacy of anti-PD1 therapy. By using mice harboring microbiota deficient for the production of AhR ligands, we further showed that microbiota-derived AhR ligands were dispensable for anti-PD1 therapy. Lack of dietary AhR ligands did not affect the baseline immune landscape, but impaired the infiltration of NK cells upon anti-PD1 treatment. Using a series of mice deficient for AhR in specific immune cells, we showed that T cells, but not NK cells or myeloid cells, are the direct cellular targets of dietary AhR ligands.

Our results indicate that homeostatic activation of AhR by dietary ligands plays a major role in immune responses to anti-PD1 therapy by favoring the crosstalk between tumor-infiltrating T cells and NK cells. Our work allows a better understanding of the role of dietary nutrients in anti-tumor immune responses and will have important implications for the design of dietary interventions for improving the efficacy of checkpoint blockade therapy.

P052

COMBINATION IMMUNOTHERAPY OVERCOMES IMMUNOSUPPRESSIVE FEATURES IN FIBROLAMELLAR CARCINOMA (FLC) TUMOR SLICE CULTURES

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Fibrolamellar carcinoma (FLC), a rare liver cancer affecting young patients without cirrhosis, has a poor prognosis and few systemic therapy options. Current evidence has uncovered numerous immunosuppressive features in the FLC tumor microenvironment (TME), including T cells that are sequestered away from carcinoma cells, dysfunctional, or inhibited. However, immune checkpoint inhibitor monotherapy has demonstrated limited efficacy in FLC. We hypothesized that overcoming multiple immunosuppressive mechanisms in the TME would be imperative to drive antitumor responses in FLC.

Patient-derived FLC and matched non-tumor liver (NTL) specimens were analyzed with flow cytometry to characterize baseline T cell phenotype. Tumor slice cultures (TSCs) from five patients were treated with IgG1 isotype control, anti-programmed cell death protein 1 antibody (aPD-1), AMD3100 (AMD, a small molecule inhibitor of CXCR4), and anti-cytotoxic T-lymphocyte-associated protein 4 antibody (aCTLA-4), alone or in combination. Slices were analyzed using immunohistochemistry (IHC), flow cytometry, and live microscopy to assess T cell migration and activation as well as carcinoma cell apoptosis.

Flow cytometric analysis of untreated FLC tumors revealed a distinct immune infiltrate compared to NTL: decreased CD45⁺ immune cell infiltration (16% vs 54% CD45⁺ of live cells, $p=0.01$); a lower CD8⁺ to CD4⁺ ratio (0.9 vs 1.8, $p<0.01$) and CD8⁺ to regulatory T cell ratio (8 vs 83, $p=0.1$); and, in some tumors, a higher percentage of PD-1⁺ and CTLA-4⁺ T cells. IHC analysis of treated slices revealed that aPD1+AMD and aPD1+aCTLA-4 significantly increased carcinoma cell apoptosis compared with control (aPD1+AMD 52% vs IgG1 32% cleaved caspase-3 (CC3)⁺ cells, $p<0.01$, $n=5$; aPD1+aCTLA-4 59% vs IgG1 40% CC3⁺ cells, $p=0.04$, $n=3$). Flow cytometry showed the highest T cell counts after aPD1+AMD compared with control (5531 vs 2362 CD3⁺, 3304 vs 1525 CD4⁺, 1952 vs 762 CD8⁺) and all treatment groups, and the highest percentage of CD69⁺ T cells after aPD1+aCTLA compared with control (65% vs 36% of CD4⁺, 78% vs 32% of CD8⁺) and all treatment groups. Time-lapse live microscopy exhibited a greater proportion of CD3⁺ T cells co-localized with apoptotic carcinoma cells after aPD1+AMD treatment compared with before treatment (44% CD3⁺ cells <20 μm from SR-FLICA⁺ CK7⁺ cells posttreatment vs 31% pretreatment, $p=0.02$).

Combination immunotherapy overcomes TME immunosuppression in FLC TSC. PD-1 combined with CXCR4 blockade increases T cell infiltration and effector function, and PD-1 combined with CTLA-4 blockade enhances T cell activation and effector function. These findings suggest that clinical trials testing available drugs targeting these pathways should be considered for patients with this challenging disease.

P053

ANDROGEN SIGNALING SHAPES THE SEXUAL DIFFERENCES OF SKIN IMMUNITY BY REGULATING ILC2 AND DENDRITIC CELLS

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The skin immune system mediates the skin health status, but the sex differences of the skin immune system are still unclear.

We investigated the sex differences in skin immunity in the context of commensal bacteria association and melanoma vaccination.

We found that female mice have a higher magnitude of adaptive immune responses during commensal bacteria association and melanoma vaccination. Correspondingly, female mice have a significantly higher level of skin dendritic cells (DCs), which play a fundamental role in commensal-induced adaptive immune responses. These sex differences were regulated by male sex hormones, that castration of males normalized the sex differences in the level of skin immune cells as well as the adaptive immune responses to bacteria. However, our scRNA-seq data reveals that the androgen receptor is not expressed in DCs, but highly expressed in skin ILC2. Females have a significantly higher level of ILC2 than males and ILC2 from females also have a more activating gene expression signature than ILC2 from males. In addition, females have a significantly higher level of ILC2-produced IL-13 and GM-CSF than male mice. In addition, our data reveal that ILC2-produced IL-13 and GM-CSF play a critical role to maintain the level of skin DCs, that IL-13/GM-CSF deficient mice have a remarkable decrease of skin DCs, and the sex differences of DCs are also largely impaired. Therefore, based on these findings, we suggest that androgen signaling negatively regulates the level of skin ILC2 and DCs, thereby shaping sex-specific skin immunity to commensals, pathogens, and/or other stimuli.

Collectively our work proposes a mechanism for the heightened immunity observed in females and has important implications for our understanding of tissue immunity in the context of infection and cancer.

P054

THERAPEUTIC HPV-16 MRNA-LPP VACCINE MEDIATE TUMOR CONTROL IN MICE

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Persistent infection with high-risk human papillomavirus can induce a variety of cancers including cervical cancer, head and neck squamous cell carcinoma, and anogenital and penile cancers. Of note, HPV16 is the most common strain that causes HPV-associated cancers among all HPV subtypes. LPP is a proprietary mRNA delivery platform, and Stemirna owns the rights to develop LPP-based mRNA therapeutics. We designed SW0128, a therapeutic vaccine composed of an antigen-encoding messenger RNA (mRNA) encapsulated in lipopolyplex (LPP) to fight against HPV16-associated cancers. The mRNA molecule encodes the E7 oncoprotein of HPV16 that serves as the tumor antigen. The aim of this study was to assess the therapeutic antitumor efficacies of SW0128.

Immunogenicity measurements

To evaluate the immunogenicity of HPV16 RNA-LPP, C57BL/6 mice were immunized subcutaneously with SW0128 at different dosages, and T cell responses were monitored by the mouse IFN- γ ELISPOT assay.

Tumor models and treatment

TC-1 tumor cell line and the related luciferase-transfected variant TC-1 luc cell line were obtained from T.C. Wu. Subcutaneous tumors were induced after the injection of 2×10^5 TC-1 tumor cells. For sublingual orthotopic tumor experiments, 5×10^4 TC-1 luc tumor cells were inoculated into the submucosa lining of the tongue. For intravaginal orthotopic tumor experiments, female mice were diestrus-synchronized via oral gavage administration of medroxyprogesterone acetate and inoculated 1×10^5 TC-1 luc tumor cells into the submucosa layer at the lateral vaginal wall of the animal 4 days later. For combination therapy experiments, 200 μ g of PD-L1 antibody or IgG2b isotype control were administered every three to four days.

C57 BL/6 mice were vaccinated with SW0128 weekly, and splenic T cells were isolated after each immunization to evaluate the T cell reactivity against vaccine encoded antigens in IFN- γ ELISPOT. Selective T cell responses against the HPV16 E7 could be observed clearly 6 days after priming immunization with even 0.3 μ g SW0128 and further enhanced upon the 2nd and the 3rd booster immunization, and even lasted stably at 3 months post priming immunization. These data fully reflect SW0128 stimulated specific T immune responses against HPV in mice are very sensitive, robust, and sustainable.

We next tested the anti-tumor efficacy of SW0128 in the TC-1 model. Complete tumor regression was observed in the majority of mice administered with 3 μ g, 10 μ g, 30 μ g SW0128 mRNA-LPP BIW or QW. All mice which achieved complete remission following the vaccination with previous 3 μ g of SW0128 remained tumor free for up to 4 months upon rechallenge with 5×10^5 TC-1 tumor cells. These data demonstrated the sustainable protective effects of SW0128 in tumor-free survival of mice. Subsequently, we tested synergize therapeutic effect of SW0128 with checkpoint-blockade anti-PD-L1 in TC-1 mouse tumor model. Tumor growth analysis showed the combination of SW0128 and aPD-L1 treatment resulted in enhanced synergistic inhibition of tumor growth compared to vaccine monotherapy alone.

In TC-1 luc sublingual tumor model, most of mice immunized with even a single dose of 10 μ g SW0128 mRNA-LPP showed a complete tumor remission within 14 days after immunization and remained tumor-free 2 months after immunization; whereas in TC-1 luc intravaginal tumor model, all mice demonstrated substantial regression of HPV16- positive tumors within 14 days after SW0128 immunization, however, tumor relapse rapidly in some mice 30 days after initial tumor inoculation,

and three doses of 10µg SW0128 mRNA-LPP vaccination exhibited the most potent protection effect.

In conclusion, our preclinical study demonstrates the potency of SW0128: SW0128 exhibits robust immunogenicity and can mediate substantial regression of HPV16–positive tumors. SW0128 immunization synergizes with checkpoint-blockade further enhances tumor remission rate and improves overall survival over vaccine only treatment.

P055

REWIRING INNATE AND ADAPTIVE IMMUNITY WITH TLR9 AGONIST TO TREAT OSTEOSARCOMA

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Osteosarcoma (OS) is the most common primary bone tumor in children and adolescent. Surgery and multidrug chemotherapy are the standard of treatment achieving 60-70% of event-free survival for localized disease at diagnosis. However, for metastatic disease, the prognosis is dismal. Exploiting immune system activation in the setting of such unfavorable mesenchymal tumors represents a new therapeutic challenge.

In immune competent OS mouse models bearing two contralateral lesions, we tested the efficacy of intralesional administration of a TLR9 agonist against the treated and not treated contralateral lesion evaluating abscopal effect. Multiparametric flow cytometry was used to evaluate changes of the tumor immune microenvironment. Experiments in immune-deficient mice allowed the investigation of the role of adaptive T cells in TLR9 agonist effects, while T cell receptor sequencing was used to assess the expansion of specific T cell clones.

TLR9 agonist strongly impaired the growth of locally-treated tumors and its therapeutic effect also extended to the contralateral, untreated lesion. Multiparametric flow cytometry showed conspicuous changes in the immune landscape of the OS immune microenvironment upon TLR9 engagement, involving a reduction in M2-like macrophages, paralleled by increased infiltration of dendritic cells and activated CD8 T cells in both lesions. Remarkably, CD8 T cells were needed for the induction of the abscopal effect, whereas they were not strictly necessary for halting the growth of the treated lesion. T cell receptor (TCR) sequencing of tumor infiltrating CD8 T cells showed the expansion of specific TCR clones in the treated tumors and, remarkably, their selected representation in the contralateral untreated lesions, providing the first evidence of the rewiring of tumor-associated T cell clonal architectures.

Overall these data indicate that the TLR9 agonist acts as an in situ anti-tumor vaccine, activating an innate immune response sufficient to suppress local tumor growth while inducing a systemic adaptive immunity with selective expansion of CD8 T cell clones, which are needed for the abscopal effect.

P056

A TRANSCRIPTOME-WIDE META-ANALYSIS REVEALS LACK OF CANCER-CELL INTRINSIC DETERMINANTS OF RESPONSE TO IMMUNE CHECKPOINT BLOCKADE

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Immune checkpoint blockade therapy (ICT) is an established class of immunotherapy that induces significant tumor shrinkage and long-term disease control in some cancer patients. However, most patients do not respond to ICT, and current biomarkers are inadequate for accurate patient stratification. Current FDA-approved biomarkers for ICT include PD-L1 expression, microsatellite instability (MSI), and high tumor mutation burden (TMB) of >10mutations/Mb. In addition, gene expression signatures of tumor immune phenotypes have been proposed to predict for ICT response. Despite these efforts, there is a lack of transcriptome-wide studies to explore potential biomarkers of ICT response conserved across cancer types. Here, we performed a meta-analysis on the complete transcriptomes of 1,486 tumors from ICT-treated and ICT responder enriched (MSI) cohorts. Using a novel computational approach, we dissected the tumor microenvironment to identify immune- and cancer-cell- intrinsic gene expression biomarkers of ICT response across cancer types.

To systematically explore transcriptome-wide biomarkers of ICT response, we obtained RNA sequencing data for 534 tumors, comprising 4 ICT-treated patient cohorts from 3 tumor types (gastric cancer, urothelial cancer and melanoma). Since about half of MSI tumors are expected to respond to ICT, as compared to <10% in microsatellite stable (MSS) tumors, we further supplemented the discovery cohort using large cohorts of treatment-naïve MSI/MSS tumors. We obtained the transcriptomic profiles of 952 tumors from 3 tumor types in TCGA with the highest frequency of MSI tumors (colorectal, gastric and endometrial cancers). In total, the discovery cohort comprised of 1,486 tumors from 5 cancer types.

We performed tumor transcriptome deconvolution to estimate the stromal- and cancer-cell expression of each gene in tumors from responders (MSI) and non-responders (MSS). Briefly, we first used inferred tumor purities based on transcriptomic and genomic data. For each ICT response group, we estimated gene expression levels in stromal and cancer cells using a non-negative least squares regression approach. Finally, we identified stroma- and cancer-cell-specific differentially expressed genes (DEGs) between ICT responders and non-responders using a permutation-based statistic. Based on the hypothesis that ICT responsive tumors share molecular features regardless of tumor type, we searched for stroma- and cancer-cell gene expression signatures associated with ICT response across distinct cohorts and tumor types.

We identified 59 stroma-specific ICT-response DEGs, which were strongly enriched in immune-related pathways and processes. Stromal expression of CXCL9, CXCL13, IFNG, and CD274 were among the top positive determinants of ICT response, consistent with current knowledge. Intriguingly, we also identified a set novel and potentially immune-suppressive genes that were negatively associated with ICT response. Using these conserved stromal-specific gene signatures, we developed a predictive model of ICT response that demonstrated improved accuracy over existing biomarkers in unseen patient cohorts and tumor types. Surprisingly, the unbiased transcriptome-wide analysis failed to identify cancer-cell intrinsic features of ICT response conserved across tumor types. These results suggest that cancer cells adopt diverse mechanisms of immune evasion, with no universal strategy adopted by cancer cells to avoid immune-mediated killing.

Our transcriptome-wide analyses identified conserved features of tumor stroma that robustly predicted ICT outcome across tumor types. Conversely, this analysis revealed a paucity of

conserved cancer-cell intrinsic determinants of ICT response, which has implications for the development of improved ICT diagnostics and treatments.

P057

ROLE OF THE IMMUNE MICROENVIRONMENT IN INDUCING LOCAL AND ABSCOPAL EFFECTS AFTER RADIOTHERAPY COMBINED WITH TLR AGONISTS

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In the last years immunotherapy has considerably improved cancer therapy. However, many patients still do not respond to it and new approaches are required. It is known that radiotherapy has immunomodulatory functions. Particularly, after radiotherapy exposure there is a local and systemic expansion of immunosuppressive cells but also a release of tumour antigens and an increase of cytosolic dsDNA that stimulate the immune response against the tumour. The activation of an anti-tumour immunity could also mediate the regression of untreated metastases, an effect called "abscopal". In order to encourage this response, combining radio- and immuno- therapy could be a promising therapeutic strategy.

In this project we are evaluating the efficacy of local administration of two different Toll-like receptors (TLRs) agonists and fractionated radiotherapy in murine models of different solid tumours. Tumour cells are injected in both flanks of mice but only one side is treated in order to evaluate the abscopal effect on the other one. Tumour growth of both treated and contralateral not-treated lesions is evaluated. At the end of the experiments, tumours are collected and processed in order to analyse the immune microenvironment through flow cytometry and immunohistochemistry.

Our observations showed that in a head and neck cancer model, TLR-9 and TLR-3 agonists reduced the growth of treated and of contralateral tumour lesions, without any additional effects with radiotherapy. Interestingly, in a poor immunogenic prostate cancer model, the TLR-9 agonist alone did not induce an abscopal effect, making the radiotherapy essential for the reduction of contralateral lesions. Additionally, in both models, despite similar readouts in terms of volume, the tumour histology and the microenvironment composition were different after radiotherapy, immunotherapy or their combination, in both treated and contralateral lesions. Therapeutic effects correlated with modifications of the tumour-infiltrating immune-milieu. Particularly, with the TLR agonists we observed an increase of CD8+ T cells in both models. Moreover, in the head and neck model, we also observed a reduction of myeloid-derived suppressor cells (MDSCs) together with an expansion of pro-inflammatory ones. Despite these encouraging results, in the head and neck model, TLR agonists induced also the expansion of some immune subsets favourable for the tumour growth. In particular, we observed an increased expression of PD-L1 in MDSCs with the TLR-9 agonist, and the activation of regulatory T cells with the TLR-3 agonist.

These preliminary results support the hypothesis that combining radiotherapy with TLR-agonists could improve the immune response against the tumour, inducing an abscopal effect that could lead to the regression of contralateral lesions, at least in the prostate cancer model. Further studies are required to validate the results and to investigate on the molecular mechanisms behind the observed therapeutic effects. Moreover, combinations with immune checkpoint inhibitors need to be considered to further improve the treatment efficacy.

P058

EPITOPE-ENGINEERED HSPCS TO ENABLE COMBINATORIAL CAR-T CELL IMMUNOTHERAPY FOR ACUTE MYELOID LEUKEMIA

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Acute myeloid leukemia (AML) is still associated with an unfavorable outcome for >50% of patients. Whereas novel immunotherapies, such as CD19 CAR-T cells, demonstrated striking efficacy when targeting a dispensable lineage antigen (Ag) the same approach cannot be exploited for AML, due to lack of safely actionable leukemia-restricted Ags. AML targets are shared with healthy progenitors or mature myeloid cells, leading to on-target/off-tumor toxicity and impairment of hematopoietic reconstitution. To address this issue, we reasoned that precise modification of the targeted epitopes in donor HSPC used in hematopoietic stem cell transplantation (HSCT) would result in loss of recognition by CAR/mAbs, without affecting protein expression, regulation and function. Differently from the KO of dispensable markers, epitope-editing allows targeting genes essential for leukemia survival regardless of expression or functional role in HSPC, minimizing the risk of Ag-negative tumor escape.

We selected the cytokine receptors FLT3, CD123 and KIT, which are expressed in >85% of AML cases. By transposon-based library screening, we identified amino-acid substitutions in the receptors extracellular-domains (ECD) that avoid detection by therapeutic Abs. We next validated the functionality of mutated receptors (ligand affinity, western-blot, proliferation response, RNAseq, phospho-protein MS, functional assays on differentiated cells) and their capacity to avoid on-target killing (mAb affinity, CAR-T cell co-culture). To design a genome editing strategy, we screened gRNAs and CRISPR-base editors to introduce these mutations and optimized them for CD34 HSPC editing. Finally, we exploited advanced in vivo models with co-engraftment of healthy HSPCs and patient derived AML xenografts (PDX) and CAR-T cell treatment to assess the selective elimination of leukemia and protection of healthy hematopoietic cells.

Cells expressing epitope variants were resistant to in vitro CAR-T killing and did not induce CAR activation. Electroporation of ABE8e mRNA and gRNAs into human CD34+ HSPCs achieved up to 90%, 85% and 75% editing efficiency on FLT3, KIT and CD123, respectively. After xenotransplant into NBSGW mice, FLT3, KIT or CD123 epitope-edited HSPC sustained long-term multilineage hematopoiesis, which was similar to AAVS1 controls also in II transplantation. Upon treatment with FLT3-CAR-T, we observed sparing of CD34+38- HSPCs, granulo-mono progenitors and B cell subsets in mice engrafted with FLT3-edited HSPCs compared to AAVS1 controls. Similarly, treatment with CD123-CAR-T showed protection of epitope-edited myeloid lineages, including granulocytes and DCs. At the same time, co-engrafted PDXs were eradicated by FLT3- or CD123-CAR-T. Despite these promising results, due to AML intra-tumoral heterogeneity and plasticity, targeting >1 surface Ag might be required to eliminate the totality of leukemia cells, including leukemia stem cells. To this end, we optimized high-efficiency multiplex epitope-editing to enable concomitant targeting of multiple Ags by CAR-T cells without overlapping toxicities on healthy hematopoiesis. We confirmed selective resistance of dual FLT3+CD123 epitope-edited HSPCs and the superior efficacy of dual-target CAR-T therapy in mice co-engrafted with a PDX that was incompletely eradicated by FLT3-CAR-T alone. Additionally, our approach was able to protect HSPCs when FLT3 CARs were combined with the clinically relevant FLT3 tyrosine kinase inhibitor Crenolanib - which promotes FLT3 surface expression - while controls showed additional toxicity.

In conclusion, transplantation of epitope engineered HSPCs endowed with selective resistance to multi-specific CAR-T-cells or Abs is a novel approach to enable more effective and safer immunotherapies for highly heterogenous tumors such as AML.

P059

REGULATORY ROLE OF B CELLS IN INTRATUMORAL MBTA IMMUNOTHERAPY OF MURINE PHEOCHROMOCYTOMA

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Intratumoral immunotherapy based on the combination of phagocytosis-promoting ligand mannan-BAM, three TLR ligands, and immunostimulatory anti-CD40 antibody (collectively called MBTA therapy) is a promising tool in the elimination of various preclinical murine tumor models, including pheochromocytoma (PHEO). PHEO is a rare neuroendocrine tumor derived from the chromaffin cells of the adrenal medulla. Even though PHEOs are considered immunologically cold tumors, MBTA immunotherapy has been shown to eliminate over 60% of PHEO tumors in mice. In our previous study, we performed a comprehensive analysis of immune cell trafficking during MBTA treatment. The results showed significantly increased infiltration of B cells (CD45⁺ CD19⁺) in intratumorally injected tumors and distant non-injected tumor lesions, pointing to its possible role in the therapy efficacy. Thus, we further focused on the role of B cells during intratumoral MBTA therapy of murine PHEO.

C57BL/6J mice and muMT B cell-compromised mice (B6.129S2-Ighmtm1Cgn/J) were subcutaneously injected with PHEO MTT cells and then intratumorally treated with MBTA therapy. Collected plasma and tumor tissues were used for further analysis by qRT-PCR and ELISA. This work has been done at the NICHD/NIH under grant number ZIAHD008735.

Interestingly, muMT had a higher rate of tumor incidence (96%) and faster tumor growth than C57BL/6J mice (60%) after MTT cell transplantation, pointing to the role of B cells in murine PHEO development. After the development of tumors, MBTA therapy was injected intratumorally in 3 daily injections every five days. Unexpectedly, almost 60% of muMT mice (7/12) treated with MBTA developed strong reactions against therapy in the second treatment cycle and consequently died or had to be euthanized due to poor conditions within 48 h from the injection. Therefore, we carefully analyzed proinflammatory cytokines in plasma and showed significantly higher levels of IL-6 and TNF alpha in both MBTA-treated groups, one of the main cytokines involved in a cytokine storm. Interestingly, cytokine levels in MBTA-treated groups were significantly higher in muMT compared to C57BL/6J mice 24 h after MBTA injection. However, 48 h after MBTA therapy, IL-6 showed significantly lower levels in muMT mice than C57BL6/J, while TNF alpha level was still significantly increased. To confirm the negative role of excessed TNF alpha release in muMT mice, we conducted the experiment with TNF alpha blockade in both groups, which led to the rescue of all muMT mice from death without any negative impact on tumor reduction during the MBTA therapy. Moreover, tissue analysis showed decreased T cell (CD3, CD4, CD8) and macrophage (CD68) infiltration in tumor samples in muMT mice, suggesting the important role of B cells in immune cell trafficking and regulation of TNF alpha.

Collectively, our data indicate that B cells display a regulatory role in the immune system response to therapy in murine PHEO. Thus, B cell's response to MBTA therapy and possibly other immunotherapies should be further studied since it may be an important part for balancing the cancer treatment efficacy and safety.

P060

IMMUNOREGULATORY ROLE OF EXTRACELLULAR VESICLES IN ADVANCED NON SMALL CELL LUNG CANCER

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Programmed death ligand-1 (PD-L1) expression on tumor cells is the only predictive biomarker used in clinical practice for Immune Checkpoint Inhibitors (ICIs) alone or in combination with chemotherapy in NSCLC. Extracellular vesicles (EVs) are lipid bilayered particles described as biomarkers for cancer progression and as modulators of anticancer immune response. We previously described that high percentage of CD81+ EVs in patients with low PD-L1 expression treated with ICIs therapy alone correlated with a better Overall Survival (OS) and outcome. Here we aimed to evaluate EVs of advanced NSCLC patients with low PD-L1 expression in order to find biomarkers for combinational therapy (chemotherapy plus ICIs) and to investigate their role as regulator of anti-tumor immune response.

Plasma-EVs were isolated using ultracentrifuge from 20 Responder (R) and 20 Non Responder (NR) advanced NSCLC patients treated with ICI plus chemotherapy. The response to therapy was evaluated based on the RECIST 1.1 criteria. EVs characterization was performed following MISEV guidelines. Tetraspanins expression was assessed via super resolution microscopy. MiRNA expression within EVs was evaluated by using miRCURY LNA miRNA Focus Panel. Proteomics analysis on EVs was done by Tymora Analytical. EVs subpopulations were sorted through FACS or immuno capture beads. T cells were isolated from healthy donor PBMC and stimulated with CD3/CD28 beads in presence of different EV subpopulations and analyzed by flow cytometry.

R- and NR-EVs did not differ in terms of size and concentration. WB analysis confirmed the expression of CD9, CD81 and Tsg101 and the presence of high density lipoprotein contaminants. Despite we did not observe differences in CD9 expression in WB analysis, super resolution microscopy revealed a slight increase in CD9+ EVs in NR samples. Class comparison analysis found 9 miRNAs differentially expressed between R and NR patients. Among them, hsa-mir-142-3p was the most expressed in R patients. Preliminary analysis revealed that miR-142-3p is expressed by T cells during the different phases of their activation. Proteomics analysis at baseline revealed high levels of CD9, integrins and adhesion molecules and we found 13 proteins upregulated in R-EVs and other 13 proteins in NR-EVs. Interestingly, 113 proteins inside EVs changed after combinational therapy. Starting from bulk plasma EVs samples, we successfully isolated different EVs subpopulations based on their expression of CD9 and CD45. In functional experiments, CD9-CD45-EVs were able to affect both the percentage and Granzyme B production of CD4+ and CD8+ T cells. No differences were observed in terms of T cells proliferation and immune checkpoint inhibitor markers expression.

Our preliminary results showed that R- and NR-EVs differ in terms of 9 different miRNAs and that potentially could be used as signature to discriminate the two groups. Proteomic analysis highlighted differences between R- and NR-derived at baseline in protein content, and that combinational therapy changed the protein profiles within EVs. Further studies are needed to elucidate the role of EV subpopulations in immune response modulation in lung cancer.

P061

DENDRITIC CELL RECRUITMENT TO THE TUMOUR MICROENVIRONMENT ENHANCES CHECKPOINT BLOCKADE THERAPY

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Immunotherapy has shown remarkable results in the treatment of certain cancers; however only a minority of patients currently respond. Identifying classes of tumour microenvironments (TMEs) that respond to immunotherapy will allow us to develop approaches to ensure more patients can respond. One key cell type in the TME is conventional dendritic cells (cDC) that have vital roles in priming and restimulating anti-tumour T-cells in the lymph node and the TME, respectively. In several cancer types being rich in type 1 cDC (cDC1s) predicts responsiveness to immunotherapy and experimentally increasing the number or activation status of cDC1s has also been shown to improve tumour immune control. cDC2, on the other hand, have more ambiguous roles during tumour progression. Thus, it is critical that we understand the mechanisms driving recruitment of cDC precursors, pre-cDC, from the blood to the TME to both understand the heterogeneity observed in terms of cDC infiltration to the TME and to potentially promote the seeding of pre-cDCs and to improve anti-tumour T-cell activation and function. Therefore, we decided to investigate the role of chemokines and their receptors in the migration of pre-cDCs to the TME.

We made use of publicly available scRNAseq, bulk RNAseq, microarray and our own scRNAseq data to identify the chemokine receptors expressed on pre-cDCs and confirmed the receptor expression using flow cytometry. To model the role of chemokines in recruiting pre-cDCs to the TME, we overexpressed chemokines in tumour cell lines and analysed the cDC compartment of their TME and determined the proliferation and activation status of T-cells in the TME. Finally, we investigated if increasing chemokine signals in the TME could be used as a therapeutic to augment checkpoint blockade therapy, we treated mice with B16zsGminOVA tumours with anti-PD-1 therapy and measured tumour growth and immune responses within the TME.

Pre-cDCs expressed a range of chemokine receptors and their expression differed between pre-cDC1 and pre-cDC2 suggesting the potential for cell type specific signals driving their recruitment. There was also significant heterogeneity within the subsets. This was confirmed by flow cytometry using both antibody staining and a reporter mouse line which allows tracking of iCCR expression. This analysis resulted in a panel of chemokine receptors with the potential to drive pre-cDC migration. The ligands for these receptors were transduced into an EL4 thymoma line and cells were implanted into mice and the composition of the TME examined at endpoint by flow cytometry. As predicted by the RNAseq analysis, several chemokines led to recruitment of pre-cDC to the TME and, indeed, some had differential impacts on pre-cDC subsets. Three chemokines specifically drove the greatest recruitment of pre-cDC1 and these were selected for further testing. A modified B16F10 line expressing the bright fluorescent protein ZsGreen and the minimal OTI and OTII recognition peptides from ovalbumin was transduced to overexpress these candidate chemokines. These were implanted subcutaneously and the composition of the TME, as well as the quality and quantity of the anti-tumour T cell response, was characterized by flow cytometry. As before these chemokines led to increased recruitment of cDC1 into the TME and this correlated with improved anti-tumour T cell priming. Finally, we showed that this translated into improved responsiveness to immune checkpoint blockade.

To conclude, we have developed a novel screen to identify the chemokine signals involved in recruiting immune cells to the TME. Using this screen and gene expression analysis we have identified the key signals that can recruit preDCs to the TME where they develop into cDCs and induce the priming of anti-tumour T-cells. By identifying the key signals involved in recruiting cDCs

to the TME, we propose that these signals can be used therapeutically to augment checkpoint blockade therapy.

P062

COMBINED ADMINISTRATION OF ENZALUTAMIDE AND EZH2 INHIBITORS RESTRAINS THE GROWTH OF CASTRATION RESISTANT PROSTATE CANCER BY ENHANCING ANTITUMOR T CELL RESPONSE

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Castration resistant prostate cancer (CRPC) is a fatal disease. Androgen receptor pathway inhibitors, like enzalutamide, are initially effective but resistance eventually occurs, often associated to the emergence of aggressive neuroendocrine variants (NEPC). Even immunotherapy induced limited results in prostate cancer, governed by an immunosuppressive microenvironment.

We propose that new approaches to revert castration resistance and to turn the immune milieu from “cold” to “hot” would be synergic against CRPC. To this aim, we investigated in preclinical models the effects of a combination between enzalutamide and the drug GSK-126, which inhibits the epigenetic modulator EZH2.

We show that enzalutamide and GSK-126 can synergize to restrain the growth of CRPC in vitro and in vivo. Moreover, this therapeutic combination efficiently reduced NEPC differentiation, in both subcutaneous and autochthonous in vivo models, increasing the rate of cured mice with regressed lesions.

The antitumor efficacy of the enzalutamide and GSK-126 combination observed in immunocompetent mice bearing subcutaneous syngeneic CRPC tumors was lost in immunodeficient mice. Furthermore, in the TRAMP spontaneous model this combination treatment restored cytotoxic activity and IFN γ production of tumor-specific CD8⁺ T cells, otherwise tolerant, and increased IL-17 production in CD4⁺ T cells. The two drugs did not modulate T cell activity in vitro, suggesting the importance of microenvironment accomplices in triggering these effects.

These results promote the combined use of enzalutamide and GSK-126 to restrain CRPC growth and NEPC differentiation, and, simultaneously, to awake antitumor T cell response, opening new possibilities for immunotherapy in prostate cancer.

P063

3-DIMENSIONAL HUMAN VASCULARIZED PANCREATIC MICROTUMORS TO STUDY IMMUNOMODULATION

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Pancreatic ductal adenocarcinoma (PDAC) is a highly aggressive form of cancer that arises from the cells lining the pancreatic duct. Despite advancements in cancer research and treatment, PDAC remains challenging, and its prognosis remains poor. One of the primary reasons for the limited progress in PDAC treatment is the paucity of suitable tumor models for preclinical screening that accurately mimic the complex tumor microenvironment (TME). The TME plays a critical role in determining the response to anti-tumor therapies. It comprises various cellular components, including immune and stromal cells, extracellular matrix components, and signaling molecules. Consequently, an effective treatment strategy must account for the heterogeneity and complexity of the TME. Most traditional preclinical models, such as cell lines or patient-derived xenografts, fail to represent the cellular interplay within the PDAC TME accurately. These models often lack immune cells or have limited stromal components, which are essential in understanding the dynamics of tumor development and treatment response.

Therefore, we generated a microfluidic-based 3-dimensional (3D) culture of human vascularized PDAC microtumors that incorporate immune cells and stromal cells, providing a more accurate representation of the TME. We characterized the heterocellular microtumors' composition, cytokine secretion, monocyte differentiation, transcriptional profiles, and vascularization. The microtumors were formed using pancreatic cancer cells (PANC-1), stellate cells (HPaSteC), endothelial cells (HUVEC), and monocytes, with a control group using non-cancer cells (HPNE).

The 4-culture microtumors containing all four cell types exhibited higher collagen I expression and increased HIF-1 α expression in their core compared to any other microtumor with lower cell heterogeneity. The complexity of cytokine patterns correlated with cellular heterogeneity of the tested microtumors, and the 4-culture microtumors showed a cytokine signature similar to the one observed in the plasma of PDAC patients experiencing poor prognosis. Only within the 4-culture microtumors, monocytes differentiated into tumor-supporting M2-like tumor-associated macrophages (TAMs). Vascularization of the microtumors within OrganiX microfluidic inserts showed that the 4-culture microtumors had an impact on the vessel formation leading to an increased peripheral blood mononuclear cell (PBMC) infiltration when compared to monoculture, suggesting the promotion of angiogenesis and leukocyte chemotaxis. The transcriptional analysis of the cancer cells in the microtumors with different compositions revealed distinct gene expression profiles. Strikingly, heterogeneity of epithelial cells and enrichment of genes related to negative regulation of cell death, migration, and stress response were observed in 4-culture microtumors rather than monoculture.

These results demonstrated that our 3D PDAC microtumor model enables the functional study of immune responses and therapeutic efficacy within a more physiologically relevant TME to understand better the mechanisms underlying PDAC response to immunotherapies and targeted therapies. Specifically, we aim to characterize the crosstalk among fibroblasts, TAMs, and T/NK cells in our 3D PDAC microtumour model derived by the interleukin (IL)-17A depletion. Our preliminary data in vivo suggested that IL-17A plays a role in modulating CAF pro-tumorigenic function by improving the recruitment of CD8⁺ T cells. In conclusion, our 3D PDAC microtumor models have the potential to improve our understanding of PDAC biology and facilitate the development of treatment strategies that consider the intricate interplay between cancer cells and the TME.

P064

MAST CELLS PROMOTE BREAST CANCER AGGRESSIVENESS THROUGH THE INDUCTION OF STEM-LIKE TRAITS

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Breast cancer (BC) is the most common type of tumor in women and, despite the great advance in research, its high heterogeneity makes the prediction of patient outcome difficult. Cancer aggressiveness is determined not only by cell-intrinsic features, but also by the crosstalk with the microenvironment. To understand how this crosstalk can modify tumor aggressiveness, we particularly focused on mast cells (MCs). MCs are immune cells well-known for their role in allergic reactions. However, the high plasticity of these cells make them relevant also in tumor context, although their role is not well characterized. Indeed, in dependence on the crosstalk that is established in the tumor microenvironment, MCs can release a number of cytokines with a pro- or anti-tumor effect. This heterogeneous behavior is reflected also in the different BC subtypes. Indeed, MCs are more infiltrated in luminal and HER2+ BC where they are also associated with worse prognosis. We hypothesized that the detrimental effect of MCs in BC can be due to induction of stem-like traits in tumor cells.

Stem-like features were evaluated both in vitro and in vivo. We used human and mouse mammary cancer cell lines to test the capacity of MCs to increase the expression of genes involved in stemness and to induce the formation of spheres, since only cells with stem-like characteristics are able to propagate as spheroid bodies. Moreover, in vivo experiments performed with C57BL/6-Kitw-sh/w-sh (Wsh) mice, which lack MCs, were instrumental to confirm the role of MCs in promoting breast cancer aggressiveness.

We found that the medium conditioned by MCs was sufficient to increase the capacity of our cell lines to form spheres and to stimulate the expression of stem-related genes. Moreover, we confirmed the role of MCs in promoting cancer initiation in Wsh mice. In fact, the co-injection of MCs with limiting dilutions of BC cells increased the engraftment rate. Since we have previously demonstrated that MCs can increase the expression of estrogen receptor (ER) in adjacent BC cells, we hypothesized that this effect could be due to the activity of heparanase (HPSE), an enzyme responsible of both ER upregulation and stemness induction. To determine the effect of HPSE in the tumor-initiating properties induced by MCs, we treated MCs with the HPSE inhibitor before collecting the conditioned medium. This medium was then employed in a sphere formation assay where the inhibition of HPSE reduced the number of spheres compared to control conditioned medium. HPSE inhibition impaired also the upregulation of ER induced by MCs. Since MC HPSE is involved in both ER and stemness induction, we hypothesize that ER may contribute to this phenotypic change. So, we silenced it and saw that the downregulation of ER caused a reduction in the formation of spheres. In the attempt to search a link between HPSE and ER upregulation, we found that MUC1 may be involved in this mechanism. MUC1 is a mucin often overexpressed in cancer and that has been shown to stabilize ER. Moreover, MUC1 is stimulated by a proinflammatory microenvironment. We found that the medium conditioned by MCs increased the expression of MUC1 while the treatment of MCs with the HPSE inhibitor reduced this effect. Then, we asked if the expression of ER induced by MCs may pass through the stimulation of MUC1. So, we silenced this mucin and saw that the effect of MCs in ER expression was impaired. The same effect was observed also in the mammosphere assay where the silencing of MUC1 reduced the number of spheres formed in the presence of MC conditioned medium.

Our findings support the hypothesis that MCs promote the formation of stem-like cancer cells in an estrogen receptor-dependent manner. Further work is necessary to understand if targeting MCs may represent a novel therapeutic strategy for BC treatment.

P065

ADDRESSING THE ROLE OF CD103+CD11b+ DENDRITIC CELLS IN PANCREATIC CANCER LUNG METASTASIS

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Conventional dendritic cells (cDCs) are key actors in the microenvironment of pancreatic ductal adenocarcinoma (PDAC). cDCs consist of two distinct subsets, namely cDC1 and cDC2. cDC1s are characterized by CD103 expression and are known for their ability to cross-present antigens to CD8+ T cells. cDC2s express CD11b and are involved in activating CD4+ T cells. Reports have showed that in mice the lack of cDC1 is associated with scarce CD8+ T cell responses to PDAC neoantigens, while less characterized tolerogenic DC populations have been described to antagonize anti-tumour immunity. Given the lung's significant role in immune regulation and its involvement in PDAC metastasis, there is a growing interest in investigating the identity and function of dendritic cells in the metastatic lung microenvironment.

To study the microenvironment of PDAC lung metastases, we transplanted cells derived from the primary tumour of the gold standard KrasG12D/+; Trp53R172H/+; Pdx1-Cre (KPC) mouse model intravenously into naïve recipients. At day 21 post-injection, resultant lung tumours were studied using flow cytometry and spectral multiplexed 3D confocal imaging to explore the immunological infiltrate and its localisation.

Immune landscaping revealed increased influx of dendritic cells, which we confirmed through large scale imaging of the lung, and T regulatory cells. Surprisingly, we observed a population of CD103+CD11b+ cDCs, which are not normally abundant in the lungs but restricted to the small intestine. SIRP α , CD86 and PDL1 staining suggested that this population is cDC2-related, activated, and immunosuppressive. Additionally, CD103+CD11b+ DCs could be found in tumour-draining lymph nodes, indicating migration of this population. We repeated this analysis in IRF4 KO mice and found they were selectively deficient in CD103+CD11b+ DCs and, importantly, tumour bearing IRF4 KO displayed a survival advantage compared to wild-type mice. In these knockout mice we found fewer Tregs, suggesting a possible role for CD103+CD11b+ DCs in the development of suppressive T cells in PDAC metastases. Finally, in vivo inhibition of ALK5 resulted in a specific decreased of CD103+CD11b+ cDCs, indicating a possible role for TGF β in the development of this cDC subset.

In summary, our findings demonstrate an increase in CD103+CD11b+ cDCs in KPC lung metastases. These cDCs exhibit an activated and immunosuppressive phenotype, with the ability to migrate to lymph nodes. Interestingly, tumour-bearing mice lacking IRF4 show a reduction in this cDCs subset and display a survival advantage compared to wild-type controls. Additionally, our results suggest a potential role for TGF β signalling in the development of this specific cDC subset. These findings contribute to our understanding of the complex interactions between cDCs, tumour microenvironment, and immune responses, offering potential avenues for targeted therapeutic interventions in cancer.

P066

INTRACELLULAR OSTEOPONTIN CONTROLS THE RELEASE OF CYTOKINES BY MAST CELLS TO RESTRAIN NEUROENDOCRINE PROSTATE CANCER

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Fatal neuroendocrine prostate cancer (NEPC) often emerges in patients relapsing after hormone therapies. Besides, de novo NEPC can rarely occur in treatment-naïve patients. Treatment-related and de-novo NEPC have different genomic alterations but share a common transcriptional profile. The processes which guide NEPC onset and development are still poorly characterized, making challenging the identification of predictive/prognostic biomarkers and of effective therapies. Tumor cell plasticity known to drive the trans-differentiation from adenocarcinoma to NEPC, can be influenced also by tumor microenvironment (TME)-derived stimuli.

TRAMP mice on C57BL/6 background spontaneously develop prostate adenocarcinoma (90% of mice) or de-novo NEPC (10% of mice). TRAMP mice rendered genetically deficient for MCs (KitWsh-TRAMP), were adoptively transferred with WT or OPN^{-/-} bone marrow-derived MCs (BMMCs). We tested OPN expression by immunofluorescence in mouse and human prostates and its release in MCs by ELISA. We cultured prostate adenocarcinoma or NEPC cell lines with MCs, measuring proliferation with trypan blue count. Also, we evaluated the cleaved-caspase-3 by Western blot analysis. Finally, we interrogated murine NEPC datasets for translation of results.

Investigating the TME, we recently found that mast cells (MCs) accumulate within hormone-sensitive prostate cancer favoring its growth, whereas are excluded by de-novo NEPC both in patients and in the transgenic TRAMP spontaneous mouse model. TRAMP mice backcrossed with MC-deficient KitWsh mice showed increased frequency of de-novo NEPC. The frequency of de-novo NEPC similarly raised also in TRAMP mice deficient for the matricellular protein osteopontin (OPN). Reconstituting KitWsh-TRAMP mice with wild type, but not with OPN-deficient MCs, lowered the frequency of NEPC to that of untreated TRAMP mice. We found that MCs stain positive for OPN in human and murine tumor sections and in vitro cultures, but release a tiny amount of OPN in supernatants if compared to NEPC cells. Notably, OPN has both secreted (sOPN) and intracellular (iOPN) forms; the latter can bind to MyD88 and regulate the signaling downstream toll-like receptors (TLRs) toward the production of cytokines. In vitro, wild type, but not OPN^{-/-} or MyD88^{-/-} MCs, inhibited the growth of NEPC cells through the production of a cytotoxic cytokine which induces apoptosis, verified by detection of activated caspase-3. Also, in silico analyses showed that genes related to inflammatory response and TLRs signaling is down regulated in human and murine NEPC. Looking for candidate ligands able to trigger the TLR pathway, we discovered that the protein SDC1 is over-expressed by NEPC cells.

Our data suggest that NEPC-derived SDC1 is the ligand prompt to activate the TLRs/MyD88/iOPN axis in MCs causing the release of cytotoxic cytokine restraining NEPC growth. This pathway could be exploited to set up innovative MC-based therapies efficiently improving NEPC targeting.

P067

STUDY OF SERUM AMYLOID A PROTEINS IN THE TUMOR MICROENVIRONMENT

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Serum amyloid A (SAA) proteins are acute phase response (APR) apolipoproteins involved in many inflammatory processes, regulating both innate and adaptive immunity. Emerging evidence suggests that SAAs participate in tumor biology via T-cell dependent and independent mechanisms. Several groups have reported pro-tumorigenic effects of SAA proteins produced locally by different cell types associated with tumors, including cancer-associated fibroblasts (CAFs), tumor-associated macrophages (TAMs) and tumor cells themselves. However, whether SAA play a protective or deleterious role in tumor growth is context-dependent, likely due to different anatomic locations, isoforms, or cellular sources of production in the local tumor microenvironment and draining lymph nodes. Additionally, how locally-produced SAA affect the immune cell recruitment and function to the tumor microenvironment is unclear. To gain a comprehensive understanding of SAA protein's regulation in tumor immune landscape and their interactions with the T cells, we generated Saa1/2 and Saa3 double-reporter mice to find the cellular sources and kinetics of SAA proteins expression in different tumor micro-environments.

Transgenic mice

Saa1/2-P2A-tdTomato and Saa3-T2A-mTurquoise2 constructs are targeted in-frame into the endogenous loci of Saa2 and Saa3 coding sequences in-trans before the stop codon in C57/BJ ES cells. Single clones are genotyped with Southern blotting and junction PCR with Sanger sequencing. ES cells are injected into B6/Albino blastocysts and obtain chimeras. Germ-line transmitting male chimeras and bred to B6/Albino females for F1 reporter mice of the following genotype: Saa1/2-P2A-tdTomato/+, Saa3-T2A-mTurquoise2, and Saa1/2-P2A-tdTomato/Saa3-T2A-mTurquoise2.

Characterization of Saa dual-reporter mice

Mice are i.p injected with 50ug LPS and sacrificed the next day. tdTomato and mTurquoise signals are measured using multiphoton microscope and flow cytometry across various organs, including the liver.

Tumor models

MC38 colon cancer cells, B16-F10 melanoma cells, KPM lung cancer cells are cultured according to manufacturer's condition. 250k cells and implanted subcutaneously into 8-10 weeks-old mice. For experiments using immune-checkpoint blockade, Tumor growth are monitored daily or at 10-14 days endpoint for volume and weight. For AOM/DSS colitis-associated colon cancer, mice are injected with 10mg/kg AOM and treated with 2% DSS water for a week and with 2-week intervals for 64 days.

In previous experiments, different tumor types are grown in SAA sufficient and deficient conditions. When treated with check-point blockade immunotherapy, different tumor types divergent responses. CD4+ T cells infiltrating B16 melanoma produce IL-17A and IFN γ in wild type hosts, while SAA deficient hosts abolished T cell IL-17A production in the tumor, indicating SAA proteins regulates the Th17 differentiation program of tumor-infiltrating T cells. Using acute inflammatory or infectious model on the reporter mice, extrahepatic SAA proteins are produced mainly by myeloid cells and stromal cells, with SAA3 being the main isoform. SAA3 positive myeloid cells and stromal cells can be detected in lymph nodes using Citrobacter Rodentium infection model and LPS acute inflammation.

SAA knockout exhibit divergent phenotypes in tumor growth dependent on tumor type, which could be mediated by altered T cell effector functions within the tumor microenvironment. This could be

due to different priming in the tumor draining lymph nodes. Using the reporter mice, we found that SAA proteins are mainly produced by myeloid cells in inflammatory conditions both in the tissue and lymph nodes. It will be informative to examine how SAA-producing myeloid cells could have altered-antigen presenting abilities that potentially affect T cell differentiation in the draining lymph nodes of different tumor types.

P068

REPEATED PERIPHERAL INFUSIONS OF ANTI-EGFRVIII CAR T CELLS IN COMBINATION WITH PEMBROLIZUMAB REMODELS THE TUMOR MICROENVIRONMENT IN DE NOVO GLIOBLASTOMA

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Treatment efficacy with chimeric antigen receptor (CAR) T cell therapy in glioblastoma (GBM) is undermined by an immunosuppressive tumor microenvironment (TME). We previously showed that CAR T cell therapy targeting epidermal growth factor receptor variant III (EGFRvIII) produces anti-tumor activity against recurrent GBM and causes upregulation of programmed death-ligand 1 (PD-L1) in the TME.

Here, we conducted a phase I trial to study the safety and tolerability of CART-EGFRvIII cells administered concomitantly with the PD-1 inhibitor pembrolizumab in patients with newly diagnosed, EGFRvIII+ GBM (n=7).

Treatment was well tolerated without incidence of dose-limiting toxicity. However, no signal of efficacy was detected with a median progression-free survival of 5.2 months (90% CI, 2.9 – 6.0 months) and overall survival of 11.8 months (90 % CI, 9.2 – 14.2 months). We aimed to elucidate reasons for limited efficacy through correlative analyses. Using BBZ qPCR, we found circulating CAR T cells in 5 out of 7 patients at the time of repeat resection, but only in one patient in the tumor. However, shared TCRs were found between the infusion product and the relapsed tumors, which could indicate an infiltration but lack of persistence of the CART. We further compared the tumor microenvironment of the tumors harvested before and after CAR+aPD1 administration using single cell RNAseq, and observed comparable proportions of the major immune cell subsets. However, the myeloid and T cells infiltrating the tumors significantly evolved, with more exhausted, regulatory and IFN-stimulated T cells at the relapse. At that time, the amount of IFN-stimulated T cells positively correlated with time from relapse to death.

Together, these findings suggest that the combination of CAR T cells and PD-1 inhibition in GBM is safe and biologically active but, given the lack of efficacy, also indicate a need to consider alternative immunotherapeutic strategies. ClinicalTrials.gov registration: NCT03726515.

P069

CO-INFECTION OF EBV SUBTYPE 1 AND 2 IS A POSITIVE BIOMARKER OF IMMUNE CHECKPOINT INHIBITOR RESPONSE AS COMPARED TO EBV SUBTYPE 1 INFECTION ALONE

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Epstein-Barr virus (EBV) is a ubiquitous pathogen that largely results in asymptomatic infections but is also linked to tumorigenesis of cancers such as lymphoma, nasopharyngeal carcinoma, and gastric carcinoma. Although some studies suggest that patients with oncoviral infections exhibit superior responses to immunotherapy, information is lacking as to whether responses differ based on viral subtype. Using real-world clinico-genomic data, we sought evaluate the association of EBV subtypes with key mechanisms of immune evasion, such as HLA class I loss of heterozygosity (HLA-I LOH), as well as overall survival (OS) after immune checkpoint inhibition (ICI).

Patients underwent comprehensive genomic profiling as part of routine clinical care. EBV detection was performed by competitively mapping sequencing reads acquired by adaptor-ligated hybridization capture to the NCBI nucleotide database. A positive status (EBV+) was assigned to samples that had a >97% identity to the BLAST sequence. Subtyping was performed by referencing the accession number of the mapped sequence to previously published EBV phylogenies. A sample was considered positive for HLA-I LOH if at least one of the HLA-I genes was predicted to be under LOH. Patient outcomes were assessed using the nationwide (US-based) Flatiron Health-Foundation Medicine gastric clinico-genomic database containing de-identified data that originated from ~280 US cancer clinics (~800 sites of care).

Of the 21050 EBV+ samples that were subtyped, 3054 (15%) were co-positive for both EBV-1 and EBV-2 (EBV1/2+) while all others were positive for EBV-1 alone (EBV1+). No sample was identified as having only an EBV-2 infection. We observed no significant difference in the median age of patients that were EBV1+ (62 years) vs EBV1/2+ (63 years). However, EBV1+ patients were more likely to be female (53%) than EBV1/2+ patients (42%) ($P < 0.001$). When assessing EBV subtype prevalence across the five genomic superpopulations, we found that East Asian patients had significantly lower rates of EBV1/2+ (12%) and South Asian patients had significantly higher rates of EBV1/2+ (17%) as compared to the other genomic ancestries (African (14%), Admixed American (15%), and European (15%)) ($P = 0.04$). Although there was no difference observed in the median TMB (3 muts/Mb) between EBV1+ and EBV1/2+ samples, EBV1/2+ samples did have a significantly higher rate of PD-L1 positivity (44%) as compared to EBV1+ samples (31%), using >1% tumor proportion score as a threshold. To investigate if EBV subtypes elicit different immune selective pressures, we compared rates of HLA-I LOH in nasopharyngeal and gastric carcinoma. In both tumor types, the rate of HLA-I LOH was significantly higher in EBV1/2+ patients as compared to EBV-negative (EBV-) patients (27% (8/30) vs 6% (3/51) in nasopharyngeal ($P = 0.01$, HR=5.8) and 30% (13/43) vs 18% (200/1123) in gastric ($P = 0.04$, HR=2.0)). In contrast, this trend was not observed in the EBV1+ patients within either tumor type (HLA-I LOH rates: 0% (0/14) in nasopharyngeal and 22% (30/134) in gastric). Lastly, we assessed OS in a subset of patients with gastric carcinoma from the start of advanced line ICI monotherapy. Patients who were EBV1/2+ ($n = 12$) had significantly longer OS as compared to patients who were EBV- ($n = 127$), tripling the median OS (mOS) from 3.2 months in the EBV- cohort to 9.5 months in the EBV1/2+ cohort ($P = 0.04$, HR=0.47). By comparison, patients who were EBV1+ ($n = 12$) had no improvement in OS as compared to patients who were EBV- (mOS=3.3 months, $P = 0.8$, HR=0.9).

Taken together, these results suggest that EBV1/2+ patients elicit a stronger immune response than EBV1+ patients, warranting future studies with larger cohorts to further evaluate EBV1/2+ status as a positive biomarker of ICI response.

P070

LOSARTAN NORMALIZES THE TUMOR MICROENVIRONMENT AND REDUCES IGF-1 LEVELS TO SENSITIZE OVARIAN CANCER TO CHEMOIMMUNOTHERAPY.

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Ovarian cancer (OvCa) is the most lethal of the gynecologic malignancies. Immune checkpoint inhibitors (ICIs), which have revolutionized the treatment of multiple malignancies, have had limited efficacy in OvCa patients. This lack of effectiveness can be attributed in part to the abnormal ovarian tumor microenvironment, characterized by a desmoplastic, highly fibrotic extracellular matrix with high levels of collagen. The accumulation of extracellular matrix components gives rise to increased compressive forces within the tumor, leading to the collapse of blood vessels, which results in reduced vessel perfusion, impaired drug delivery, and compromised trafficking of cytotoxic T-cells. The resulting tumor microenvironment is hypoxic and immunosuppressive. We aim to test the strategy of normalizing the tumor microenvironment with losartan to improve immunotherapy for OvCa. Previously, we discovered that losartan, an FDA-approved, widely prescribed angiotensin blocker to control hypertension, is capable of normalizing the extracellular matrix, which alleviated compression of the tumor blood vessels to improve chemotherapeutic drug delivery and efficacy.

In two syngeneic mouse OvCa models, we examined the losartan effects on drug delivery using fluorescently labeled anti-PD1 antibodies; and examined the losartan effects on immune cell infiltration and function by flow cytometry. We used targeted gene expression, single-cell RNASeq, and angiotensin II receptor 1 knockout mice (*Agtr1^{-/-}*) to investigate the losartan direct effect on IGF-1 expression and signaling.

In this study, we discovered that losartan exhibits dual effects on both the tumor microenvironment and cancer cells. Losartan treatment i) reduces matrix content in the tumor microenvironment, resulting in increased vascular perfusion and drug delivery, and ii) reprograms the tumor immune microenvironment, resulting in increased intratumoral infiltration and function of immune effector cells. We further discovered that losartan treatment via suppressing the IGF-1 expression and signaling, enhanced OvCa cancer cell chemosensitivity. As a result of the combined tumor and stromal effects, losartan treatment augments the efficacy of chemo-immunotherapy in models of OvCa.

We propose novel combination treatment strategies that have the potential to enhance chemo- and immunotherapy efficacy. Our study represents a major paradigm shift in the treatment of this uniformly fatal disease and opens doors for improving the treatment of other malignant diseases. As one of the most commonly prescribed drugs for hypertension, the safety and low cost (less than \$1-2/day) of losartan warrants rapid translation of our research to patients with OvCa.

P071

NEDD8 LOSS CAUSES CANCER VULNERABILITY TO IMMUNE CHECKPOINT BLOCKADE IN TRIPLE-NEGATIVE BREAST CANCER

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Triple-negative breast cancer (TNBC) is an aggressive form of breast cancer with limited treatment options. Over recent years, immunotherapy using immune checkpoint blockade (ICB) against the PD-1/PD-L1 axis has shown promising data, suggesting immunotherapy as a good option for the treatment of TNBC. However, challenges remain as clinical responses to ICB therapy in TNBC patients are limited, potentially due to reduce anti-tumor immunity driven by cancer intrinsic mechanisms.

In this study, we employed genome-wide CRISPR screens integrated with a Tumor Immuno co-Culture System (TICS) in the presence of an approved anti-PD-1 ICB drug, nivolumab, in order to identify novel intrinsic mechanisms in TNBC upon the ICB therapy. For this, a library of gRNAs was transduced into Cas9-expressing TNBC cells to target the entire human genome. After addition of immune cells and anti-PD-1 antibodies that trigger cancer cell death, Next-Generation Sequencing (NGS) analysis was performed to determine the frequencies of gRNAs in TNBC cells that survived/resisted immune-mediated killing. Our screen results were validated using in vitro assays and in vivo studies performed in syngeneic mouse models to assess any significant improvements in anti-tumor immunity.

Analysis of our CRISPR screens has revealed NEDD8 was among the most significantly depleted genes in TNBC cells when nivolumab was present, indicating NEDD8 as a key resistance gene to treatment. Removal of the NEDD8 gene by CRISPR/Cas9 in TNBC cells confirmed the strong immunogenic effects and anti-tumor efficacy after PD-1 blockade. In particular, when NEDD8 knock-out (KO) human TNBC cells were co-cultured with primary human lymphocytes, we observed significantly increased responses to nivolumab, as compared to the wild type counterparts. We observed the same with an anti-PD-L1 ICB drug, durvalumab. Moreover, proteomics data further confirmed that NEDD8 KO cells were more immunogenic. For example, HLA-DR was upregulated upon NEDD8 deletion in TNBC cells. Strikingly, NEDD8 deletion in a murine breast cancer cell line, EO771, enhanced response to ICB treatment and resulted in curative effects and prolonged survival mediated by CD8+ cells in tumor-bearing mice. We observed activation of multiple immunological pathways in KO tumors treated with PD-1 blockade. Because NEDD8 is a common essential gene, it was surprising to observe that TNBC NEDD8 KO cells remained proliferative. We performed CRISPR screens in ctrl/NEDD8 KO TNBC cell line pair to confirm the loss of essentiality in NEDD8 KO cells. Combining CRISPR screens and proteomics, our data revealed that the activation of alternative pathways, such as mTOR, DNA repair, UBEs mechanisms, contributed to the cell reprogramming upon NEDD8 deletion.

Altogether our work provides evidence that NEDD8 is conditionally essential in TNBC and uncovers its intrinsic resistance mechanisms against ICB therapy.

P072

PRESENCE OF T CELLS SPECIFIC TO TRANSPOSABLE ELEMENTS IN GLIOBLASTOMA TUMORS

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Glioblastoma (GBM) are the most common primary brain tumors in adults. They have a devastating prognosis with a 5-year survival of 5% with standard-of-care treatments. As such, new treatments are heavily needed. Recently, peptide coding, GBM-specific, transposable elements (TEs) have been described as potential tumor-specific targets for cancer immunotherapies. These TEs are highly recurrent among patients, which hold important promises for broadly-applicable therapeutic strategies.

Here, we tested whether TEs-derived peptides are immunogenic in GBM patients. From a list of 34 HLA-A2-restricted TEs peptide candidates, we identified 21 strong binders that we used to further refold peptide-MHC tetramers. Using these in a combinatorial strategy, we assessed TEs-specific responses within aCD3/IL2-expanded tumor-infiltrating lymphocytes (TILs) of five HLA-A2+ GBM patients.

Five over 21 tested peptides led to a detectable antigen-specific T cell population in at least one patient, with a frequency up to 1/104 after expansion. 2/5 peptides were found in all tested patients, suggesting that they are immunogenic in a majority of patients.

These results confirm the presence of TEs-specific T cells within tumor-infiltrating T cells from glioblastoma patients, and open interesting opportunities for therapeutic vaccination and cellular immunotherapy.

P073

TUMOR CONTEXT DICTATES RELIANCE ON TCF1 FOR RESPONSE TO IMMUNOTHERAPY

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Immune checkpoint blockade (ICB) therapy represents a breakthrough in cancer treatment with the potential to induce long-term remission. Nonetheless, clinical benefit of ICB remains elusive in many patients. Stem-like CD8 T cells have emerged as key players in the response to ICB. These cells depend, at least in part, on the transcription factor TCF1. Genetic ablation of TCF1 in mature CD8 T cells compromises, but does not completely abrogate, their function and maintenance thereby limiting the efficacy of ICB in pre-clinical cancer models. Furthermore, patients' responsiveness to ICB cannot be reliably correlated with the frequency of TCF1+ CD8 T cells. These findings indicate that the dependence on TCF1+ stem-like T cells for ICB efficacy may not be equal across patients and in different tumor contexts and raises the question of what determines reliance on TCF1 for effective responses.

Here we leveraged conditional knock-out (cKO) mice that delete TCF1 in mature CD8 T cells to investigate how TCF1 instructs the early fate and functions of CD8 T cells upon ICB therapy in tumors that differ for immunogenicity and levels of tumor antigens expression.

Notably, we discovered that TCF1 is required for ICB efficacy in poorly immunogenic B16OVA melanomas but is dispensable in highly immunogenic MC38OVA colorectal tumors. Single-cell RNA sequencing (scRNA-seq) and immunophenotyping in the tumor draining lymph node (TDLN) revealed defective priming and expansion of tumor-specific TCF1 cKO T cells in B16OVA tumors treated with ICB but not in highly immunogenic MC38OVA tumors. In vitro, we observed defective proliferation, reduced PD-1 and CD28 up-regulation, and reduced phosphorylation of key signaling molecules downstream the T cell receptor pathway in TCF1 cKO T cells when stimulated with low but not high TCR signals. These data indicate that TCF1 poises T cells for optimal antigen responsiveness in suboptimal priming conditions such as those found in low antigen expressing tumors. Moreover, transcriptional profiling of T cells in the TDLN revealed the accumulation of a subset of tumor-specific naïve T cells poised to give rise to short-lived effectors in TCF1 cKO mice. This naïve subset is therefore less efficacious in poorly immunogenic tumors where expansion of T cells retaining memory potential is required for the deployment of durable anti-tumor responses. In tumors, scRNA-seq and immunophenotyping revealed that highly and poorly immunogenic tumors differentially instruct the differentiation trajectory of CD8 T cells. Both WT and TCF1 cKO mice implanted with MC38OVA tumors showed an expanded population of CD8 cells sharing a gene signature with highly cytotoxic transitory effector cells that mediate ICB efficacy in chronic viral infection models. Expansion of these cells accounted for the strong anti-tumor response observed in both WT and TCF1 cKO mice. Conversely, B16OVA tumors failed to expand transitory effectors and accumulated TOX+ dysfunctional CD8 T cells. Importantly, in the absence of TCF1 TOX+ dysfunctional T cells became destabilized, failed to persist, and acquired a gene signature found in both pre and post treatment tumor specific CD8 T cells from patients that do not respond to ICB.

Together, the defective priming of stem-like T cells in the TDLN combined with a destabilized dysfunctional T cell state in the tumor contributed to the failure of TCF1 cKO mice to sustain effective ICB responses in poorly immunogenic tumors. Improving T cell priming by therapeutic vaccination or enhancing antigen presentation on melanoma cells could rescue the defective ICB responses of TCF1 cKO CD8 T cells. Our study highlights a role for TCF1 during the priming and early stages of

the anti-tumor CD8 T cell response. These findings have important implications for guiding therapeutic interventions in cancers with low frequency of TCF1+ stem-like T cells and low neo-antigen expression.

P074

STAT5A COUNTERACTS EXHAUSTION AND DIRECTS A DURABLE EFFECTOR/NK-LIKE PROGRAM IN CHRONICALLY STIMULATED CD8 T-CELLS: IMPLICATIONS FOR CANCER IMMUNOTHERAPY

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CD8 T-cell exhaustion is a leading cause of cancer immunotherapy failure. Overcoming this process therapeutically remains an unmet challenge due to the epigenetic stability of the exhaustion lineage. However, flexibility in the exhaustion program was recently highlighted with evidence of an “effector-like” subset of exhausted CD8 T-cells (TEX intermediate; TEXint) suggesting opportunities for interventions. Here, we discovered a reciprocal antagonistic circuit between the transcription factor Tox (the master instigator of the exhaustion program in CD8 T-cells) and Stat5a in exhausted CD8 T-cells (TEX) including preferential re-engagement of Stat5a activity in the “effector-like” TEXint subset.

By leveraging gold-standard murine model for the study of CD8 T-cell exhaustion (Lymphocytic Choriomeningitis virus; [LCMV]), combined to next generation epigenomic technologies and leading-edge immunological tools, we investigated the impact of Stat5a on TEX cells development and evaluated therapeutic opportunities for manipulating this axis to rewire TEX cells toward better cell-states.

Here, we found that Stat5 directed TEXint cell development, re-activated the effector machinery in this subset and enabled TEX responses to PD-L1 blockade. Enforcing constitutive Stat5a activity (STAT5CA) antagonized Tox, provoking an epigenetic and transcriptional rewiring of antigen-specific CD8 T-cells towards a durable “effector-like” state expressing NK receptors and displaying superior anti-tumor potential. Enforcing Stat5 activity in mature TEX using an orthogonal IL-2/IL2RB pair system boosted TEXint cells formation, particularly in combination with PD-L1 blockade. Finally, temporal reactivation of Stat5 in TEX progenitors (TEXprog) reversed key epigenetic features of exhaustion allowing de novo accessibility at TEF/TMEM-related open chromatin regions and restored polyfunctionality.

Together, we identify Stat5 as a key regulator of TEX differentiation, antagonizing Tox-driven terminal exhaustion and fostering improved effector activity and durability in the setting of chronic antigen (Ag) stimulation. Moreover, therapeutic augmenting IL-2/Stat5 signals specifically in TEX in combination with PD-1 blockade not only expanded the TEXint population but also rewired these TEX cells towards a more protective differentiation state with features of durability under chronic antigenic stress and enhanced effector biology.

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P075

PROGRAMMABLE BACTERIA SYNERGIZE WITH PD-1 BLOCKADE TO OVERCOME GENETIC IMMUNE RESISTANCE MECHANISMS

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Tumors employ a variety of genetic resistance mechanisms to evade immune responses and immunotherapies such as PD-1 blockade. The pleiotropic cytokine interferon-gamma (IFN γ) is a potent immune effector and critical for patient response to PD-1 blockade, yet conventional systemic delivery is hindered by severe dose limiting toxicities. As such, the effects of exogenously introduced IFN γ either as monotherapy or in combination with PD-1 blockade in the context of different tumor genetic background remain poorly understood.

Synthetic biology allows programming of microbes for tumor-specific delivery of therapeutic candidates that are otherwise not possible using conventional administration strategies. Here, we engineered a strain of probiotic bacteria for localized and sustained delivery of IFN γ . We validated the efficacy of our therapeutic strain, either as monotherapy or in combination with PD-1 blockade, in multiple murine tumor models.

We demonstrate that engineered probiotic bacteria can locally produce and release IFN γ within the tumor microenvironment (TME), such that a single infusion of IFN γ -producing bacteria is sufficient to generate a neoantigen-specific adaptive immune response in both primary and distal untreated lesions via activation of CD8 $^+$ T cells and type 1 conventional dendritic cells. Furthermore, bacteria-derived IFN γ overcomes primary resistance mechanisms to PD-1 blockade via activation of cytotoxic CD4 $^+$ and CD8 $^+$ T cells and acquired tumor cell-intrinsic genetic immune resistance mechanisms, including loss of function mutations in the IFN γ signaling and antigen presentation pathway, via activation of NK and CD4 $^+$ T cells.

Collectively, these results demonstrate the promise of combining IFN γ -producing bacteria with PD-1 blockade as a novel neoadjuvant therapeutic strategy for patients suffering from locally advanced, immune resistant and metastatic disease.

P076

ANTI-TUMOR EFFECT OF INTRANASAL VACCINATION UTILIZING MULTI-EPI TOPE LONGER PEPTIDE WITH D-OCTAARGININE-LINKED PNVA-CO-AA IN MICE

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Recent advances of cancer immunotherapy including immune checkpoint inhibitors greatly contribute the clinical success in treatment for cancer, however, the route for drug delivery and types of antigens can be improved. Many researches showed that mucosal immunization such as oral, nasal or sublingual route could induce systemic immune responses effectively by stimulating mucosa-associated immune system especially in infectious disease area, but few in cancer immunotherapy. In this study, we investigated the feasibility of intranasal administration of cancer vaccine with multi-epitope longer peptide and cell membrane penetrating D-octaarginine-linked copolymer of N-vinylacetamide and acrylic acid (VP-R8) as a candidate of novel cancer vaccine.

Four kinds of CD8T cell epitopes from human papillomavirus (HPV) type 16 E6E7 proteins were linked by arginine linkers to synthesize HPV E6E7 longer peptide. To investigate anti-tumor effect of the intranasal administration of the mixture of longer peptide with VP-R8, 1×10⁶ of TC-1 tumor cells stably expressing HPV E6E7 protein were subcutaneously injected into C57BL/6 mice. Intranasal administrations of the mixture were performed once a week for 4 weeks (n=5). To determine antigen-specific T cell immune responses, spleen cells were isolated and intracellular cytokine staining, MHC class I tetramer assay and LDH-release cytotoxicity assay were performed. To expand the feasibility for the drug delivery system, 4 kinds of CD4T and CD8T cell epitopes from Wilms' tumor 1 (WT1) protein and VP-R8 were mixed and intranasally administrated into mice bearing C1498-mWT1 which were stably expressing mouse WT1 protein, and the anti-tumor efficacy was determined.

Intranasal administrations of the mixture of HPV E6E7 longer peptide with VP-R8 significantly decreased the tumor growth of TC-1 after 4-week treatment compared to longer peptide alone (p<0.05). The intranasal administration significantly prolonged the survival compared to intraperitoneal administration of longer peptide with incomplete Freund's adjuvant (p<0.05). Intracellular cytokine staining showed that the number of HPV E6E7-epitope specific CD8T cells secreting IFN-gamma were significantly increased compared to longer peptide alone (p<0.05, respectively). The intranasal administration significantly increased the E6E7-specific cytotoxicity against TC-1 cells compared with the intranasal administration of longer peptide alone at effector:target ratio 40:1 (p<0.05). For WT1 study, intranasal administration of the mixture of WT1 longer peptide with VP-R8 remarkably decreased tumor growth and prolonged survival against C1498-mWT1 compared to intraperitoneal administration of WT1 peptide with incomplete Freund' adjuvant in mice.

We demonstrated that intranasal administrations of multi-epitope-linked longer peptide with VP-R8 could induce robust tumor-specific immune responses in mice. Intranasal administration with cell penetrating polymer could be less invasive and more available route than current subcutaneous cancer vaccines to deliver cancer antigen. Our findings have significant implication for intranasal cancer vaccines that deliver antigen to nasal-associated lymphoid tissues and elicit strong anti-tumor T cell immune responses.

P077

LOCAL IL-4 SIGNALING WITHIN BONE MARROW CONTROLS PATHOGENIC MYELOPOIESIS IN LUNG CANCER

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Non-small cell lung cancer (NSCLC) accounts for over 1.6 million annual deaths worldwide. A key driver of cancer progression is thought to be the tumor microenvironment (TME), which in NSCLC is dominated by myeloid cells that support tumor growth through diverse mechanisms. Myeloid cells, in particular monocytes and macrophages, are well-described to suppress the antitumor immune response. The majority of myeloid cells arise from bone marrow (BM) progenitors in response to inflammatory signals, and are thought to adopt immunosuppressive states upon exposure to local tumor cues. However, the molecular mechanisms controlling immunosuppressive myeloid cell states are ill-defined, hampering the development of myeloid-targeted therapies for cancer.

Here, we transcriptionally profiled over 500,000 tumor infiltrating leukocytes in NSCLC patients and in the KrasG12DTP53-/- (KP) murine lung adenocarcinoma model. In both species, the Th2 cytokine IL-4 was computationally predicted to be the primary driver of tumor infiltrating myeloid cell phenotype, despite the fact that lung tumors were largely devoid of IL-4 producing cells. Correspondingly, antibody blockade of IL-4 in tumor-bearing mice profoundly reduced tumor burden. We subsequently utilized a panel of conditional knockout mice and in vivo approaches to define the mechanism by which IL-4 signaling controls tumor progression. Finally, we designed and initiated the first clinical trial of dupilumab, a humanized IL-4Ra blocking antibody commonly used for atopic disease, given in conjunction with PD-(L)1 blockade in relapsed/refractory NSCLC patients who had progressed on standard chemoimmunotherapy combinations and longitudinally analyzed the clinical and cellular response to this treatment.

Using a panel of conditional knockout mice, we found that only deletion of IL-4Ra within early myeloid progenitors in BM reduced lung tumor burden, while deletion of this receptor in downstream mature myeloid cells had no effect. Detailed transcriptional analysis followed by mechanistic studies in vivo revealed an essential role for local BM IL-4 signaling in reprogramming myelopoiesis in cancer. Mechanistically, basophils and eosinophils within BM upregulated IL4 production upon sensing distal tumor cues; local BM IL-4 acted on granulocyte-monocyte progenitors to enhance myelopoiesis and transcriptionally program the development of immunosuppressive myeloid cells, which then homed to the tumor and promoted tumor growth. Consequentially, specific depletion of basophils, which were enriched in BM but absent from the tumor, profoundly reduced tumor burden and normalized myelopoiesis in experimental lung tumors. In our clinical trial, dupilumab drove a major reduction in circulating monocytes coupled with an expansion of circulating and tumor-infiltrating cytotoxic T cells and B cells. Remarkably, one out of the six patients who enrolled in Phase 1b of this trial exhibited significant decrease in his tumor burden after two months of dupilumab treatment, and the clinical response deepened further to a near complete response even after cessation of dupilumab, suggestive of successful reprogramming of the antitumor response.

Here, we define IL-4 as a central driver controlling monocyte and mo-mac immunosuppression in NSCLC, and make the surprising discovery that the relevant site of IL-4 signaling is not the tumor itself, but the BM, where it acts on myeloid progenitors to imprint myeloid cell fate. Collectively, our

study defines a role for IL-4 signaling in lung tumor progression, identifies a central axis controlling immunosuppressive myelopoiesis in cancer, and highlights a novel combination therapy for immune checkpoint blockade in humans.

P078

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Most investigations of the molecular and cellular circuits governing the differentiation of immune cells in cancer have focused on tumor bed cells. However, emerging data now highlight the importance of immunological events in the tumor-draining lymph node (tdLN) as well as in distinct intra-tumoral niches. Recent work has revealed that various human cancers have regions within or adjacent to the tumor that contain antigen-presenting cells and resemble the T cell zone of secondary lymphatic tissues. These regions are enriched for stem-like CD8 T cells, which provide a proliferative burst following checkpoint blockade therapy and are associated with a better prognosis. Unfortunately, while extremely useful for studying tumor growth and for preclinical development of antitumor therapies, murine tumor models do not fully capture the true tumor structure complexity. Similarly, classic two-dimensional cultures lack the necessary complexity to replicate such an architecture. In contrast to other 3D options such as organ on a chip, organoids, and injectable lymphoid structures, we propose to employ a novel three-dimensional (3D) in vitro bioprinting approach to generate a high-resolution immune niche tumor model. This will be combined with a highly complex and multi-layered, tumor-draining lymph node model, to obtain systems that can be easily manipulated, scaled up, integrated, and can be geometrically customized to address spatially related questions.

Together with advanced immune profiling and bioinformatics, these 3D models will provide an opportunity to examine how spatial organization, cellular composition, and relationships between different cells (immune and non-immune) can impact immune cell differentiation and function.

I will discuss our recent advances and future goals working with this innovative technology.

The research proposed here will advance the basic biological understanding and confirm the importance of intratumor and tdLN spatial organization of cells in immune niches. This research will produce revolutionary new mechanistic insights into a fundamental problem in tumor immunology and provide innovation far beyond the current state of the art. The novel three-dimensional (3D) in vitro bioprinted immune niche tumor model and highly complex, multilayered tumor-draining lymph node model, systems introduced here can be easily manipulated, scaled up, integrated and geometrically customized to address spatially related questions. Finally, these approaches will provide a platform for the study of more generalized interactions within a tumor and draining lymph node and the crosstalk between these two compartments.

P079

SENSITIZATION TO CHEMO-IMMUNOTHERAPY BY TARGETING TGF-B IN PRECLINICAL TRIPLE NEGATIVE BREAST CANCER MODELS

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Metastatic triple-negative breast cancer (mTNBC) is refractory to anti-PD-(L)1 immunotherapy. In order to sensitize it, preclinical data show the value of adding chemotherapy treatment for its ability to induce immunogenic cancer cell death (Kroemer et al, 2013). This would enable the recruitment of cytotoxic T-CD8+ lymphocytes (CTL) and thus initiate an antitumor immune response to sensitize tumors to immunotherapy. Nevertheless, the results of clinical trials using chemo-immunotherapy combinations are divergent (IMpassion 130 and 131). Indeed, the IMpassion 130 trial presenting the results of a combination of nab-paclitaxel and atezolizumab (anti-PD-L1) shows a benefit of chemo-immunotherapy in PD-L1+ patients. Unfortunately, IMpassion 131 with paclitaxel does not confirm the results obtained with Nab-paclitaxel. The choice of chemotherapy to combine with immunotherapy appears to be crucial for inducing an antitumor immune response. Optimizing chemo-immunotherapy combinations is a major challenge in the management of TNBC.

The initial aim of this project was to evaluate the immune effects of an experimental chemotherapy doublet, based on two drugs used in mTNBC: cisplatin (CDDP) and eribulin. The idea was to find an alternative to the use of taxanes in combination with anti-PD-(L)1. In fact, the results of the TONIC trial had supported the idea of using a platinum salt to induce an immune response, and eribulin has been described to play a role in tumor vascularization and thus potentially enhance CDDP-induced immune recruitment. In addition, we also wanted to explore the immunological properties of eribulin, a recently approved antimitotic recently approved in mTNBC.

The effect of the two molecules, alone or in combination, was evaluated on immunogenic death stigmata, modulation of effector or immunosuppressive immune populations and stromal populations (endothelial cells and fibroblasts), and sensitization to anti-PD-L1 immunotherapy in different preclinical models of mTNBC, in particular the immunologically "cold" model: 4T1. The in vivo studies has been realized by generation of orthotopic 4T1 tumors, the analysis of the immune infiltration was performed by RT-qPCR, IHC and flow cytometry.

In vitro, our results indicate that CDDP, and more intensely in combination with eribulin, significantly induces the various immunogenic death stigmas. In vivo, the therapeutic effect of chemotherapies is limited, with a superior effect of CDDP and the combination. Nevertheless, CTL recruitment and functionality induced by CDDP are significantly enhanced by eribulin. Despite these data pointing to a potential therapeutic synergy with anti PD-L1 immunotherapy, chemoimmunotherapy treatment fails to induce a superior treatment effect. The significant increase in a TGF β signature induced by the combination in parallel with CTL recruitment explains this resistance. The results show that inhibition of TGF β , combined with chemoimmunotherapy in the 4T1 model, leads to a reduction in tumor immunosuppression and intratumoral fibrosis, as well as an increase in CTL infiltration and cytotoxicity, enabling tumor sensitization. The deleterious effect of CDDP via TGF β was also found on a panel of human TNBC lines.

Overall, this project highlights the benefits of using an experimental doublet of CDDP and eribulin-based chemotherapies. The combination enhances tumor immunogenicity, promoting CTL recruitment and cytotoxicity. Nevertheless, our chemoimmunotherapy is not therapeutically synergistic, which is explained by the increase in TGF β induced by CDDP, which paralyzes the anti-tumor immune response and limits the therapeutic effect of the combination. Targeting TGF β to

reduce immunosuppression in the tumor microenvironment helps overcome the resistance of the 4T1 model to chemoimmunotherapy. This project demonstrates that management of chemo-induced immunosuppression mechanisms is necessary to overcome tumor resistance to immunotherapy.

P080

B-CŪR: A UNIVERSAL TARGET AND ANTIBODY DISCOVERY PLATFORM INTERROGATING THE B CELL REPERTOIRE FROM EXCEPTIONAL RESPONDERS

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The use of human B cells for antigen and antibody discovery is on the rise due to the emergence of new techniques to tap into the human antigen-experienced B cell repertoire. Here, we describe a universal platform that enables the discovery of novel therapeutic target and antibody pairs by functional screening of the B cell repertoire of exceptional responders.

Starting with PBMCs of exceptional responders, B cells are transduced to express BCL6 and BCL-xL to overcome the limited proliferative lifespan of primary B cells by effectively preventing terminal differentiation and apoptosis. B-CŪr B cells are amenable to high-throughput processing through indefinite expansion capabilities, expression of the cognate B cell receptor on the cell surface and concomitant secretion of mAbs. In addition, B-CŪr B cells are more stable than EBV-transformed cells and the produced mAbs show excellent safety profiles and physicochemical properties. The B-CŪr platform shows >85% immortalization efficiency in isolated antigen-experienced B cells. Furthermore, the induction of activation-induced cytidine deaminase (AID), resulting in ongoing mutation in the B cell receptor/Ab sequence, allows for in vitro selection of affinity and stability variants with labeled antigens. This process yields information on critical and non-critical residues for binding and can generate surrogate mAbs for in vivo studies. Next to PBMC-derived B cells, tissue- or tumor-derived B cells are easily applied in our platform. This allows for novel target discovery by interrogation of tertiary lymphoid structures (TLS) and tumor-infiltrating B cells. B-CŪr has been successfully applied to B cells from various animal species, including rabbits, mice, rats, pigs, non-human primates, and llamas. Transduced B cells from these species show the same phenotype as transduced human B cells.

Recently, the EMA granted market approval for the anti-RSV mAb nirsevimab (Beyfortus®) that was discovered using the B-CŪr platform. Nirsevimab recognizes a new and unique epitope on the prefusion state of RSV F protein and is superior to the current standard of care (Palivizumab).

Kling Biotherapeutics has discovered several unique cancer targets and their corresponding human antibodies that are currently in preclinical development. KBA1412, a CD9-targeting human antibody is currently being evaluated in a Phase 1b dose escalation and dose expansion study in patients with advanced solid tumors (ClinicalTrials.gov: NCT05501821).

Kling Biotherapeutic's target-agnostic approach enables the identification of novel disease targets and corresponding fully human antibodies from exceptional responders who have shown a highly successful immune response leading to virus clearance or tumor eradication. Our approach provides a significant competitive advantage compared to traditional target and antibody identification approaches.

P081

BLINATUMOMAB AND TYROSINE KINASE INHIBITORS IN B-ALL: FRIENDS, OR ENEMIES?

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Blinatumomab, a bi-specific T cell engager (CD3/CD19), is primarily used in the treatment of relapsed and refractory B-acute lymphoblastic leukemia (B-ALL) or as a salvage therapy before transplantation. It has been observed that relapses after blinatumomab treatment often manifest as extramedullary disease, indicating potential changes in cell adhesion specificity. Tyrosine kinase inhibitors (TKIs) are widely employed in the therapy of chronic myeloid leukemia, targeting the BCR-ABL oncogene and Src family kinases while also influencing leukemia cell adhesion. Recent clinical trials have successfully combined blinatumomab with TKIs for treating B-ALL. Although theoretically the combination may not be effective due to TKIs inhibiting the essential SRC kinases involved in T cell activation, promising results have been obtained with the combined therapy.

In our study, we utilized lymphocytes from healthy controls (either freshly isolated or expanded in vitro) and B-ALL cell lines (RAJI, REH, ARH-77) as experimental models. These cells were subjected to treatment with blinatumomab and/or TKIs (dasatinib and ponatinib). Through the use of flow cytometry and western blotting techniques, we evaluated: 1) the effectiveness of blinatumomab- and TKI-induced cytotoxicity, 2) alterations in the expression of cell adhesion molecules prompted by the treatment, and 3) the activation status of both effector T cells and target leukemia cells.

Our findings revealed that tyrosine kinase inhibitors hinder the efficacy of blinatumomab-mediated cytotoxicity in a dose-dependent manner. While the highest clinically achievable concentrations completely impeded the cytotoxic effect, the lowest clinically relevant levels did not significantly impact it. Additionally, TKIs influenced the levels of CD62L (L-selectin) on the surface of B-ALL cells, and this effect seemed to occur independently of Src family kinases.

In summary, our study demonstrates that even though TKIs inhibit SRC kinases necessary for T cell activation, the lowest clinically relevant levels of TKIs do not impede the cytotoxic effect of blinatumomab. Moreover, TKIs have an impact on the levels of CD62L on the surface of B-ALL cells, indicating potential changes in cell adhesion specificity. These findings provide valuable insights into the mechanisms underlying the effectiveness of combination therapy and emphasize the importance of further investigating the interplay between blinatumomab, TKIs, and cell adhesion molecules in the context of B-ALL treatment.

P082

INVESTIGATING THE MECHANISM OF ACTION OF AN ANTIGEN-PRESENTING CELL TARGETED DNA VACCINE AGAINST CANCER NEOANTIGENS

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Tumor-specific mutations give rise to unique cancer neoantigens that can be recognized by the immune system, making them appealing targets for therapeutic cancer vaccines. While neoantigen plasmid-DNA (pDNA) vaccines have shown promising results in preclinical models, demonstrating effective T cell-mediated tumor eradication, the immune responses induced by pDNA vaccines have varied in the clinic. Thus, DNA-based vaccines can benefit from inducing more robust and long-lasting effector T-cell responses, especially when targeting close-to-self antigens like neoantigens. To improve the effectiveness of a cancer neoantigen pDNA vaccine, we included the immune stimulatory molecule C-C motif ligand 19 (CCL19) to deliver the neoantigens to antigen-presenting cells (APCs) selectively. To this end, we genetically fused the antigen payload of 13 neoantigens to CCL19, which can bind the CCR7 receptor on the surface of APCs. In mouse cancer models, the APC-targeting pDNA vaccine, EVX-03, induced stronger T-cells responses and anti-tumor efficacy than a vaccine that encodes only the cancer neoantigens.

The research presented here investigates the mechanisms of action of the EVX-03 pDNA vaccine. We hypothesized that the EVX-03 protein is secreted by pDNA-transfected cells and recruits additional APCs to the immunization site. Moreover, EVX-03's interaction with CCR7 could induce receptor internalization, increasing the chances of neoantigens internalization and presentation by APCs.

We investigated the levels of EVX-03 protein product encoded by the pDNA vaccine in muscle lysates and serum from intramuscularly immunized mice using a CCL19 sandwich ELISA.

To demonstrate the ability of the EVX-03 protein to attract APCs, we performed an in vitro trans-well migration assay interrogating human monocyte-derived dendritic cells (moDCs) migration towards the supernatant derived from cells transfected with EVX-03 pDNA.

The ability of CCL19 as part of an APC-targeting vaccine to activate CCR7 was tested in vitro using a reporter system based on bioluminescence resonance energy transfer (BRET) in cell lines transfected with CCR7 and the BRET reporter CAMYEL.

We have taken the first steps to investigate the mechanism of action by which the APC-targeting pDNA cancer vaccine, EVX-03, improves the immunogenicity and anti-tumor efficacy of cancer neoantigens. By deploying the assays mentioned above, we detected the secretion of the protein product in immunized mice and demonstrated its ability to recruit APCs. Moreover, we showed EVX-03's ability to activate CCR7 in vitro, an essential step in increasing neoantigen internalization and presentation by APCs.

This study contributes to our understanding of how improving the interaction with APCs can enhance the immunogenicity and efficacy of cancer neoantigens. The elucidation of the molecular mechanism of action behind APC-targeting vaccines contributes to developing strategies that improve the effectiveness of therapeutic cancer vaccines and ultimately contribute to better patient outcomes.

P083

GERMLINE HOMOZYGOSITY AND ALLELIC IMBALANCE OF HLA-I ARE COMMON IN ESOPHAGO-GASTRIC ADENOCARCINOMA AND IMPAIR THE REPERTOIRE OF IMMUNOGENIC PEPTIDES

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The individual HLA-I genotype is associated with cancer, autoimmune diseases and infections. This study elucidates the role of germline homozygosity or allelic imbalance of HLA-I loci in esophago-gastric adenocarcinoma (EGA) and determines the resulting repertoires of potentially immunogenic peptides.

HLA-genotypes and sequences of either (1) ten relevant tumor-associated antigens (TAAs) or (2) patient-specific mutation-associated neoantigens (MANAs) were used to predict good-affinity binders using an in-silico approach for MHC-binding (www.iedb.org). Imbalanced or lost expression of HLA-I-A/B/C alleles was analyzed by transcriptome sequencing. FluoroSpot assays were used to determine peptide-specific T cell responses.

We show that germline homozygosity of HLA-I genes is significantly enriched in EGA patients (n=80) compared to an HLA-matched reference cohort (n=7605). Whereas the overall mutational burden is similar, the repertoire of potentially immunogenic peptides derived from TAAs and MANAs was lower in homozygous patients. Promiscuity of peptides binding to different HLA-I molecules was low for most TAAs and MANAs and in-silico modelling of the homozygous to a heterozygous HLA-genotype revealed normalized peptide repertoires. Transcriptome sequencing showed imbalanced expression of HLA-I alleles in 75% of heterozygous patients. 33% of these showed complete loss of heterozygosity, whereas 66% had altered expression of only one or two HLA-I molecules. In a FluoroSpot assay we determined that peptide-specific T cell responses against NY-ESO-1 are derived from multiple peptides, which are often exclusively binding only one HLA-I allele.

The high frequency of germline homozygosity in EGA patients suggests reduced cancer immunosurveillance leading to an increased cancer risk. Therapeutic targeting of allelic imbalance of HLA-I molecules may improve immune response in EGA.

P084

MECHANISMS AND MRNA THERAPIES BYPASSING INTERFERON (IFN)-DEFICIENCIES IN IMMUNOTHERAPY RESISTANT BREAST TUMORS

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Ligand-dependent corepressor (LCOR) specifically mediates tumor immunogenicity independently of interferon signaling and overcome immunotherapy resistance in triple-negative breast cancer (Pérez-Núñez et al. 2022).

ChIP-seq, ATAC-seq, and mass spectrometry analysis were used to elucidate the mechanistic insights by which LCOR bind and transcriptionally activate the APM/MHC supercluster in the human chromosome 6, and thus trigger tumor cell immunogenicity in breast cancer models and other cancer types. Specific antigen recognition assays have been performed using OVA with OT-I T-cells and EGFP with JEDI T-cell mouse model systems in different genetic background. Preclinical functional assays have been addressed in syngeneic immunocompetent mouse models and further validated in humanized mouse models when using human cancer cells. For protein replacement therapies, we have optimized an innovative mRNA therapy approach in experimental metastasis settings using extracellular vesicles and specific poly-b-aminoester nanoparticles for delivery in combination with immune-checkpoint immunotherapies.

We unveiled an unexpected function of LCOR as a master transcriptional activator of antigen presentation machinery (APM) genes binding to IFN-stimulated response elements (ISREs) in an IFN signaling-independent manner. In addition, IFN requires the presence of LCOR to induce tumor immunogenicity through APM modulation. IRF1- and STAT1-deficient cells can still activate APM through LCOR-mediated APM. LCOR requires of chromatin remodeling partners to displace H3K27me3 marks and open the chromatin in the APM genomic cluster region (chr. 6), which is normally closed in poorly differentiated tumors. Remarkably, Lcor-mRNA therapy in combination with anti-PD-L1 overcame resistance and eradicated breast cancer metastasis in preclinical models. In addition in estrogen-positive breast cancer, using LCOR in combination with anti-hormonal therapy we have converted cold tumors into hot tumors and make them vulnerable to anti-PDL1/PD1 immunotherapy, which is a new milestone of ICB therapies in ER+BC immunity.

Collectively, these data support LCOR as a promising target for enhancement of ICB efficacy in TNBC and ER+BC by boosting tumor APM independently of the IFN pathway. Therefore, we are developing a first in class mRNA therapy with nanoparticle delivery of LCOR mRNA to increase tumor antigen presentation across all tumor cells and render them visible to immune detection, as an essential partner for immune-checkpoint blockade therapy in breast cancer.

P085

BND-35, AN ANTI-ILT3 MONOCLONAL ANTIBODY INHIBITS THE IMMUNOSUPPRESSIVE EFFECTS OF ILT3 REMODULATE THE SUPPRESSIVE TME AND ENHANCES ANTI-TUMOR ACTIVITY OF IMMUNE CELLS IN PRECLINICAL IN VITRO, EX VIVO, AND IN VIVO MODELS

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Ig-like transcripts (ILTs) are a family of immuno-modulating receptors expressed on immune cells. ILT3 is expressed in various suppressive myeloid cells including tumor associated macrophages (TAM), myeloid derived suppressor cells (MDSCs) and tolerogenic dendritic cells (DCtol). ILT3 binds proteins in the tumor microenvironment (TME) and tumor extracellular matrix (ECM) like Apolipoprotein E (APOE) and Fibronectin. The binding of ILT3 to its ligands induces an immunosuppressive phenotype in myeloid cells, mediates the inhibition of T cells activity and generates an immunosuppressive TME which supports tumor growth and proliferation. The inhibition of ILT3 can restore the anti-tumor activity of myeloid cells and T cells, and thus remodulate the tumor microenvironment from immunosuppressive to pro-inflammatory.

Here we describe preclinical characterization of BND-35, a humanized IgG4, ILT3 antagonist antibody developed for the treatment of solid tumors.

BND-35 binding to ILT3 was evaluated by flow cytometry, ELISA and surface plasmon resonance. We investigated the ability of BND-35 to block ILT3 interactions with APOE and fibronectin, to enhance the pro inflammatory activity of various myeloid cells and reverse ILT3-mediated immune suppression of T cells by MDSCs and DCtol using ELISA and in vitro and ex vivo cell-based assays. The anti-tumor activity of BND-35 was also evaluated in vivo in hILT3 transgenic mouse tumor models as well as in tumoroid systems generated from cancer patients

BND-35 binds ILT3, but not other ILT-family receptors, with low nanomolar affinity and blocks its interaction with APOE and fibronectin in a concentration-dependent manner. ILT3 blockade with BND-35 restored the pro-inflammatory activity of FcR-stimulated DCs and THP1 cells inhibited by fibronectin. BND-35 was also shown to restore an M1 phenotype in cancer patients' derived monocytes differentiated in the presence of autologous tumor cells.

In addition, BND-35 restored T cell activity (both, CD4 and CD8 T cells) inhibited by either DCtol or MDSCs as a single agent and in combination with anti PD-1. Ex- vivo tumoroid system is an experimental system in which the original tumor's cell composition and tumor ECM are preserved in the tumoroid particles. Tumoroid samples are showing enhanced secretion of proinflammatory cytokines following treatment with BND-35. In vivo, blocking ILT3 activity with BND-35 resulted in decreased tumor growth and induced a pro-inflammatory phenotype in tumor resident T cells and myeloid cells populations.

BND-35 is an anti-ILT3 antagonist antibody that was shown to induce potent pro inflammatory activity of myeloid cells and enhance T cells activity inhibited by ILT3 expressing myeloid suppressive cells in multiple in vitro, ex vivo and in vivo models. By doing so, BND-35 can lead to TME remodeling from immunosuppressive to proinflammatory. Safety, tolerability, and anti-tumor activity of BND-35 will be explored in a first-in-human clinical trial in cancer patients with solid tumors.

P086

UNRAVELLING THE ROLE OF LIPID-LOADED MACROPHAGES IN RESISTANCE TO ANTI-CANCER THERAPIES

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The efficacy of immune checkpoint inhibitors (ICIs) in cancer relies on the presence of an effective T cell-mediated immune response. However, multiple features of the tumor microenvironment constrain T cells activation thus contributing to therapy resistance. Importantly, emerging studies have shown that ICIs efficacy is in part dependent on tumor associated macrophages (TAMs). On this line, we recently discovered that lipid-laden macrophages (LLMs) infiltrate tumors, including prostate cancer and melanoma.

We applied single cell RNA sequencing to the immune infiltrate of prostate cancer and melanoma. We then implemented a multiparametric flow cytometry strategy to confirm transcriptional findings. In vivo, we set up transgenic and transplantable models of prostate cancer and melanoma to investigate the immune composition of tumors. Finally, we isolated tumor infiltrating lipid-loaded macrophages and we performed bulk RNA sequencing and mass spectrometry to dissect the transcriptional features and the proteome profile of LLMs.

We demonstrated that the abundance of LLMs correlates with tumor size and we discovered that LLMs promote cancer progression in association with immune evasion and poor response to chemotherapy. Lipid intake in TAMs is partially dependent on scavenging by MARCO and MARCO neutralization in vivo deplete LLMs both in prostate cancer and melanoma models. Mechanistically, we also found that TAMs display a dysfunctional autophagy provoked by a deregulation of the TFEB-dependent CLEAR signalling pathway that is responsible of lipid accumulation. Notably, LLMs sorted from tumors display a specific transcriptional landscape and protein profile that distinguish them from other TAMs subsets. Finally, a gene signature enriched in LLMs appear in anti-PD1 and anti-CTLA4 refractory melanoma patients and this enrichment is even observed prior to treatment initiation, suggesting that LLMs may be predictive of ICIs response.

Together, our findings identify a heterotypic crosstalk involving LLMs and cancer cells that drives tumor aggressiveness and is implicated in resistance to immunotherapies.

P087

ENGINEERING SMART MOLECULES TO STIMULATE THE IMMUNE SYSTEM

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Activating the immune system against cancer is becoming an increasingly effective therapeutic option that can result in dramatic and durable responses. One approach to achieve the reactivation of endogenous antitumor T cells is by disrupting interactions between PD-1/PD-L1 on T cells. However, only limited cancer patients (15-25%) respond to anti-PD-1/PD-L1 immunotherapy. The expression of PD-L1 in tumours correlates with response of immunotherapy. Interestingly, it was found that tumour cells exposed to DNA reactive species express high levels of PD-L1. Tumours with high mutation rates "tumour mutation burden" (TMB), tend to be more sensitive to immunotherapy than their counterparts. Furthermore, current inhibitors of PD-1/PD-L1 interaction are expensive antibodies with limited applications as single agents. Hence, several small molecule inhibitors against PD-1/PD-L1 are being investigated as oral bioavailable agents that can be used in combination therapy. In this context, we developed a new strategy that consists of engineering smart small molecules to behave as inhibitors of PD-1/PD-L1 and DNA damaging agents, with the purpose of increasing TMB and tumour associated antigens (TAA), thereby enhancing the immunogenicity of refractory tumours. Here we report on the biological activities of such compounds synthesized in our laboratory.

Melanoma cell line B16-F10 was used to evaluate the potency the new molecules using the sulforhodamine B (SRB) assay. Homogenous time-resolved fluorescence (HTRF) binding assay was used to determine the IC50 values associated with inhibition of PD-1/PD-L1. Drug metabolism in extracted cells was measured by LC-MS.

Structure activity relationship in a series of smart molecules show that: (1) a biphenyl scaffold is more tolerated than their sulfonamide counterpart (2) the PD-1/PD-L1 potency was increased by 24-fold when the sulfonamides were replaced by a biphenyl (3) analysis of intracellular metabolites revealed three main metabolites that can only result from the release of the short-lived promutagenic species.

Smart molecules with the biphenyl scaffold could be used to make the proof-of-concept of the approach. The biphenyl scaffold is optimal for maintaining strong PD-L1 binding potency and contributing to cell death. Hydrolytic cleavage of the alkylating agent is indirect evidence of the formation of the alkylating species required to induce promutagenic lesions.

P088

THE ROLE OF ADAT3 IN CYTOTOXIC T CELL FUNCTION FOR CANCER IMMUNOTHERAPY

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Cancer immunotherapy shows great promise in utilizing the patient's immune system to treat cancer achieving long-lasting cures. Unfortunately, most patients do not respond well to this growing approach. A key aspect to promote effective immunotherapy is better understanding our immune cells, particularly the critical cytotoxic T cells (CTLs) within the tumor microenvironment. Dysfunctional states in CTLs, including impaired proliferation, activation, and killing, pose significant challenges to obtain successful treatment. To optimize CTLs functions, such as rapid proliferation, activation, and killing capabilities, robust protein synthesis is essential. Yet, the precise basic regulation of protein synthesis in T cells remains poorly understood. Therefore, our objective is to comprehensively explore the various steps involved in protein synthesis by targeting tRNA modification enzymes, which play a pivotal role in the translational process and are critical for robust anti-tumor immunity. Specifically, we investigate ADAT3, an evolutionarily conserved tRNA modification enzyme that converts adenosine to inosine at position 34 of tRNAs through deamination.

To investigate the role of ADAT3 *in vivo*, we generated ADAT3 flox/flox mice using CRISPR-Cas9 technology and crossed them with CD4 cre-recombinase mice to obtain T cell-specific ADAT3 knockout (T-ADAT3 KO) mice. Subcutaneous tumor models were established using MC38 colon cancer cells, Yummer melanoma cells, and B16 melanoma cells, with tumor growth closely monitored. Tumor-infiltrating lymphocytes were analyzed using flow cytometry. For proliferation assessment, isolated T cells were labeled with cell trace violet dye (CTV), cultured with anti-CD3/CD28 antibodies, and analyzed for CTV dilution by flow cytometry. Antigen-specific T cell responses were evaluated by intravenous injection of *Listeria monocytogenes* expressing OVA, followed by sacrifice on day 7 for spleen analysis. MHC tetramers loaded with OVA peptide were employed for tetramer staining to identify antigen-specific T cells. Additionally, a homeostatic proliferation assay was conducted by isolating T cells from both littermate control and T-ADAT3 KO mice, labeling them with CTV, and adoptively transferring them into RAG lymphopenic recipient mice. After 3 days, proliferation and expansion of the transferred T cells were assessed.

In various mouse tumor models, we observed accelerated tumor growth in T cell-specific ADAT3 knockout (T-ADAT3 KO) mice. Further analysis of tumor-infiltrating lymphocytes from T-ADAT3 KO mice revealed reduced OVA-specific T cells compared to littermate control mice in OVA-expressing tumor models. Defective proliferation was also observed in ADAT3-knockout T cells upon activation, as well as normal homeostatic proliferation of naïve T cells under lymphopenia conditions. Additionally, infection with *Listeria monocytogenes* expressing OVA resulted in significantly reduced antigen-specific T cells in T-ADAT3 KO mice compared to littermate control mice on Day 7 of the peak response.

In summary, our findings highlight the significance of ADAT3 in promoting T cell proliferation and the control of tumor growth. Understanding the role of ADAT3 and other aspects of translation will be crucial for the development of better effective cancer treatments.

P089

BACTERIOPHAGE EVASION OF CYCLIC NUCLEOTIDE-BASED IMMUNITY

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Viral infection is a ubiquitous threat to all known cellular life forms. To overcome this threat, prokaryotes and eukaryotes encode a diverse array of defense systems that prevent viral replication or transmission. Since their initial discovery, eukaryotic innate immune pathways have been considered unique to eukaryotic organisms. However, recent studies have overturned this idea and it is now clear that many eukaryotic innate immunity pathways are evolutionarily derived from prokaryotic genes. CBASS is an anti-phage defense system in diverse bacteria that is homologous to the eukaryotic cGAS-STING pathway and uses cyclic nucleotide signals to induce cell death and prevent viral propagation. Phages use multiple strategies to defeat host CRISPR and restriction-modification systems, but mechanisms to evade CBASS immunity are just now beginning to be understood.

To identify mechanisms of CBASS evasion, we devised a biochemical screen to identify phages capable of degrading cyclic nucleotide immune signals. We then used activity-guided biochemical fractionation to identify specific viral genes responsible for cyclic nucleotide cleavage.

Using a biochemical screen of 57 phages, we discover Acb1 from phage T4 as a founding member of anti-CBASS evasion proteins that counteract defense by specifically degrading cyclic nucleotide signals that activate host immunity. Crystal structures of Acb1 in complex with 3'3'-cGAMP define a mechanism of metal-independent hydrolysis 3' of adenosine bases, enabling broad recognition and degradation of cyclic di- and trinucleotide CBASS signals. We further show that Acb1 blocks downstream effector activation and protects phages from multiple CBASS defense systems in vivo.

We conclude that the primary role of Acb1 is a CBASS evasion protein. I will also present recent results that uncover a broader role of Acb1 in replication of T4-like phages, and highlight additional layers of complexity in the interaction between bacteriophages and CBASS immunity.

P090

FUNCTIONAL ACTIVITY OF CARCIK-CD19 CELLS COMPARED TO UNMODIFIED CIK CELLS COMBINED WITH BISPECIFIC ANTIBODY BLINATUMOMAB

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Although cytokine Induced Killer (CIK) cells showed anti-tumor activity in vitro and in vivo, there remains a need to increase their efficacy and specificity. This could be reached using either bispecific antibodies or chimeric antigen receptor (CAR) modifications. The aim of this study was to compare the function of CIK cells combined with soluble CD3xCD19 bispecific antibody Blinatumomab (CIK-Blina) or modified with two different anti-CD19 CAR molecules (CARCIK-MNZ and CARCIK-BG2). The CARCIK-MNZ are being used by our group in clinical trials (FT01CARCIK and FT03CARCIK; Eudract n. 2017-000900-38 and 2020-005025-85) and the CARCIK-BG2 was designed on the structure of Tisagenlecleucel CAR (Novartis), cloned however in a transposon vector.

Unmodified CIK cells were generated in 21 days cultures, starting from peripheral blood mononuclear cells (PBMCs) in the presence of IL-2. To generate CAR-modified CIK cells, PBMCs were co-transfected on day 0 with the two plasmids encoding the Sleeping Beauty (SB) transposase and the CAR-CD19 molecules and then followed CIK cells expansion protocol. In vitro functional assay were performed in response to the CD19+ target cell lines REH and Daudi, at different effector to target ratios (E:T). The immune synapsis was evaluated by fluorescent microscopy live imaging. In vivo experiments were conducted on NSG mice inoculated with Daudi cell line and then infused with CARCIK-CD19 cells or CIK cells, the latter alone or followed by daily Blinatumomab infusions for the first three weeks.

Either CARCIK-MNZ, CARCIK-BG2 or CIK+Blina demonstrated to be cytotoxic against CD19+ target cells (mean cytotoxicity of 55.8%, 30.9% and 36.1%, respectively versus 11.2% for CIK alone against REH cell line at E:T 10:1)($p < 0.05$ in all cases). Proliferation of CIK+Blina and CARCIK-MNZ was slightly higher than that of CARCIK-BG2 (mean proliferation indexes in presence of REH cell line 11.4, 12.6 and 6.4 at E:T 1:1, respectively)($p < 0.05$). All cell types released IFN- γ , TNF- α and IL-8 in response to REH cells (E:T 1:1). IFN- γ production was significantly higher for CIK-Blina compared to all the other effectors ($p < 0.05$) and IL-2 was significantly higher for CARCIK-MNZ ($p < 0.05$). All the effectors activated NFAT and NF- κ B signaling pathways in response to antigen binding at similar levels. Looking at the immune synapsis, CIK+Blina, CARCIK-MNZ and CARCIK-BG2 generates a similar frequency of synaptic events, significantly higher compared to unmodified CIK cells alone (respectively 21.8%, 15.7%, 34% and 4.3%, E:T 3:1 against REH cell line)($p < 0.05$). Looking also at the speed of the effector cells in co-culture with REH cell line, we confirmed the same ratios between effectors. In vivo animal models infused with unmodified CIK cells and receiving Blinatumomab for the first three weeks showed a significant increase of survival compared to CIK cells alone ($p < 0.01$), moreover the occurrence of circulating CD19+ cells was delayed. However, mice receiving CARCIK-MNZ and CARCIK-BG2 showed a good expansion of circulating CD3+ cells, persisting until sacrifice, and also the absence of CD19+ cells, both circulating and in the other organs analyzed (bone marrow, spleen and kidney).

These data suggest that the addition of Blinatumomab renders CIK cells as efficacious in vitro as gene-modified CARCIK-CD19 cells, generated using different CAR-CD19 molecules. Blinatumomab improve the ability of CIK cells to generate immune synapsis following target addition in a similar manner compared to the CAR expression. Finally, in vivo mouse model demonstrated a more

lasting response to CARCIK-CD19 cells compared to the combination of CIK cells and Blinatumomab, comparable between the two CAR constructs. Overall, these data demonstrated that the combinatorial use of unmodified cells with bispecific antibodies can be a valid alternative to genetically modified CARCIK cells.

P091

MODULATING THE TUMOR MICROENVIRONMENT BY TARGETING CD40 – FROM THE BENCH TO THE BEDSIDE AND BACK

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While immunotherapy has revolutionized the treatment of several cancer types, some patients do not respond to current therapies, necessitating new ways to mobilize the immune system to fight cancer.

CD40 is a TNF receptor superfamily member expressed on antigen-presenting cells (APCs, e.g. monocytes and dendritic cells) and is one of the most potent activators driving the adaptive immune response. Several first-generation CD40 agonistic antibodies (Abs) have been tested in clinical trials, however, their efficacy has been limited and accompanied by dose-limiting systemic toxicities (thrombocytopenia and transaminitis).

Our hypothesis is that CD40 is expressed on dendritic cells within the tumor and tumor-draining lymph nodes. Engagement of CD40 by optimized agonistic anti-CD40 Ab will lead to maturation and activation of the APCs, enhance antigen presentation and recruitment of cytotoxic CD8 T cells, which will result in tumor elimination.

Efficient CD40 agonism requires multimerization of the protein, which is challenging to achieve by agonistic Abs. We have previously demonstrated that when the Fc portion of anti-CD40 Abs binds to the inhibitory Fc-Receptor (FcR) FcRIIB - the Abs provide the necessary crosslinking, support CD40 trimerization and promote maturation and activation of the CD40-expressing APCs. Therefore, we engineered the Fc portion of a human IgG1 anti-CD40 agonistic antibody (clone 2141) by introducing several point mutations (termed herein: the V11 modification) to selectively increase its binding affinity to FcRIIB. To mitigate systemic adverse events - we switched the administration route from systemic to in situ (injecting the Abs directly into tumors). Finally, we generated a unique murine strain that allows evaluating Abs designed for the clinic in an immunocompetent setting – mice that express human CD40 and human FcRs, while lacking murine CD40 and murine FcRs (termed herein: hCD40/hFcR mice). We implanted syngeneic murine tumor cells in these mice, treated with CD40-targeting Abs and characterized their impact on both tumor growth kinetics and the immune landscape.

Following pre-clinical toxicology studies in Non-Human Primates, we have conducted a Phase I 3+3 dose escalation trial (n=12), in which the Fc-engineered CD40-agonistic antibody (2141-V11) was injected into one/two lesions, per study protocol (NCT04059588).

Our in vivo findings demonstrate that in situ administration of CD40-targeting Abs leads to complete elimination of orthotopic murine breast tumors. Moreover, in a bilateral tumor challenge, both tumors fully regress, despite administering the Ab only to a single tumor. This abscopal effect suggests a robust systemic immune activation, which was supported by an increase in serum cytokine levels such as IFN γ , TNF α , IL-15, IL-18, CXCL10, CCL7 and IL-6. Multiplex IHC staining of regressing tumor sections demonstrated formation of tertiary lymphoid structures (TLS). Furthermore, mice that rejected the tumors upon treatment were fully protected from a tumor re-challenge, suggesting development of a long-term anti-tumor immune memory. Depletion studies and adoptive cell transfer experiments showed that this memory response was mediated by CD4 and CD8 T-cells, but not B-cells.

In the clinical trial, there have been no DLTs, including no changes to platelets or transaminases. Adverse events included G1-G2 injection site reaction and G1-G2 fever. Moreover, biopsy and serum analyses from treated patients confirmed our in vivo findings and both TLS formation and an

increase in cytokine serum levels. Most importantly, two patients achieved Complete Response (CR).

These in vivo and patient data jointly demonstrate the efficacy and safety of intra-tumoral administration of Fc-enhanced agonistic CD40 antibody therapy in cancer and describe a potential unique mechanism of action compared to other intralesional immunotherapies.

P092

RADIATION-INDUCED CXCL10 RELEASE FROM TUMOR-ASSOCIATED MACROPHAGES SHAPES T CELL INFILTRATION

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Radiotherapy (RT) induces antitumor immunity in preclinical models fostering priming and recruitment of CXCR3⁺ effector T cells. One of the most abundant immune cell populations in the TME are macrophages which are important regulators of leukocyte trafficking. Here, we investigated the effects of RT on macrophage cancer cell interactions and their influence on immune cell migration.

We used a bilateral syngeneic subcutaneous tumor mouse model to assess radiation-induced immune infiltrates in ipsilateral and abscopal tumors by flow cytometry. Moreover, we established an in vitro coculture model of human monocyte-derived macrophages with cancer cells to study radiation-induced effects on cell-cell interactions. RNA-Seq, RT-qPCR and flow cytometry were used to evaluate macrophage phenotype. Chemokine secretion was quantified using multiplex immunoassays and ELISA. Leukocyte migration toward conditioned media was assessed using modified Boyden chamber assays.

In vivo, RT increased the number of CXCR3⁺ T cells in the TME of irradiated tumors but not at the contralateral tumor site. Macrophages were the most abundant immune cell population in the post-RT TME, while dendritic cell numbers remained low. In vitro, we also observed CXCR3⁺ lymphocyte migration towards irradiated macrophage cancer cell cocultures. RT evoked an IFN response gene signature in macrophages and shaped polarization states with increased expression of costimulatory molecules. CXCL10 was specifically upregulated in supernatants of irradiated cocultures but not in monocultures. Mechanistically, CXCL10-release depended on direct macrophage cancer cell contact and CXCL10 neutralization abrogated directional migration of CXCR3⁺ immune cells.

Taken together we identified macrophages as important regulators of CXCR3⁺ effector T cell recruitment through CXCL10 release after RT. Modulating macrophage cancer cell interactions may be of help increase effector T cell infiltration and shape anti-tumor immunity.

P093

GASDERMIN E ANTAGONIZES IMMUNE-DRIVEN MUTAGENESIS BY LIMITING SURVIVAL FROM SUBLETHAL CASPASE ACTIVATION

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Apoptosis was traditionally synonymous with cellular demise. However, in recent years, numerous studies have demonstrated that cancer cells can survive apoptosis induction, resulting in mutagenesis and enhanced aggression in the progeny of cells that have survived caspase activation. Amongst the substrates of caspase-3 is the Inhibitor of Caspase Activated DNase (ICAD/DFFA/DFF45), who primarily functions as the chaperone of Caspase Activated DNase (CAD/DFFB/DFF40). Cleavage of ICAD by caspase-3 results in subsequent ICAD degradation, thus releasing CAD to initiate DNA fragmentation. In cells that survive apoptosis induction, CAD dependent DNA nicks thus serves as a source of mutagenesis. Despite the initial report of the rapidity of CTL in killing leukemic cancer cells, it has been observed since the 2000s that adherent tumor cells are highly resistant to CTL killing. These observations suggest an intriguing implication: that the majority of CTL contacts with target cells may not result in target cell demise. We thus wondered whether CTL's failure to kill tumor cells may still trigger sufficient caspase activity to drive DNA damage, resulting in mutagenesis in cells that survived CTL attack. Another factor that may impact tumor cell sensitivity to apoptosis inducers is the recently discovered pore-forming protein Gasdermin E (GsdmE). Cleavage of GsdmE by Caspase-3 releases the N-terminal cytotoxic domain to oligomerize to form pores in the plasma membrane, disrupting osmotic balance and resulting in rapid lysis of the cell after Caspase-3 activation. GsdmE is frequently downregulated or silenced in tumor tissues and cell lines alike, positing it as a putative tumor suppressor, although its exact mechanism of tumor suppression is unknown.

B16F10 murine melanoma line and Neuro2a murine neuroblastoma cell line used in vitro and in vivo.

In the present study, we elucidate failed apoptosis downstream of CTL attack as a potential source of tumor mutagenesis and contributing to possible immune escape and tumor evolution. We found that tumor cell expression of GsdmE, a pore forming executioner protein activated by Caspase-3, is a major determining factor to tumor cell resistance to CTL attack. GsdmE expression truncated the apoptotic process and diminished the recovery of cells undergoing cytotoxic cell-induced apoptosis. Consequently, loss of GsdmE allowed enhanced recovery of cells undergoing apoptosis, resulting in increased subsequent mutagenesis resulting from the activation of Caspase Activated Nuclease (CAD) triggered by Caspase-3 activation. We found that immune surveillance hindered the growth of GsdmE expressing tumors in vivo, with immune memory development correlating with the dependency upon CD8 T cells for initial tumor elimination. Using human TCGA datasets, we confirmed a negative association between tumor GSDME expression and genetic instability, as measured by higher mutational burden as well as rates of insertion/deletion. This negative correlation grew in strength with increasing scores for CD8 T cell gene signature. Furthermore, an inverse relationship regarding tumor mutational burden could also be established between GSDME and CAD expression, suggesting an interplay between these two proteins in contributing to human tumor genetic instability as well. Lastly, we found that in a model of forced GsdmE expression, tumors that outgrew immune surveillance enriched for cells that have lost GsdmE expression, a phenotype heavily contributed by CD8 T cells.

We conclude that GsdmE functions to truncate the apoptotic process under immune surveillance during tumorigenesis, thus limiting immune-driven tumor mutagenesis. Our results suggest the possibility that the known relationship between tumor mutational burden and immunotherapy response is not necessarily due to the number of mutations needed to enlist an immune response. Rather, pre-existing immune pressure may also play a role in driving tumor cell mutagenesis.

P094

STAT3 OPERATES AS A RHEOSTAT REGULATING SENSITIVITY OF NK CELLS TO INFLAMMATORY CYTOKINES

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Natural killer cells are innate lymphocytes that respond to a variety of cytokines during viral infection. Several homeostatic and inflammatory cytokines bind to receptors that signal via STAT3. Previous studies have suggested that STAT3 suppresses NK cell function in tumors. However, other studies have found STAT3 to be important for NK cell function, suggesting that STAT3 may play a context-dependent role in NK cells. In our study we seek to dissect the mechanism of the context-specific function of STAT3 in NK cells.

To study context-dependent STAT3 function, we use conditional STAT3^{-/-} mice (NKCrt STAT3^{flox/flox}) in a model of mouse cytomegalovirus (MCMV) infection. For a mechanistic analysis, we use RNA-seq, ATAC-seq and CUT&RUN to analyze binding of STAT transcription factors.

We found that NK cells deficient in STAT3 expand less than wildtype (WT) NK cells during mouse cytomegalovirus (MCMV) infection. IL-21R and IL-10R are the only receptors expressed on NK cells that signal through STAT3 homodimers. However, IL-21R or IL-10R-deficient NK cells showed no or little expansion differences, respectively, compared to WT controls, suggesting additional cytokine receptors may be signaling through STAT3 during viral infection. To evaluate whether STAT3 regulates the sensitivity of NK cells to cytokines that signal through the heterodimerization partners STAT1 and STAT5, we performed cytokine titration experiments. We observed that sensitivity towards IFN- α and IL-15 was markedly reduced in STAT3-deficient NK cells.

We have previously found that NK cells require inflammatory cytokines for antiviral expansion but will proliferate poorly in hyper-inflammatory environments. Because STAT3 appears to regulate the sensitivity of NK cells to inflammatory cytokines, we hypothesized that STAT3-deficient NK cells would be shielded in settings of hyper-inflammation. Indeed, using high-dose MCMV infection, we observed that STAT3-deficient NK cells expanded better than WT NK cells. In high-dose infection, we found earlier expression of IL-10 and higher amounts of type-I interferon. Furthermore, NK cells during high-dose infection were characterized by higher type-I-interferon signaling which was attenuated in STAT3-deficient NK cells. Thus, we hypothesized that STAT3 might confer its context-dependent role by regulating sensitivity of NK cells to type-I interferon.

Mechanistically, *in vitro* stimulation revealed that IL-10 but not IFN- α induces sufficient STAT3 phosphorylation and genomic binding assessed using STAT3 CUT&RUN. IL-10 stimulation of WT and STAT3-deficient NK cell showed that only a limited number of genes are induced by STAT3, but IL-10 stimulation induced rapid and STAT3-dependent epigenetic remodeling including at regions bound by STAT1.

In summary, we believe STAT3 operates as a rheostat, modulating the sensitivity of NK cells to type-I interferon predominantly through epigenetic remodeling. Our data indicate that STAT3 inhibitors tested in cancer patients under the assumption that STAT3 suppresses NK cell function might differently affect NK cells in 'cold' and 'hot' tumors.

P095

DIRECTING CAR T CELLS TO THE BRAIN OR OTHER SPECIFIC TISSUES WITH COMPUTATIONALLY PREDICTED OPTIMAL SENSING ANTIGENS

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In principle, therapeutic cells could be programmed to recognize specific tissues or organs, providing a novel approach for increasing therapeutic specificity and minimizing off-target toxicities. We have been developing sensors for engineering CAR T cells to recognize specific tissues as part of a multi-factor disease recognition program. However, the human body is composed of diverse organs, tissues, and cell types, each having distinct yet complex gene expression patterns. Given the complexity and similarity of the transcriptomic and proteomic profiles of these tissues, it remains challenging to compartmentalize the human body based on genetic signatures.

Here, we computationally developed an antigen-based catalog of humans that has the ability for engineered T cells to distinguish between different tissues. Specifically, we introduced a machine learning-based framework using large-scale omics datasets to navigate the composition of human tissues to uncover tissue-specific single and combinatorial antigen circuits.

We have experimentally tested the top predicted antigens for the ability to precisely and flexibly direct T cells towards or away from tissues when mapping the human body and delivering immunotherapies. We have also validated the efficacy of specific brain recognition T cells, and demonstrated how these can be incorporated into more effective and robust anti-brain tumor CAR T circuits.

In summary, by harnessing machine learning techniques, we are able to accurately predict discriminatory candidate multi-antigen circuits for the designing of next generation CAR T cell therapies for challenging solid tumors with tissue-specific recognition strategies.

P096

GPR35 AND MEDIATOR(S) FROM PLATELETS AND MAST CELLS IN INNATE IMMUNE CELL MIGRATION DURING (AIRWAYS) INFECTIONS AND CANCER

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Cell migration is crucial for immune responses to infections or cancer, and G-protein coupled receptors (GPCRs) play a significant role in regulating this process. Recently, we showed that the orphan GPCR GPR35 is upregulated in activated neutrophils to facilitate their recruitment upon inflammation and infections, in response to platelet and mast cell derived 5-hydroxy-indole-acetic acid (5-HIAA) (De Giovanni et al., Cell 2022).

However, whether GPR35 plays a role in more clinically relevant contexts is unknown.

To study the role of GPR35 during murine airways infections and cancer, we applied a combination of advanced imaging approaches, *in vivo* airways infection experiments, spectral flow cytometry and *in vitro* assays.

We have now obtained evidence indicating the involvement of GPR35 and 5-HIAA in promoting pathogenic eosinophil recruitment to fungal-infected airways. In this regard, our data suggest that GPR35-mediated eosinophil accumulation influences Th1/Th2 balance within infected lungs and supports fungal replication (De Giovanni et al., Immunity 2023). Thus, inhibition of the GPR35/5-HIAA ligand receptor system may be beneficial during fungal airways infections. Intriguingly, there is emerging evidence linking GPR35 expression in cancer and immune cells to tumor growth, suggesting its translational potential in cancer. Consistent with this notion, our preliminary data indicate that GPR35 supports tumor growth and regulates the accumulation of innate immune cell subsets within tumors.

In conclusion, our findings shed light on GPR35 as an important regulator of immune cell recruitment during disease and uncover its translational potential in the context of inflammation, airways infections and cancer.

P097

PYK2 SIGNALING IN GBM TUMOR CELLS CONTRIBUTES TO THE REGULATION OF AN IMMUNOCOMPETENT MICROENVIRONMENT

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Glioblastoma (GBM) is a highly aggressive brain cancer that is typically fatal within a year of diagnosis. Our previous studies demonstrated a positive relationship between the activation of a protein called proline-rich non-receptor tyrosine kinase (Pyk2) in GBM cells and the expression of cytokines by tumor-associated myeloid cells (TAM cells), leading to a sustained increase in the expression levels of monocyte chemoattractant protein 1 (CCL2), granulocyte-macrophage colony-stimulating factor (GM-CSF), IL10, IL12, and vascular endothelial growth factor (VEGF), as well as an increase in the CD86+/CD206+ TIM population. We hypothesize that Pyk2 signaling in GBM cells is involved in the regulation of the release of inflammatory cytokines, which in turn suppresses the immune response in the tumor microenvironment.

CRISPR/Cas9 knock out the Pyk2 (Pyk2KO) and wild-type (Pyk2WT) GL261 mouse glioma cells and GL261-C57Bl/6 GBM model were used in the study. RT-PCT and flow cytometric analysis of TAM cells were used for used to identify cytokines expression profile, TAM cell composition and activation state.

Analysis of the cytokines present in the cell culture medium conditioned by Pyk2KO and Pyk2WT cells identified a decrease in the release of CCL2 and CCL5 in the Pyk2KO cells compared to the Pyk2WT cells. RT-PCR analysis of myeloid cell population, isolated from Pyk2WT and Pyk2KO tumors in the GL261-C57Bl/6 GBM model, identified a significant down-regulation of key pro-tumorigenic factors such as CCL2, CCL12, CCL5, tumor necrosis factor (TNF), VEGF and epidermal growth factor (EGF) in the Pyk2KO tumors compared to the Pyk2WT tumors. Additionally, flow cytometric analysis revealed a reduction in myeloid-derived suppressor cells (MDSCs), an increase in lymphoid dendritic cells (DC), and up-regulation in TNF/IFN γ expressing and antigen-specific CD8+ T cells in Pyk2KO tumors. Furthermore, a significant decrease in pro-tumorigenic Ly6C+/CD206+ and an up-regulation of inflammatory Ly6C+/CD86+ myeloid cells were found in the Pyk2KO tumors.

These findings suggest that Pyk2KO tumors display an immunocompetent microenvironment with enhanced phagocytic and cytotoxic function compared to Pyk2WT tumors.

P098

EPIGENETIC REPROGRAMMING OF TUMOR-ASSOCIATED MACROPHAGES

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Tumor-associated macrophages (TAMs) are associated with poor prognosis in multiple solid tumor types and can block the efficacy of existing immunotherapies. Re-programming TAMs from an immunosuppressive “tumor-promoting” to a “tumor-fighting” phenotype has shown promise for treating cancer in pre-clinical models. We hypothesized that tumor-promoting macrophages can be converted into tumor-fighting macrophages by targeting epigenetic regulators.

The BAF (Brg/Brahma associated factor) complex is a multi-subunit nucleosome-remodeling complex that controls cell type-specific chromatin accessibility and gene expression, including regulating inflammatory gene networks. To study the role of the BAF complex in tumor-associated macrophages, we used a mouse model with deletion of ARID1A (the largest subunit of the BAF complex) driven by the myeloid-lineage specific LysM-cre allele.

Tumor growth was slowed in mice lacking myeloid-ARID1A compared to controls. RNA-sequencing of TAMs sorted from subcutaneous MC38 colon cancer tumors, revealed that deletion of ARID1A leads to an enrichment of an interferon-responsive gene expression signature, including upregulating of the gene-encoding the co-stimulatory CD86 molecule and the immunosuppressive PD-L1. Mice with myeloid-specific deletion of ARID1A displayed heightened sensitivity to anti-PD-L1 therapy compared to control mice. Increased sensitivity to anti-PD-L1 was correlated with increased cell surface expression of MHC class II and CD86 on TAMs and increased infiltration of CD8+ T cells in myeloid-specific-ARID1A-deficient hosts. We used an in vitro co-culture system and determined that the upregulation of these molecules is driven by a cell-intrinsic mechanism.

Our results indicate that the canonical BAF complex is a critical regulator of tumor-macrophage transcription and function and that myeloid-targeted ARID1A inhibition may boost response to anti-PD-L1 immunotherapies.

P099

ACETYLCHOLINE/A9 NICOTINIC ACETYLCHOLINE RECEPTOR AXIS REGULATES THYMOCYTE NEGATIVE SELECTION

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Signaling pathways previously thought to be specific to the nervous system are increasingly being discovered to play important roles in the immune system. Acetylcholine (ACh) and its receptors (AChRs) are widely expressed in various kinds of immune cells and fine-tune both innate and adaptive immune responses. However, whether cholinergic signaling regulates immune cell development is unclear.

In this study, our objective was to determine the roles of ACh and AChRs in thymocyte development. To achieve this, we employed a comprehensive approach involving bioinformatics, mouse genetics, as well as biochemistry and molecular biology techniques to systematically investigate the functions of ACh and AChRs in thymocyte development.

We show that mouse CD4+CD8+ double positive (DP) thymocytes express high levels of $\alpha 9$ nicotinic (n) AChR, the expression of which is dependent on transcription factor Heb, but is repressed by TCR signaling. Although *Chrna9*^{-/-} mice lacking expression of $\alpha 9$ nAChR possess seemingly normal thymocyte populations, defective thymocyte development is evident in a mixed bone marrow chimera experimental setting. In addition, using OT-I and OT-II TCR transgenic mice and transgenic mice expressing ovalbumin under the control of the rat insulin promoter (RIP-mOVA), we demonstrate that $\alpha 9$ nAChR-mediated signaling does not affect positive selection, but prevents excessive negative selection of both CD4 single positive (SP) and CD8 SP thymocytes. Mechanistically, $\alpha 9$ nAChR signaling regulates calcium homeostasis in thymocytes and helps to maintain proper surface levels of TCR molecules during TCR stimulation. Furthermore, we demonstrate that thymic tuft cells, B cells, and a small number of T cells express choline acetyltransferase (ChAT) and are sources of ACh in the mouse thymus.

Our results reveal a new mechanism of how a cholinergic signaling pathway (ACh/ $\alpha 9$ nAChR axis) can regulate immune cell development and thus may be implicated in the etiology of autoimmune disorders and cancer.

P100

CLINICAL SIGNIFICANCE OF PLASMA AMINO ACID PROFILING FOR PREDICTING PROGNOSIS IN NSCLC PATIENTS TREATED WITH IMMUNE CHECKPOINT INHIBITORS

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Immune checkpoint inhibitors (ICI) have significantly improved the prognosis of non-small cell lung cancer (NSCLC). However, only a limited proportion of patients can benefit from this therapy, and clinically useful biomarkers for stratifying patients who will benefit from ICI treatment remain to be elucidated. Amino acid metabolism is essential for tumor cell proliferation and regulation of anti-tumor immunity. Nevertheless, the clinical significance of amino acids and tryptophan-related metabolites in plasma has not been fully understood in patients with NSCLC who receive ICI.

Peripheral blood samples were collected from 53 patients with NSCLC before treatment with PD-1 (Programmed cell death-1) inhibitor. The plasma concentrations of 21 amino acids, 14 metabolites, and neopterin were measured by liquid chromatography-mass spectrometry. Cox proportional hazard analysis was performed to establish a multivariate model with these variables for stratifying patient overall survival (OS). Gene expression in peripheral blood mononuclear cells (PBMCs) was compared between the high-risk and low-risk patients stratified by this multivariate model.

Cox hazard analysis demonstrated that higher concentrations of seven amino acids (glycine, histidine, threonine, alanine, citrulline, arginine, and tryptophan) as well as lower concentrations of three metabolites (3h-kynurenine, anthranilic acid, and quinolinic acid) and neopterin in plasma were significantly associated with better OS ($p < 0.05$). In particular, the multivariate model, composed of a combination of serine, glycine, arginine, and quinolinic acid, was most efficient for stratifying patient OS (concordance index=0.775, HR=3.23, 95% CI 2.04 to 5.26). The transcriptome analysis in PBMCs showed that this multivariate model was significantly associated with the gene signatures related to immune responses, such as CD8 T-cell activation/proliferation and proinflammatory immune responses. In addition, 12 amino acid-related genes, including SLC1A3, HAAO, PHGDH, AANAT, ALAS2, FAH, BCAT1, SLC11A1, GLUL, DCT, SLC6A13, and TPH1, were differentially expressed between the high-risk and low-risk groups. Notably, among them, higher expressions of 3 amino acid-related genes, including, SLC11A1, HAAO, and PHGDH, were negatively correlated with OS (Log-rank test, $P < 0.05$)

The profiling of amino acids and metabolites in plasma might be useful for stratifying patients who will benefit from ICI treatment. The next phase of clinical trials to validate this multivariate model have been ongoing as a multicenter cohort study in patients with NSCLC and gastric cancer who receive ICI.

P101

IMMUNOTHERAPY TARGETING TUMOR-SPECIFIC ENDOGENOUS RETROVIRAL ELEMENTS LEADS TO STRONG IMMUNE-MEDIATED TUMOR CONTROL IN PRECLINICAL MODELS

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Personalised immunotherapies that selectively target unique tumor mutations, so-called neoantigens, have demonstrated promising results in early clinical trials. However, the successful design of these therapies requires a relatively high number of tumor mutations, thereby rendering many patients ineligible for such treatments. Therefore, there is a need to broaden the accessibility of personalised therapies. In the presented work, we explored the feasibility of using an alternative source of cancer antigens, Endogenous Retroviral Elements (EVEs), to design cancer immunotherapies. EVEs are remnants of ancient viruses incorporated into the human genome and usually remain transcriptionally silent. However, cancer can trigger their expression, and hence, EVEs can potentially serve as promising cancer targets. To this end, we developed an in silico, AI-guided target discovery platform that analyses the tumor transcriptome to identify, rank, and select the best EVE-derived epitopes for vaccine design based on their predicted likelihood to be presented on MHC molecules and elicit an immune response. We applied this platform to design immunotherapies tailored to the EVE profile of established tumor models and tested the ability of EVE-targeting therapies to protect mice from tumor challenge. Finally, to explore the clinical relevance of EVE-targeting immunotherapy, we investigated whether the number of expressed EVEs can stratify cancer patients receiving immunotherapy based on their overall survival.

To assess whether EVE-based immunotherapy can confer tumor protection, mice were immunised prior to subcutaneous tumor challenge and subsequently monitored for tumor growth. To explore the induction of vaccine-specific CD8⁺ T cells, blood was collected and stained with fluorescently labelled antibodies and a multimer specific to a vaccine EVE epitope followed by analysis using Flow Cytometry. To investigate the presence of functional T cells, isolated splenocytes were re-stimulated with vaccine-matching peptides in an IFN γ ELISPOT assay. For analysis of the tumor compartment, single cell suspensions of isolated tumors were stained with antibodies specific for leukocytes, CD4⁺ and CD8⁺ T cells and analysed with Flow Cytometry. For stratification of metastatic melanoma patients based on their EVE burden, sequencing data from pre-treatment tumor biopsies and healthy tissue were used to compute the number of expressed EVEs and somatic mutations.

Our work demonstrated that vaccination with in silico selected EVEs leads to strong anti-tumor effect in preclinical models. Complementary immune analyses indicated an expansion of EVE-specific CD8⁺ T cells in the blood and highlighted the presence of functional T cells in vaccinated mice. Furthermore, immune characterisation of isolated tumors from vaccinated mice revealed strong enrichment of leukocytes, driven by an increased influx of CD4⁺ and CD8⁺ T cells in the tumor microenvironment. Finally, analysis of pre-treatment tumor samples of patients receiving immunotherapy revealed that the number of expressed EVEs could specifically stratify patients with few somatic mutations into groups of differential survival. This suggests that EVEs may compensate for the scarcity of targetable neoantigens and serve as alternative targets for clinically meaningful T-cell responses.

These data provide strong argumentation that immunotherapies targeting tumor-specific EVEs can lead to anti-tumor effects in preclinical models and that EVEs may serve as a complementary source of potent T-cell targets in patients with few somatic mutations, thus expanding the applicability of personalised cancer immunotherapies.

P102

VIRAL PRE-EXISTING IMMUNITY: A “FRIEND” OF ADENOVIRAL BASED THERAPY

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Oncolytic vaccines, as highly immunogenic platforms, re-gained momentum leading to public and private investments. Indeed, oncolytic viruses propagate and kill selectively cancer cells. Simultaneously, the virus infection acts as adjuvant recruiting anti-tumor immunotherapy effectors within the cancer bed. Historically oncolytic viruses have been co-developed together with their non-replicating counterpart viral vectors, that are used for gene therapy and delivery. For classical gene therapy approaches, a high level of tissue target transduction is needed hence the presence of a pre-existing anti-viral immune response, diminishes the efficacy of that vector, hampering the entire clinical protocol. On the other hand, in the field of oncolytic viruses, and specifically oncolytic immunotherapy, it is still not clear whether pre-existing immunity to the virus is deleterious or efficacious; this aspect of the oncolytic viruses needs to be elucidated. Here, we addressed the role of viral pre-existing immunity upon treatment with a platform previously developed in our lab named PeptiCRAd.

In this study, in vitro pulsing of DCs and Macrophages was used. In vivo animal model of melanoma, breast and colon cancer. Immunophenotyping was performed by flowcytometry and IFN γ Elispot assay.

First, we investigated the impact of viral neutralizing antibodies on PeptiCRAd uptake in APC (DCs and macrophages). To this end, we used SIINFEKL as a model and we pulsed primary DCs or macrophages either with PeptiCRAd-SIINFEKL or with PeptiCRAd-SIINFEKL pre-incubated with naïve (Naïve-PeptiCRAd) or adeno positive (PEI-PeptiCRAd) murine serum. In presence of PEI-PeptiCRAd-SIINFEKL, we observed an enhanced presentation of H2Kb bound SIINFEKL and CD86 in the macrophagic population, indicating that the immune adenoviral serum directed PeptiCRAd-SIINFEKL internalization toward cross-antigen presentation pathway in an immunogenic fashion. As we sought to better characterize the uptake of PEI-PeptiCRAd in macrophages, we adopted Surface Plasmon Resonance (SPR) method to analyze the intake of PEI-PeptiCRAd in RAW 264.7, a murine macrophage cell line. Confirming our previous observations, PEI-PeptiCRAd showed enhanced interaction with RAW 264.7 in comparison to Naïve-PeptiCRAd. Next, we wanted to examine the anti-tumor effect of adenoviral pre-existing immunity in vivo in three different immunological murine cancer models. For these studies, we selected the poor immunogenic melanoma model B16.OVA, the immunogenic colon cancer model CT26, and the immunosuppressive triple-negative breast cancer model 4T1. As mice are naïve to adenoviral infections, to generate adenoviral pre-existing immunity, we subcutaneously injected mice with adenovirus. Before engrafting the tumors, we confirmed the presence of an anti-viral immune response by the detection of anti-adenovirus IgG in the serum of pre-immunized mice and by anti-adenoviral T cell in a ELISPOT IFN- γ assay. A cohort of not-adeno preimmunized mice (naïve mice) was used as a control as well. In the poorly immunogenic B16.OVA model, the tumor growth was delayed in both Naïve and PEI mice upon PeptiCRAd treatment either using the model peptide SIINFEKL or the more clinically relevant peptide TRP2. Indeed, both flow cytometry and ELISPOT IFN- γ analysis showed the generation of antigen-specific T cells in mice treated with PeptiCRAd to the same extent in naïve and PEI mice. In contrast, in the immunogenic tumor model CT26, tumor growth control was observed in Naïve and to a better degree in PEI mice. In this latter, the presence of antigen-specific T cells was enhanced in presence of vector pre-existing immunity. Currently, 4T1 tumor model is under investigation.

Overall, our data suggest that the presence of anti-adenoviral antibodies enhances the internalisation of PeptiCRAd complex in APCs, bolstering the efficiency of a vaccine platform adeno-based coated with MHC-I restricted tumor peptides.

P103

COMBINATION THERAPY WITH STEM CELL DERIVED NATURAL KILLER CELLS AND MONOCLONAL ANTIBODIES LEADS TO POTENT ADCC THROUGH ENGAGEMENT OF ENDOGENOUS CD16

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Natural killer cells (NK) are gaining traction as cell therapy products with potent anti-tumor potential for use in combination with therapeutic agents, such as monoclonal antibodies (mAb), for further enhancement of cytotoxic efficacy.

Glycostem's ex vivo expansion and differentiation method in a fully closed automated manufacturing platform (uNiK™), leads to the generation of GTA002 (oNKord®), an "off-the-shelf" allogeneic cryopreserved NK cell product derived from umbilical cord blood CD34+ hematopoietic stem cell progenitor cells, which is currently under clinical evaluation in a Phase I/II clinical study in AML, WiNK (NCT04632316). Safety and tolerability of a non-cryopreserved predecessor was demonstrated in an earlier Phase I trial in elderly AML patients (Dolstra et al. 2017). One of the important outcomes of this study was the notable increase in the CD16 expression of infused NK cells. Thus, we next exploited the potential of further enhancing and focusing cryopreserved NK cell anti-tumor responses in an antigen (Ag)-specific manner via antibody-dependent cellular cytotoxicity (ADCC) in pre-clinical models of both hematological and solid malignancies.

Similar to its predecessor non-cryopreserved NK cells, GTA002 significantly upregulated CD16 expression in vivo in immunodeficient NCG mice. This spurred the optimization of the culture process to upregulate CD16 expression in order to study the ADCC potential of GTA002 in vitro. ADCC was assessed against CD19+ and HER2+ targets at low effector-to-target (E:T) ratios by end-point flow cytometry assays as well as impedance- and live imaging- (2D & 3D) based real time analysis. Next, we engineered CD16-NK cells by introduction of a lentiviral transduction step to the uNiK™ platform, to evaluate the effect of CD19-targeted ADCC of NK cells expressing engineered or endogenous CD16. Furthermore, expression of important activating and inhibitory receptors and intracellular levels of TNF, IFN γ , perforin and granzyme B were measured by flow cytometry to investigate their role in efficient cytotoxicity of GTA002 cells. We detected simultaneous tumor targeting by GTA002, both via preserved innate NK cell responses as well as Ag-specific targeting via ADCC at low E:T ratios.

Moreover, GTA002 cells were tested for their ability to mediate killing of an ovarian cancer cell line in the presence of an Fc-active monoclonal antibody targeting a tumor associated antigen expressed by SKOV-3 cells. Impedance-based cytotoxicity assays revealed that GTA002 exerted potent ADCC upon CD16 engagement. Addition of cytokine support further enhanced both baseline cytotoxicity as well as ADCC, leading to complete eradication of the SKOV-3 tumor cells.

Overall, the enhancement of the inherent potency of GTA002 by harnessing ADCC through combination therapy with mAbs achieved efficient Ag-specific responses demonstrating the great potential of multimodal targeting against a variety of challenging cancers using a highly safe "off-the-shelf" NK cell-based cellular therapeutic.

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TUMOR CELL PLASTICITY SUPPORTS IMMUNE EVASION AND RESISTANCE TO IMMUNE CHECKPOINT BLOCKADE BY SHAPING THE IMMUNE MICROENVIRONMENT IN MELANOMA PATIENTS

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The immune contexture plays a key role in the control of tumor progression and response to immunotherapies and is tightly regulated by immune-intrinsic factors. However, tumor cell-intrinsic features can also impact the immune fitness. Although melanoma has one of the highest response rate to immunotherapies, about half of patients still show primary or acquired resistance. Melanoma is characterized by a high intra-tumor heterogeneity relying on an exacerbated tumor cell plasticity. We previously demonstrated that phenotypic transitions from proliferative/differentiated to invasive/stem-like phenotypes are regulated by Transcription Factors of the Epithelial-to-Mesenchymal Transition (EMT-TFs), hence ensuring rapid and reversible phenotypic transitions, and are associated with tumor progression and resistance to targeted therapy. However, whether melanoma cell plasticity regulates the immune contexture and the response to immunotherapy remains to be elucidated. Our recent results showed that the EMT-TF ZEB1 is associated with a defect in CD8 T cell recruitment in tumors, by rewiring of CXCL10 expression in melanoma cells, and that ZEB1 loss-of-function was sufficient to sensitize anti-PD1-resistant melanoma mouse models (Plaschka et al. JITC, 2022).

To follow up on this work, we are investigating the impact of melanoma plasticity on i) other tumor infiltrating immune cells and ii) response to immunotherapy. First, we developed multiplexed immunofluorescence (multi-IF) panels for in situ analysis of macrophages and DC subsets, alongside with melanoma cell state.

Analysis of cutaneous melanoma lesions from patients treated with curative (n=40) or adjuvant (n=60) immunotherapy revealed that, besides CD8 T cells exclusion, ZEB1 expression is also associated with a shift towards immunosuppressive tumor-associated macrophages. Strikingly, a high score of melanoma cell de-differentiation correlates with resistance to immunotherapy. Finally, we uncovered that specific DC subsets, showing patterns of aggregation in melanoma lesions, are associated with response to immunotherapy by favoring interactions with anti-tumor immune cells. Underlying signaling pathways and soluble mediators regulating the crosstalk between melanoma cell plasticity and macrophages or DC subsets are currently under investigation.

Overall, we evidence melanoma phenotype plasticity as a driver of immune escape, which can be targeted to overcome resistance to immunotherapy. We therefore propose to develop and evaluate the power of a composite score including tumor-intrinsic plasticity markers alongside with immune parameters to better predict patients' response to immunotherapy in melanoma.

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MODULATING THE GUT MICROBIOTA TO IMPROVE TREATMENT BENEFIT IN HER2+ BREAST CANCER

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Trastuzumab represents the standard of care for the treatment of HER2-positive breast cancer (HER2+BC) and its use strongly improved the outcome of patients. Nonetheless, a high grade of heterogeneity is observed in patients' response to trastuzumab and only 50% of them achieve pathologic complete response (pCR), even in those patients with tumors classified as the most sensitive to trastuzumab (i.e. HER2-enriched and with high immune infiltration). The importance of the host immune system in its mechanism of action has become increasingly clear indicating that trastuzumab, not only inhibits HER2-triggered signal transduction, but also has immunomodulatory properties and may act as immunotherapeutic agent. Recently, we demonstrated the role of the gut microbiota as a tumor extrinsic factor influencing the efficacy of anti-HER2 treatment by regulating the trastuzumab-elicited antitumor immune response. In this study, we aim to investigate the possibility to exploit gut commensal bacteria to improve trastuzumab activity.

Gut microbial composition was analyzed by shotgun DNA sequencing in patients with HER2+BC treated with neoadjuvant trastuzumab. Differences between responsive (R) and non-responsive (NR) patients were assessed and the metabolic potential of gut bacteria was investigated in silico. Metabolomic analysis was also carried out in plasma of patients. In mice, the gut microbiota was manipulated by using diet with different amount of fibers. Upon alteration of intestinal homeostasis by vancomycin, mice were fed a diet that was enriched in fiber (cellulose, 20%, and fibrulose, 5%) or a normal control diet.

The gut microbiota of R as compared to NR was characterized by a higher α -diversity and a major abundance of bacteria belonging to the Lachnospiraceae, Bifidobacteriaceae and Paraprevotellaceae taxa. The microbial metabolic potential assessed by KEGG Orthology (KO) revealed 121 genes significantly (p -value <0.05) different between R and NR, with the principal component analysis of KO discriminating R from NR (ANOSIM p -value=0.04). In R patients, an upregulation of 68 genes involved in the metabolism of oligosaccharides and carbohydrates was observed. These metabolic activities are generally associated with fiber consumption suggesting the possibility to exploit dietary intervention with fibers to shape the gut microbial composition towards communities associated with a higher trastuzumab efficacy. In support to this hypothesis, we found that in mice unresponsive to trastuzumab, the increase of fiber intake restored the antitumor activity of trastuzumab by improving trastuzumab-mediated recruitment of DCs and cytotoxic cells in the tumor microenvironment.

The obtained results support the potential adjuvant effect of fiber consumption to promote the growth of commensal communities favorable for trastuzumab immune-mediated antitumor activity.

P107

TARGETING MYELOID CELLS AND MODULATION OF THEIR FUNCTION BY FULLY SYNTHETIC ANTIBODY MIMETICS

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In certain circumstances, monocytes, especially macrophages, contribute to the onset of autoimmune diseases, are involved in various inflammatory disorders, and also have significant impact on the development and progression of tumors, metastasis as well as drive tumor microenvironment towards suppression of immune responses. As a result, numerous therapeutic strategies are currently being investigated to target and modulate the activity along with the specific function of these myeloid cells. One of the promising areas of investigation within therapeutic development is a specific targeting of the high affinity IgG FcγRI receptor, CD64, primarily expressed on monocytes and macrophages.

Recently, we have introduced the development of fully synthetic antibody mimetics called iBodies. These conjugates based on N-(2-hydroxypropyl)methacrylamide (HPMA) copolymer have gained captivated consideration in the fields of polymer-drug delivery and biomedicine. iBodies are non-immunogenic, stable, and highly modular copolymers able to passively target tumor tissues through the enhanced permeability and retention effect. In addition, iBodies excel in their versatility, low production cost, easy preparation, and fine-tuning of their pharmacokinetics and pharmacodynamics.

The anti-CD64 iBodies proved a significant improvement in binding potency to CD64, compared to the CD64 ligand alone. Using flow cytometry and confocal microscopy, we detected a specific binding of anti-CD64 iBodies to human monocytes and monocytes derived macrophages even with subnanomolar effectiveness. The anti-CD64 cytotoxic iBodies, simultaneously decorated with a specific ligand targeting CD64 and a cytotoxic moiety connected to the HPMA copolymer by a cleavable linker, showed selective depletion of CD64-positive cells compared to the control cytotoxic iBodies without the CD64 ligand and CD64-negative cells. Furthermore, iBodies proved limited cytotoxicity and no off target specificity within a whole fraction of peripheral blood mononuclear cells in vitro. In monocyte-derived macrophages, the anti-CD64 cytotoxic iBodies modulate their immune function by causing cell death pointing towards apoptosis, alter the anti-inflammatory phenotype and interfere with binary M1/M2 macrophage polarization.

In conclusion, we have developed anti-CD64 cytotoxic iBodies that are able to specifically eliminate monocytes and macrophages and modulate their phenotype. iBodies may provide novel therapeutical strategies and opportunities for drug development and delivery in cancer immunotherapy.

P108

MOLECULAR MIMICRY AND CANCER VACCINE DEVELOPMENT

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The gut microbiota profile is unique for each individual and is composed of different bacteria species according to individual birth-to-infant transitions. In recent years, microbiota's local and systemic effects on cancer onset, progression, and response to treatments, such as immunotherapies, have been extensively described. Here we offer a new perspective, proposing a role for the microbiota based on the molecular mimicry of tumor-associated antigens by microbiome-associated antigens.

In the present study, we looked for homology between published TAAs and non-self microbiota-derived (MoAs) epitopes. Blast search for sequence homology was combined with extensive bioinformatics analyses and immunological ex vivo validation. In particular, T cell responsiveness to TAAs and MoAs has been assessed ex vivo on PBMCs from healthy subjects and cancer patients.

Several evidence for homology between TAAs and microbiota-derived antigens have been found. Strikingly, three cases of 100% homology between the paired sequences have been identified. The predicted average affinity to HLA molecules of microbiota-derived antigens is very high (<100nM). The structural conformation of the microbiota-derived epitopes is, in general, highly similar to the corresponding TAA. In some cases, it is identical and contact areas with both HLA and TCR chains are indistinguishable. Moreover, the spatial conformation of TCR-facing residues can be identical in paired TAA and microbiota-derived epitopes, with precisely the same values of planar and dihedral angles. T-cell responsiveness to TAAs, MoAs or cross-reactivity to both types of antigens has been observed in both healthy subjects and cancer patients.

The data reported in the present study show for the first time the high homology in the linear sequence as well as in structure and conformation between TAAs and peptides derived from microbiota species of the Firmicutes and the Bacteroidetes phyla, which together account for 90% of gut microbiota. Cross-reacting CD8+ T cell responses have been observed in both healthy subjects and cancer patients. Therefore, the anti-microbiota T cell memory may turn out to be an anti-cancer T cell memory, able to control the growth of cancer developed during the lifetime if the expressed TAA is similar to the microbiota epitope. This may ultimately represent a relevant selective advantage for cancer patients and may lead to a novel preventive anti-cancer vaccine strategy.

P109

SUPERIOR CYTOTOXIC SIGNATURES OF CORD BLOOD STEM CELL-DERIVED NATURAL KILLER CELL THERAPEUTICS IDENTIFIED WITH BULK AND SINGLE-CELL TRANSCRIPTOMICS ANALYSIS

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Glycostem Therapeutics has developed a closed, automated, and feeder-free system (uniK™) for ex vivo expansion and differentiation of umbilical cord blood-derived CD34+ stem cells into highly functional, cryopreserved, truly off-the-shelf GTA002 NK cells, currently evaluated in a Phase I/II clinical study in AML, WiNK (NCT04632316).

GTA002 batches are each generated from a different stem cells donor, however, show similar patterns of expression of cell surface receptors and potent in vitro cytotoxicity against a variety of tumor cell lines. Nevertheless, the interindividual variability between donors may result in heterogeneity of functionality, which could not be predicted beforehand. Therefore, we investigated the transcriptome profile of multiple GTA002 batches, comparing the bulk (population level) and single cell (sub-population level) RNA-sequencing profiles. We actively selected batches with biological heterogeneity by comparing their in vitro cytotoxic potential against various cancer cell lines, evaluated under challenging low E:T ratio conditions.

First, we selected 10 GTA002 pre-clinical batches, for which we monitored relevant biological parameters as surface receptor profile and potency, to distinguish a group of superior killers (4/10), compared to the rest (6/10). Next, we used bulk RNA-seq to investigate transcriptome differences between the two groups. Interestingly, unsupervised clustering based on gene expression distinguished the same two groups identified based on cytotoxicity, suggesting that intrinsic gene expression is linked to cytotoxic capacity. Gene set enrichment and pathway analysis revealed the enrichment of functional effector pathways in the excellent group, including the Natural Killer cell mediated cytotoxicity pathway and the Notch signaling pathway. Conversely, monocyte/macrophage-mediated immunity signatures were found in the rest group. In parallel, we pursued in-depth investigation of batch sub-populations by performing topological and clustering analysis via scRNA-Seq. Notably, although the same sub-populations were identified from all 10 donors, their distribution was specific, with a larger cluster of active cytotoxic NK cells, and a smaller proportion of myeloid-like cells in the superior killers. These data show that intrinsic differences in sub-population distribution are linked to differences in donor functionality.

By generating a deep understanding of underlying donor variability, this research contributes to the improvement of allogeneic NK cell therapies, towards more standardized products.

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VECTOR AIDED MICROENVIRONMENT PROGRAMMING (VAMP): REPROGRAMMING THE TME WITH MVA VIRUS EXPRESSING IL12 FOR EFFECTIVE ANTITUMOR ACTIVITY

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Despite the success of immunotherapy in cancer treatment, its efficacy is restricted to a limited number of patients. Resistance to immunotherapy can be attributed to the tumor microenvironment (TME). To overcome TME-mediated resistance, different strategies have been developed, including depletion of tumor-promoting cells or skewing of immune-suppressive cells towards an immune-stimulating phenotype. IL12 is a potent pro-inflammatory cytokine that demonstrates striking immune activation and tumor control but causes severe adverse effects when systemically administered. Thus, local administration is considered a potential strategy to achieve high cytokine concentrations at the tumor site while sparing systemic adverse effects.

We developed a novel approach called Vector Aided Microenvironment Programming (VAMP), based on intratumoral (IT) delivery of Modified Vaccinia Virus Ankara (MVA) encoding IL12 to remodel the TME and enhance the anti-tumor response. MVA is a safe and highly attenuated poxvirus that efficiently expresses encoded genes. MVA-IL12 IT administration in different pre-clinical models achieved high cytokine concentrations at the tumor site while sparing systemic adverse effects, leading to significant efficacy. RNA sequencing analysis was performed to assess changes in the TME in treated and distal tumors and the effect on the intratumoral T-cell receptor repertoire.

IT injection of MVA-IL12 is effective in various tumor models, including those resistant to the activity of checkpoint inhibitors (CPI) both alone and in combination with the immune checkpoint inhibitor α PD1. MVA-IL12 induces a shift in the TME from a suppressive to a pro-inflammatory state by promoting an increase in pro-inflammatory M1 macrophages, a reduction in immunosuppressive M2 macrophages, recruitment of cDC1 cells, and an increase in CD8 T effector cells. Importantly, the combination of MVA-IL12 and α PD1 resulted in increased cytotoxicity and memory phenotype of intratumoral CD8 T cells, expansion and diversification of the intratumoral TCR repertoire and affected the TME signature, enhancing antigen presentation, CD8 proliferation, and cytotoxic effector functions.

These results indicate that MVA-IL12 holds promise for overcoming TME-mediated resistance to immunotherapy and provides a novel strategy for improving cancer treatment outcomes.

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ANTIBODIES AGAINST TUMOR-ASSOCIATED ANTIGENS ARE ASSOCIATED WITH RESPONSE TO IMMUNE CHECKPOINT THERAPY FOR NON-SMALL-CELL LUNG CANCER

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Immune checkpoint inhibitor (ICI) therapy is a mainstay in the treatment of non-small-cell lung cancer (NSCLC) without driver genes. However, there have never been robust biomarkers predictive and monitoring of response except for imperfect tumor PD-L1 expression levels in clinical practice. On the other hand, solid cancers including NSCLC express various tumor antigens (Ags) of tumor-associated Ags (TAAs) and tumor-specific Ags. TAAs include oncofetal proteins and cancer-testis antigens (CTAs), which spontaneously elicit antibody (Ab) and/or cellular responses in cancer patients. Recently, comprehensive tumor analyses demonstrate that B and plasma cells and TLS are associated with clinical benefits from ICI therapy for various solid cancers including NSCLC. B cells produce Abs against TAAs (TAA-Abs, so-called autoantibody), which are frequently detected in cancer patients. Here, various TAA-Abs including CTA-Abs were measured to explore the relations between TAA-Abs and ICI therapy response in NSCLC.

To date, immune responses against tumor Ags remain explored and understood during ICI therapy, because there have never been indicative markers specific for tumor Ags. We previously reported that NY-ESO-1 and XAGE1 CTA-Abs were detected in approximately 25% of NSCLC patients and predicted ICI monotherapy response. Here, we serially measured NY-ESO-1 and XAGE1 Abs of IgG and IgA isotype to monitor Ab response during ICI therapy, using ELISA.

This study was prospectively designed, and patients' sera were collected from 131 NSCLC patients received ICI therapy and 39 nonmalignant controls. TAA-Abs against 74 TAAs including 48 CTAs were measured by Luminex beads assay before ICI therapy. Positive TAA-Ab was defined as ≥ 95 percentile of each control TAA-Ab. NY-ESO-1 and XAGE1 Abs of IgG and IgA isotype were measured during ICI therapy by ELISA, as previously reported. XAGE1 protein in tumors and NSCLC cell lines with driver genes was detected by immunohistochemistry and immunoblotting, respectively.

Out of 131 NSCLC patients, 98 (75%) patients had multiple TAA-Abs (≥ 2 ; median 4, range 0 - 40), and 81 (62%) did multiple CTA-Abs (≥ 2 ; median 2, range 0 - 23). NY-ESO-1 and XAGE1 Abs with ELISA were positive in 33 (25%) patients. Notably, patients with NY-ESO-1/XAGE1 Abs had significantly more TAA-Abs than those without the Abs (7 vs 2, $p < 0.005$). The responders ($n=32$) for ICI monotherapy had significantly more TAA-Abs than the non-responders ($n=74$) (7 vs 3, $p = 0.02$), irrespective of tumor PD-L1 expression levels. Similarly, the responder had significantly more CTA-Abs than the non-responders (4 vs 2, $p = 0.01$). These trends were also observed in overall patients analyzed here. On the other hand, NY-ESO-1 and XAGE1 IgG-Abs were decreased and increased corresponding to clinical responses during ICI therapy, and the IgA-Abs were increased with disease progression.

Here, 12 patients had EGFR/ALK alterations, and five patients (3 EGFR, 2 ALK) had XAGE1 Ab with tumor XAGE1 expression using available specimens. These five patients showed two CR, one PR, one SD, and one PD in ICI therapy after targeted therapy. Interestingly, XAGE1 protein was expressed in 8 of 9 cell lines with driver genes (EGFR/ALK/ROS1) and increased in targeted-drug resistant cells. Thus, these findings suggest that some of NSCLC with driver genes and XAGE1 Ab would respond to ICI therapy.

This study suggests that TAA-Abs including CTA-Abs are associated with ICI therapy response in NSCLC. NY-ESO-1 and XAGE1 Ab levels correlated with clinical response during ICI therapy,

suggesting monitoring markers of response across NSCLC. XAGE1 was expressed in NSCLC cell lines with driver genes and increased in targeted-drug resistant cells. Thus, TAA-Abs including NY-ESO-1/XAGE1 Abs would be biomarkers to predict and monitor ICI therapy response, even in NSCLC with driver genes.

P112

IDENTIFICATION OF CXCL10-INDUCING CHEMOTHERAPY/TARGETED THERAPY COMBINATIONS FOR PD-1 BLOCKADE SENSITIZATION IN “COLD” TRIPLE NEGATIVE BREAST CANCER

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Metastatic triple negative breast cancer (mTNBC) is currently the subtype with the poorest prognosis, and treatment options are very limited. Because of its molecular characterization, current targeted therapies don't work and the only treatments available are chemotherapy combinations. Immunotherapy by anti-PD-(L)1 (ICIs) used as monotherapy has revolutionized the management of certain cancers such as melanoma or lung but, in mTNBC, results are disappointed. One strategy for increasing the response of tumors to ICIs is the addition of immunogenic chemotherapy. Results of IMpassion130 and KEYNOTE 522 studies show a benefit of taxanes-based chemo-immunotherapy only in patients whose tumors PD-L1. In majority of case, PD-L1 expression is linked with TILs infiltration, in particular cytotoxic T lymphocytes (CTLs) and their activation in the tumor. Poorly CTLs-infiltrated tumors, called “Cold” phenotype can be explained by the lack of induction of CXCR3-associated chemokines (CXCL9, 10 and 11) which allow CTLs recruitment tumor microenvironment. The very limited therapeutic effect of chemo-immunotherapy in PD-L1-negative tumors may be explained by the inability of chemotherapy to efficiently induce cancer cell immunogenic death, CXCR3-associated chemokines and CTL recruitment. Our internal data shown in 4T1 TNBC murine model that taxane (paclitaxel) is not able to induce CXCL10, CTLs recruitment and sensitization to PD-L1 blockade. In this context, identifying chemotherapies capable of inducing CXCL10 expression and looking for strategies to amplify this phenomenon is an important issue for boosting the response to ICIs in “cold” TNBCs.

The aim of the preclinical research project was therefore to identify a combination therapy, inspired by the current clinical setting, capable of inducing CXCL10 chemokine and therefore restoring CTLs recruitment in 4T1 “cold” mTNBC model. For this, based on CXCL10 induction, we first screened standard chemotherapy used in TNBC. We selected carboplatin for its potential to induce CXCL10 production in vitro and its clinical use in combination with ICIs in TNBC. Next, we screened in combination to carboplatin a targeted therapies library comprising more than 400 molecules and looked for inhibitors which can amplified CXCL10 secretion by tumor cells. Finally, we explored the immunological and therapeutic properties of the combination.

Our drug screening was carried out in vitro on the “cold” TNBC 4T1 model. CXCL10 secretion was analyzed by flow cytometry using LegendPlex technology (Biolegend). The in vivo studies has been done by generation of orthotopic 4T1 tumors and analysis of the immune infiltration was performed by RT-qPCR, IHC and spectral flow cytometry.

Our screening revealed different epigenetic drugs, such as histone deacetylase (HDAC) inhibitors, which significantly induce CXCL10 secretion in combination with carboplatin. In vivo, this combination is therapeutically additive and increased immune recruitment, particularly of CD8+ T lymphocytes. CXCR3 blockade reduces therapeutic effect of our combination. By exploring the biological mechanism in vitro, we concluded that the combination activated the type I interferon pathway to induce secretion of the chemokine CXCL10. To complement these elements, the involvement of the type I interferon pathway and CXCL10 in the efficacy of the combination was confirmed in vivo.

These data describe a combination of chemotherapy and HDAC inhibitor capable of inducing CTL recruitment in the tumor via increased CXCL10 production. The remainder of the project will focus on analyzing the mechanism of action of HDACi on CXCL10 production and the capacity of our combination to sensitize tumor to ICIs. Overall, this project shows that epigenetic modifications in tumor cells could help to amplify carboplatin-induced CXCL10 secretion and turn “cold” ICIs resistance tumor to “hot” phenotype.

P113

ESTABLISHMENT OF AN EX VIVO MODEL USING PRECISION CUT TUMOR SLICES (PCTS) TO EVALUATE NOVEL IMMUNE CELL TARGETS IDENTIFIED BY SINGLE-CELL RNA SEQUENCING

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Immuno-oncological (I-O) treatment methods have revolutionized cancer therapy. Despite that bladder cancer has characteristics generally associated with response to I-O treatments, i.e. immune infiltration and high tumor mutational burden, only around 15-30 % of the patients benefit from current immune checkpoint blockade. The low response rate indicates that there are additional immunosuppressive hurdles to overcome. To improve patient survival, novel immune cell drug targets need to be identified and explored to achieve efficient elimination of cancer cells. Additionally, a reliable test assay is needed that reflects the complexity of the tumor microenvironment (TME) for evaluation of new targets and drugs that interfere with these. Furthermore, there is a need for suitable test systems that can precisely predict each patient's response to I-O treatments based on the intricacies of each individual patient's disease.

CD45+ (~30 000 cells) or CD3+ cells (~5500 cells) were isolated from bladder tumor biopsies (n=20) and control bladder tissue (n=1) by fluorescent-activated cell sorting and subjected to single-cell RNA sequencing (scRNAseq) using the 10x Genomics or Smart-seq3 protocols, respectively. For the latter, parallel protein assessment (index sorting) was performed. The transcriptional data was analyzed to identify immune populations, define transcriptional profiles and to identify novel potential targets selectively expressed by specific populations. To validate findings from the scRNAseq, including evaluation of candidate targets, an ex vivo bladder tumor model was established by using precision cut tumor slices (PCTS). Fresh tumor tissue (n=8) was cut into multiple slices with a thickness of 300 µm using a vibratome, covering 20-40 cell layers and maintaining the original tissue composition. Different growth medias and time points of up to six days were tested to establish a protocol that maintains the primary tissue structure of tumor and stromal regions, as well as leucocyte composition. Cell viability and composition were assessed using fixable viability stain and multiplex flow cytometry, as well as hematoxylin and eosin (H&E) staining of formalin-fixed paraffin-embedded (FFPE) tumor slices. A flow cytometry-based assay was used to investigate antibody penetration into the tumor tissue when treating the slices with an anti-CD3 antibody.

The transcriptional profiles of the infiltrating immune cell populations were explored, indicating selective expression of various markers. In particular, a potential novel target was identified and further confirmed on protein level. Regarding the ex vivo model, a reproducible workflow was established that enables the cultivation of PCTS for several days without altering the original tissue architecture. A cell viability of up to 80 % could be achieved, both for cancer cells and several immune cell subsets, even after six days of cultivation. These included T cells, B cells, and myeloid cells which are crucial in regulating a possible I-O response. Additionally, tumor heterogeneity observed across patients did not affect the maintenance of the tissue, with both tumor and immune rich tissue successfully maintained ex vivo. Treatment of slices with an anti-CD3 antibody resulted in a complete labelling of CD3+ cells after 24 h, demonstrating antibody penetration within the tissue sections, supporting the use of PCTS and emphasizing their fast and effective use in testing antibody-based therapies.

The immune cell landscape in bladder tumors was outlined and a novel potential target, expressed on the cell surface and suitable for antibody-based targeting strategies, was identified. The

implementation of PCTS enables fast and reliable investigation of treatments by conserving both the tumor and its surrounding microenvironment. Taken together, we have established a workflow for evaluating novel treatment strategies in bladder cancer.

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THE COMMON H232 VARIANT OF TMEM173 REPRESENTS A HYPOMORPHIC ALLELE IN DNA/VIRAL SENSING AND IN A MONOCYTE TO MACROPHAGE DIFFERENTIATION MODEL: IMPLICATIONS FOR CANCER IMMUNOTHERAPY

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GMP-AMP synthase (cGAS)-Stimulator of interferon genes (STING) axis is the key pathway involved in sensing of cytosolic DNA to trigger type I interferon (IFN) response. Outside microbiology, this axis gained extremely broad interest for human health. Beyond somatic mutations often reported in cancer as result of immunoediting, the human gene TMEM173 encoding STING protein holds great genetic heterogeneity in human population. Three different main allelic variants have been reported, known as wild-type (R232), REF (R232H), HAQ (R71H, G230A, R293Q) with different distributions in individuals of distinct ethnic populations (H232 variant is distributed in 13% world population and 20% of East Asians). While some studies aimed to define the functionality of these alleles, the use of cellular models with different genetic background made the interpretation of results conflicting and still debated.

To shed light on the functionality of these alleles, we implemented different genetic background matched cell models to investigate whether, and how, R232, HAQ and H232 alleles affect STING function.

We showed that R232 and HAQ are equally functional, whereas H232 variant is severely hypomorphic. We also demonstrated a novel role of STING in modulating monocytic cell function and differentiation into macrophages, where the loss of functionality of H232 allele reflects an impaired differentiation, interferon response and antigen presentation. Beyond synthetic stimuli, we also corroborated these finding with actual susceptibility to clinically relevant viruses, where H232 bearing cells were significantly more susceptible to HSV-1 and were unable to trigger type I IFN response upon MVA infection.

These results could support a potential contribution of these genotypes in inter-individual susceptibility to microbial infection, but they could also be of interest in different fields where STING pathway is involved, spanning from cancer to autoimmunity up to senescence-associated diseases. where the sensing of cytoplasmic DNA could be beneficial or detrimental for disease outcome. These findings could represent a starting point to improve precision medicine in cancer immunotherapy and to predict illness severity for diseases where STING pathway is involved.

P115

OVERCOMING CHALLENGES IN GLIOBLASTOMA RESEARCH: INTEGRATING TUMOR MICROENVIRONMENT AND TUMOUR HETEROGENEITY IN AN INNOVATIVE IN VITRO MODEL FOR IMPROVED CHEMOTHERAPY AND IMMUNOTHERAPY VALIDATION

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Glioblastoma multiforme (GBM) presents significant challenges due to its aggressive nature and limited treatment options. Approved therapies like Temozolomide (TMZ) and Bevacizumab have shown only modest success in improving patient outcomes, with TMZ resistance being a common issue and Bevacizumab failing to enhance overall survival. The heterogeneous nature of GBM and the selective permeability of the blood-brain barrier (BBB) hinder the effective use of conventional chemotherapeutic agents. Furthermore, the development of innovative therapies such as CAR-T, which holds promise for targeting GBM, is impeded by the lack of preclinical models that accurately mimic the key features of GBM relevant to therapy resistance and drug delivery.

To address this critical need, we have developed an advanced in vitro 3D model that faithfully replicates the major components of the GBM tumor microenvironment (TME) involved in immunotherapy. This model captures the dense tumor core and invasive front observed in clinical tumors, surrounded by a functional BBB with physiological permeability. Our investigations have revealed that GBM tumors induce increased permeability of the tumor-associated vasculature, causing mislocalization of crucial endothelial junction proteins and compromising BBB integrity near the tumor site. Notably, these dysregulated BBB changes are also observed in clinical GBM samples.

In our study, we have focused on unraveling the angiogenic nature of GBM and exploring the impact of tumor organization and the TME on TMZ resistance and immunosuppression. Our findings demonstrate a remarkable correlation between the complexity of tumor 3D organization and TMZ sensitivity. Leveraging next-generation single-cell proteomics and genomics approaches, we have identified key proteins and molecular signatures associated with these phenomena, providing valuable insights into potential pathways contributing to therapy resistance.

Crucially, we have discovered that this compromised vasculature acts as a formidable physical barrier, hindering the extravasation and efficacy of CAR-T cell therapy, a promising immunotherapeutic approach for GBM. To overcome this hurdle, we have employed our model to screen a diverse CAR library of costimulatory domains, specifically targeting IL13Ra2. Our aim is to identify novel co-stimulatory domains that enhance CAR-T cell extravasation, persistence, and cytotoxicity, particularly in the challenging GBM TME. This approach holds tremendous promise for improving the efficacy of CAR-T cell therapy, enhancing our understanding of T cell biology, and overcoming the limitations posed by the GBM TME.

In summary, our study represents a significant advancement in GBM research by developing a physiologically relevant in vitro model that faithfully replicates the complex features of the GBM TME involved in tumorigenesis and therapy resistance. Leveraging this model, we have gained important insights into the mechanisms driving TMZ resistance, immunosuppression, and the impact of tumor-associated vasculature on CAR-T cell therapy. Our findings pave the way for functional validation and the development of novel therapeutic strategies, opening new avenues for investigating GBM tumor biology and ultimately improving treatment outcomes for patients.

P116

LOCAL ABLATION BREAKS IMMUNE TOLERANCE IN PANCREATIC CANCER

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Pancreatic cancer (PC) is a devastating disease characterised by late diagnosis, neoplastic heterogeneity, poor T-cell infiltration, a highly immunosuppressive tumour microenvironment, which results in poor clinical outcomes. Local ablative techniques have been proposed to treat unresectable PC patients, although their impact on activating host immune system and overcoming resistance to immunotherapy remain elusive.

We enrolled a cohort of patients with unresectable locally advanced PC and longitudinally evaluated the impact of tumour ablation on circulating immunological parameters. Additionally, we established a preclinical platform recapitulating systemic and localised inflammation induced by radiofrequency ablation (RFA) to evaluate the efficacy of the treatments in vivo.

Local ablation induced a short-term inflammatory process increasing circulating myeloid cells and plasma levels of high mobility group box (HMGB)-1 molecule, which were associated with better prognosis. We evaluated the therapeutic efficacy of RFA alone or in combination with immune checkpoint-based therapy in mice bearing orthotopic tumors. We demonstrated that RFA synergises with immunotherapy to restrict tumour progression, and improving the survival of PC-bearing mice through activation of a T lymphocyte-dependent anti-tumour immune response. Tumour immune landscape characterisation revealed that RFA in combination with immunotherapy sculpts an immune hostile milieu towards an effective anti-tumour environment characterised by an increased infiltration of cytotoxic T lymphocytes in spite of CD206-expressing tumour-associated macrophages.

The study reveals that tumour ablation allows immunotherapy effectiveness by breaking tumour immune tolerance and unleashing the full cytotoxic abilities of tumour-specific T-cells. Thus, RFA might circumvent the current limitations of immunotherapy in patients with PC.

P117

A PLASMACYTOID DENDRITIC CELL-BASED VACCINE APPROACH TO GENERATE SPECIFIC CD8+ T CELL DIRECTED AGAINST HEPATOCELLULAR CARCINOMA

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Hepatocellular carcinoma (HCC) affects around 900.000 people each year in the world, and is the third leading cause of cancer-related death. Few curative treatments are efficient since chemotherapies, monoclonal antibodies and immune checkpoint blockers-based immunotherapies have a limited efficacy. In addition, natural immune responses against the tumor are insufficient, with low tumor-specific T cell frequencies and an immunosuppressive tumoral microenvironment affecting immune cells, including T cells and dendritic cells. Hence, it is necessary to develop new therapeutic approaches and we hypothesize that targeting T cells could constitute an efficient way to improve patient survival considering their central role in antitumor immunity. Our objective is to design a therapeutic vaccine based on plasmacytoid dendritic cells (pDCs) in order to amplify large numbers of specific CD8+ T cells directed against HCC-specific antigens.

We used a patented clinical-grade pDC line (WO2009138489A1) known for its strong antigen-presentation ability. This cell line can be loaded in-vitro with diverse specific peptide epitopes, derived from different tumoral or viral diseases. This vaccine strategy has already been tested in two clinical trials in the context of melanoma (NCT01863108) and non-small-cell lung cancer (NCT03970746) with good tolerance and promising results. For HCC, we searched for the best antigenic combination of epitopes to amplify specific T cells directed against HCC tumor cells. Transcriptomic databases of liver cancer samples (TCGA/NCBI) allowed identifying genes having an ectopic activation in HCC and being expressed in more than 50% of the samples. By bioinformatic analysis, protein sequences were extracted and HLA*02:01 restricted 9-mer peptides with strong predicted affinity (IC50 < 50 nM) were analyzed and selection regarding affinity, hydrophobicity and predicted immunogenicity. pDCs were incubated with each peptide individually and were equally pooled as subunit vaccines. Co-culture with peripheral blood mononuclear cells (PBMCs) from healthy donors and HCC patients were performed during 28 days in the presence of IL-2 with weekly pDC-restimulations. Frequencies of specific T-cells were measured by flow cytometry using tetramer staining every week.

Forty-seven genes were identified from the databases and 22 were chosen according to their implication in cancer pathophysiology. Some had already been described as tumor-associated antigens in HCC (AFP, GPC3) and others like FBXW10 or SMC1B have been associated with poor outcomes. From their protein sequences, we extracted 167 HLA*02:01-restricted 9-mer peptides and 30 were chosen according to our selection pipeline. PBMCs from 6 healthy donors and 11 HCC patients were stimulated in-vitro with the pDC-vaccine. To date, 4/30 tested peptides were able to significantly amplify peptide-specific T cells after 28 days of culture. The maximum fold-increase at day 28 from baseline was of 187 for the peptide "FLW" (MAGE-A3 gene), 125 for the peptide "SLY" (PRAME gene), 37 for the peptide "KMF" (LRC46 gene) and 32 for the peptide "FMN" (AFP gene).

The current approach associating in silico identification of tumor-specific peptides and co-culture of peptide-loaded pDC line together with PBMC allowed us to test the immunogenicity of these peptides. This selection will allow designing a multi-epitope vaccine that could improve patient survival in combination with immune checkpoint blockers, by reestablishing a robust and functional T response against HCC. In addition, the generation of functional T cells in vitro could allow designing new TCR-transgenic T cells for future adoptive cell transfer therapies.

P118

IO PRIME: RESTORING IMMUNE FITNESS WITH ORAL SALMONELLA TYPHI ZH9 TO UNLOCK THE FULL POTENTIAL OF CANCER IMMUNOTHERAPIES

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Immunotherapy has revolutionized cancer treatment by harnessing the immune system's power to combat tumors. However, majority of patients fail to benefit, emphasizing the need for innovative approaches to unlock its full potential. Recent evidence suggests that orthogonal strategies targeting the myeloid compartment may be effective in driving deep and durable responses to approved therapies.

Tumor-infiltrating myeloid cells suppress anti-tumor immunity within the tumor microenvironment (TME). Their plasticity presents an opportunity for systemic treatments for reprogramming. Prokarium is developing a live-attenuated *Salmonella enterica* Typhi (ZH9), aiming to create living cures. This study sought to determine whether *Salmonella* can restore immune fitness through myeloid cell training and long-term reprogramming to support T cell-targeting immunotherapies.

The effect of oral *Salmonella* on myeloid cells was investigated by phenotyping of splenic myeloid cells using flow cytometry and assessment of their cytokine production after *ex vivo* re-stimulation. The impact on tissue immunosurveillance was measured in syngeneic subcutaneous (MC38) and experimental metastasis (4T1) models. Combinations with established therapies were examined by utilizing *in vitro* co-culture system with cancer patient samples and demonstrating combination treatment efficacy *in vivo* using syngeneic murine models.

Oral *Salmonella* treatment induced long-term phenotypic and functional changes in myeloid cells, including upregulation of co-stimulatory and MHC molecules on systemic DCs, monocytes and macrophages, and increased responsiveness to secondary stimuli in CD11c⁺ splenocytes, suggesting systemic reprogramming of myeloid cells by oral *Salmonella*. As a monotherapy, oral *Salmonella* treatment resulted in enhanced tissue immunosurveillance, leading to delayed tumor growth in subcutaneous and experimental metastasis models, indicating that *Salmonella*-induced trained myeloid phenotype could positively impact the myeloid compartment of the TME. Additionally, immune training by *Salmonella* complemented the effects of other cancer therapies. *In vitro*, *Salmonella*-trained human monocytes from healthy donors and cancer patients overcame the suppressive M2 phenotype, synergizing with checkpoint inhibitors to drive T-cell proliferation. *In vivo*, oral *Salmonella* treatment synergized with anti-PD-L1 to suppress the growth of checkpoint-refractory subcutaneous MC38 tumors, and with chemotherapy to reduce primary tumor growth and metastasis.

Our study demonstrates that oral *Salmonella* treatment can reprogram myeloid cells to induce trained immunity for re-sensitization of solid tumors to different therapies. This exciting finding opens new avenues for combination therapy, leveraging trained immunity to improve immune fitness in cancer patients and expand the reach of current treatments. As immunotherapy moves into earlier therapeutic settings, *Salmonella*-mediated immune training holds significant potential for enhancing cancer interception strategies and warrants clinical investigation.

P119

ALLOGENIC TUMOR CELL-BASED VACCINE TO TREAT COLORECTAL CANCER: DEVELOPMENT AND PRECLINICAL VALIDATION

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In recent years, tumor immunotherapy has emerged as a new approach for eliminating malignant tumors. However, the weak immunogenicity of tumor cells is underlying their immune escape. Therefore, the search for vaccines with strong immunogenicity has become a key issue in immunotherapy. In that context, Brenus-Pharma has developed allogenic Stimulated Tumor Cells (STC) vaccine technology¹ aiming to increase the immune recognition of tumor cells and overpass the limitations of strategies focusing on select tumor antigens. The STC approach has yielded promising results in immunocompetent mouse models.

Herein, we developed an STC-1010 human therapeutic vaccine composed of three cell lines (LoVo, HCT116 and HT-29) stressed and haptened to form an immunogenic complex that educates the immune system to target colorectal cancer (CRC) cells. Each of these cell lines was stressed physically (low-dose irradiation and heat shock) or chemically (chemotherapy exposure) before haptening, and the STC-1010 vaccine was a pool of these drug substances.

Using a transcriptomic approach, we identified a single signature of each drug substance and transcriptome data reflected a balanced mixture in the STC-1010 vaccine relying on deconvolution strategies. In addition, the proteomic analysis demonstrated that each of the physical and chemical stresses, modified protein expression which confirms the rationale for using 3 cell lines with 2 stresses to cover the heterogeneity of the CRC. Approximately 200 cancer-related proteins from the Atlas proteins' database were identified in the STC-1010 final product including CRC-specific linked proteins, tumor plasticity and tumor-associated antigens. We suggest that these overexpressed proteins in addition to neoantigens carried by the cell lines (more than 3000 neo-epitopes identified from the TRON Cell Line Portal) are the main drivers of immunogenic response. We next evaluate STC-1010 for the potential to favor an immune stimulatory response on human monocyte-derived dendritic cells (moDCs). We showed that STC-1010 induced IL-8 and IL-12 production, and reduced IL-10 during moDCs maturation. In addition, dendritic cells (DCs) exposed to STC-1010 during maturation enhanced Interferon gamma (IFN γ) production by CD8⁺ T cells in combination with LPS/IFN γ and CD40L. T cells primed with STC-1010-treated DCs promote massive apoptosis of human colorectal tumor cells HCT116, HT-29 and SW620 in an allogeneic approach. The benefit of STC-1010 was not detectable with LS174T cells characterized by a low expression of HLA-ABC and used as a negative control in this study.

These results showed that Brenus STC-1010 vaccine is an efficient strategy to educate the immune system and promote CRC cell death in vitro. Immune checkpoint inhibitors can be used in combination with the STC-1010 vaccine to promote immune activation and generate a strong multi-specific immune response.

P120

IN VITRO CHARACTERIZATION OF MACROPHAGE PHENOTYPE UPON TREATMENT WITH TLR9 AGONIST AND ANTI-PD-1 ANTIBODY

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TLR9 agonists have been extensively tested for the treatment of different tumors but no satisfied results have been achieved so far. Indeed, the activation of the immune response mediated by TLR9 stimulation is paralleled by the induction of negative feedback mechanisms, such as PD-1 up-regulation, exploited to prevent uncontrolled inflammatory reactions. PD-1 is reported to be expressed by adaptive and innate immune cells and it is associated with development of cellular dysfunction. Inhibiting PD-1 can restore the activity of immune system but only 30-40% of patients with advanced cancer benefit of this kind of therapy. Therefore, a combinatorial immunotherapeutic regimen based on TLR9 agonist administration and anti-PD 1 antibody may have the potential to be effective. However, we previously observed a substantial decline in the effectiveness of a TLR9 agonist when combined with anti-PD-1 antibody in an immunodeficient mouse model of ovarian cancer. We also demonstrated that macrophages exposed to this combination acquired an immunosuppressive and pro-tumor phenotype.

In the present study, we attempted to better define such phenotype through different approaches. RAW264.7 mouse macrophage cell line was exposed to TLR9 agonist, anti-PD-1 antibody alone or in combination and the effect of the combinatorial treatment on macrophage phenotype was assessed by gene expression analysis, multiplex ELISA assay and metabolomic profile. Untreated cells served as controls.

Principal component analysis performed on microarray data showed the existence of four separate clusters. While macrophages treated with single agents were found in close proximity, RAW264.7 cells incubated with both TLR9 agonist and anti-PD-1 antibody exhibited a completely different gene expression profile and segregated apart from all the other experimental groups. Functional analysis revealed a significant enrichment of pathways related to IL-6, IL-4, IL-13, IL-10 and IL-12 signaling in RAW264.7 treated with TLR9 agonist and anti-PD-1 antibody, suggesting that these macrophages may acquire a polarization status that is "mixed" between M1 and M2 phenotype. Cytokines important for macrophage activation and function were quantified by multiplex ELISA assays on RAW264.7 supernatants treated as described above. When multiplex ELISA data were collectively considered by Partial Least Squares Discriminant Analysis (PLS-DA), we identified four different clusters, closely resembling those observed for genomic data. Measurement of cytokine release showed a statistically significant increase of IL-4, IL-6 and IL-12b and a parallel reduction of TNF- α release in RAW264.7 exposed to concomitant TLR9 agonist and anti-PD-1 antibody administration compared to that incubated with single agents, strengthening microarray results. Finally, mass spectrometry was carried out on RAW264.7 supernatants to characterize the secretome profile. We observed an overall increase of metabolite levels in macrophages exposed to the combination therapy, in particular of polyamines belonging to arginine catabolism, several aminoacids, and carnitine derivatives. These results indicate that TLR9 agonist and anti-PD-1 antibody combination is also able to promote a metabolic rewiring in macrophages.

Collectively our analysis suggests that, when TLR9 signaling is activated, the co-administration of anti-PD-1 antibody induces in macrophages the acquisition of a phenotype with M1/M2 features. As previously reported, these macrophages possess enhanced immunosuppressive activity, eventually promoting tumor growth. Since TLR9 stimulation and PD-1 blockade combinatorial immunotherapy is under investigation in different clinical trials, the impact of both agents on macrophages should be

taken into consideration to avoid potentially harmful adverse effects, especially in tumors where the infiltration of macrophages is particularly abundant.

P121

PRECLINICAL DEVELOPMENT OF A THERAPEUTIC MRNA-BASED MULTIEPITOPE VACCINE FOR GLIOBLASTOMA

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Peptide vaccination against peptides of tumor-associated antigens (TAAs) over-presented via HLA class I and II molecules on glioblastomas (GBM) has shown some promise in clinical trials. To leverage the immunogenic potential of messenger ribonucleic acid (mRNA) vaccines, we developed a mRNA-based multiepitope vaccine candidate for GBM, CVGBM.

The investigational vaccine consists of an mRNA with unmodified nucleotides, which is formulated in lipid nanoparticles. It encodes a single fusion protein comprising eight TAA-derived epitopes that have demonstrated immunogenicity as peptide vaccines, each encoded with its flanking sequences in a stretch of 29 amino acids. Five of the peptides can be presented on HLA class I molecule HLA-A*02:01 to CD8+ T cells, while the other three peptides can be presented on various HLA class II HLA-DR molecules to CD4+ T cells. Following vaccination, the mRNA is taken up by cells at the injection site and in local lymph nodes and translated into the fusion protein, which is then processed into peptides.

To assess correct translation and processing of the fusion protein and presentation of the encoded peptides, two human cell lines endogenously expressing HLA-A*02:01 (HEK293T and THP-1) were transfected with CVGBM mRNA and HLA-presented peptides were isolated by immunoprecipitation and identified by LC-MS/MS. This immunopeptidomic analysis confirmed presentation of four of the six HLA-A*02:01-presented peptides encoded by CVGBM. Notably, no additional peptides derived from other parts of the fusion protein, such as the epitope junctions, were detected. Analysis of HLA class II-bound peptides was only performed for the THP-1 cells and no CVGBM-derived peptides were detected, potentially due to the overall low HLA class II expression on THP-1 cells and the low number of eluted HLA class II-bound peptides.

As CVGBM encodes human sequences that are foreign to mice, a murine surrogate mRNA vaccine was used to assess immunogenicity and anti-tumoral efficacy of the mRNA and protein design used for CVGBM. The surrogate mRNA vaccine contained the same non-coding mRNA elements as CVGBM but encoded for a fusion protein with similar structural design containing 10 epitopes derived from the murine B16.F10 tumor model and additionally the synthetic T-helper epitope PADRE. This allowed the anti-tumoral efficacy of the surrogate vaccine to be assessed in the syngeneic B16.F10 tumor model, while avoiding limitations inherent to xenogeneic tumor models. Intramuscular vaccination of naïve C57BL/6 mice with three doses of the surrogate multiepitope mRNA vaccine at weekly intervals induced CD8+ T cell responses against five of the epitopes as well as CD4+ T cell responses against the T-helper epitope PADRE. In B16.F10 tumor-bearing mice, survival was significantly extended by this vaccination schedule from a median survival of 23.2 days in mice vaccinated with a control mRNA vaccine encoding an irrelevant antigen to 30.9 days in mice vaccinated with the surrogate mRNA vaccine.

These data supported the initiation of a first-in-human, phase 1 trial (CV-GBLM-001) assessing CVGBM in HLA-A*02:01-positive patients with newly diagnosed and surgically resected MGMT-unmethylated glioblastoma (CNS WHO Grade 4).

P122

IMMUNOTHERAPY IN THREE DIMENSIONS: VIDEO-MICROSCOPY OF IMMUNE CELL KILLING IN PATIENT-DERIVED MICROTUMORS

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We have engineered an in vitro 3D system for immuno-oncology experiments with extended preservation (>20 days) of patient tumor explant micro-tumoroids and patient matched immune cells (TILs, PBMCs, and CAR T) that uses high speed in situ laser scanning confocal microscopy to create time-lapse videos enabling precise tracking and quantification of immune cell action (T-cell killing, proliferation, and motility) and tumor responses (invasion, proliferation, extravasation, and collective cell behavior). This unique preclinical platform has been designed specifically for 3D immunotherapy and has been used on colorectal cancer, melanoma, renal cell carcinoma, small cell lung cancer, pancreatic cancer, and glioblastoma. We will present numerous high resolution movies of effective immunotherapy approaches in 3D including aPD1 checkpoint inhibitors, TILs, and CAR T cells.

Tumor microexplants¹ (200-400 μm) were created from resected cancers obtained from properly consented donors. Immune cells were labeled with CellTrace CFSE and co-cultured in 3D Liquid Like Solid (LLS)² with Organoid liquid media³ and supplements. Steady perfusion flow of 40-60 $\mu\text{L/hr/well}$ was maintained. Average cytokine production was measured using Meso Scale Discovery Small Spot assay and confirmed by metabolomics analysis (GC-MS⁴). Cell viability, motility, and immune activation were assessed by multi-immunofluorescence cell phenotyping using a Nikon A1R laser-scanning confocal microscope. In situ imaging of immune cell interactions with cancer cells was performed at various intervals to create sequential time-lapse images enabling detailed tracking of 3D positions, motions, infiltration dynamics, and immune cell killing. These data were analyzed using cell tracking AI algorithms and confirmed by immuno-phenotyping using flow cytometry⁵. Spatiotemporal cytokine profiles were measured by taking advantage of LLS stability; using 3D printing in LLS we are able to precisely print patterns of ELISA beads or create large random fields of ELISA beads across the experiment to measure local concentrations of cytokines in situ. Sophisticated spatiotemporal reaction-diffusion models quantify the tumor's production rate, tumor margin concentrations, and the entire immuno-regulatory micro-environment.

Immunofluorescence viability assays coupled with flow cytometry assessment and GC-MS analysis revealed that cancer microexplants and autologous PBMCs can be co-cultured for more than 2 weeks with viability > 90%. Flow cytometry data showed the presence of CD4+, CD8+, EpCAM, and CD14+ cells and the preservation of heterogeneous populations of metabolically active epithelial, endothelial, and immune cell subsets from the original tumors. The presence of aPD1 immune-checkpoint inhibitors (ICI) induced an activated immune killing response measured through in situ imaging and flow cytometry by granzyme B, CD25, FoxP3, CD45RA, CCR7, and PD1 labeling. Tracking of immune cells and analysis of mean squared displacement revealed both chemotaxis and chemokinetics of CD8+ cells which had an average migration speed of > 2.8 $\mu\text{m}/\text{min}$. Average CD8+ T cell killing rates were ~3 cancer cells/h, which decreased monotonically after 12 hours to approximately 1 cancer cell/h. An 11-day study of ICI treatment showed a statistically significant increase in IFN γ production as compared to both the untreated negative control groups and tumors with an immune-excluded phenotype. Fitting spatiotemporal data of cytokine concentrations revealed production rates of 2 IL8 molecules per cell per second giving tumor margin concentrations of over 2ng/ml after 10 hours.

The in vitro immuno-oncology platform with in situ fast scanning fluorescence microscopy was able to quantify immune cell migratory patterns and speeds as well as immune cell infiltration and killing.

The addition of ICI treatment led to measurable increases in immune cell activation, motility, activity, and killing.

P123**NEUTRALIZATION OF NET-ASSOCIATED HUMAN ARG1 ENHANCES CANCER IMMUNOTHERAPY**

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Pancreatic ductal adenocarcinoma (PDAC) is a leading cause of cancer-related deaths in the United States, with a current 5-year survival rate of only 10%. Most patients present with late-stage metastatic disease, and even patients diagnosed at earlier stages, who are eligible for potentially curative tumor resection, have disease recurrence rates exceeding 80%. Standard-of-care cytotoxic therapies only modestly extend life expectancy; thus, different therapeutic combinations are urgently needed. Immunotherapies targeting immune checkpoint molecules have transformed clinical outcomes for patients with many types of solid tumors; however, these have yet to affect outcomes for PDAC. Poor immunogenicity of PDAC, a highly immunosuppressive tumor immune microenvironment (TME), and dense stroma represent hurdles to immunotherapeutic efficacy. A better understanding of the human PDAC immune contexture and heterogeneity is needed to inform rational drug design. Myeloid cells can restrain antitumor immunity by metabolic pathways, such as the degradation of L-arginine, whose concentrations are regulated by the arginase 1 (ARG1) enzyme. Results from preclinical studies indicate the important role of L-arginine metabolism in PDAC progression, suggesting a potential for clinical application. Important differences in ARG1 biology exist between rodents and humans, which have halted the clinical translatability of many findings. Understanding human ARG regulation is essential to develop effective strategies of intervention.

Humanization of mice; immunosuppressive assay; ex vivo PDAC assay; nuclear magnetic resonance spectroscopy; ARG1 "interactome" in NETs; isothermal titration calorimetry; NET isolation and quantification; systemic treatment of humanized mice with hTERT865–873 TCR–engineered T cells; systemic treatment of humanized mice with checkpoint inhibitors; human ARG1 specific mAb generation; ARG1 neutralization assay; ARG1 epitope mapping.

We show that neutrophil extracellular traps (NETs), released by spontaneously activated neutrophils isolated from patients with PDAC, create a microdomain where cathepsin S (CTSS) cleaves human ARG1 into different molecular forms endowed with enhanced enzymatic activity at physiological pH. NET-associated ARG1 suppresses T lymphocytes whose proliferation is restored by either adding a ARG1-specific monoclonal antibody (mAb) or preventing CTSS-mediated cleavage, whereas small-molecule inhibitors are not effective. We show that ARG1 blockade, combined with immune checkpoint inhibitors, can restore CD8+ T cell function in ex vivo PDAC tumors. Furthermore, anti-hARG1 mAbs increase the frequency of adoptively transferred tumor-specific CD8+ T cells in tumor and enhance the effectiveness of immune checkpoint therapy in humanized mice.

Neutrophils and macrophages are the most abundant immune cell type within the TME and are associated with poor clinical prognosis because of their immunosuppressive properties and roles in sustaining therapeutic resistance. Whereas different strategies have been set and are currently being investigated in clinical trials, a limited number of therapies targeting neutrophils, both as a number and as a function, have been developed so far. Here, we showed that low-density and normal-density neutrophils from patients release NETs as a result of their constitutive activation that might be contributed by the high serum levels of IL-8 and TNF- α . In NETs, we found that ARG1 is present in different molecular forms that contribute to ARG1 gain of function at physiological pH. The

presence of ARG1 active in the blood at physiological pH might be a marker for patients' stratification and prognosis during disease follow-up. Our studies suggest that neutralization of ARG1 could be an effective way to support ICB efficacy and support the option to block CTSS with an antagonistic Ab, especially in tumors where this enzyme is present at higher amounts like PDAC and colorectal carcinomas.

P124

CLINICAL AND GENOMIC DETERMINANTS OF THE EFFICACY OF IMMUNE CHECKPOINT BLOCKADE THERAPY IN HEAD AND NECK SQUAMOUS CELL CARCINOMA

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Head and neck squamous cell carcinoma (HNSCC) that is recurrent, metastatic (R/M), or both, is an incurable disease, with patients experiencing median survival of under ten months and often significant morbidity. While immune checkpoint blockade (ICB) drugs are effective in ~20% of patients, the remaining 80% experience limited clinical benefit and are exposed to potential adverse effects and financial costs. Clinically approved biomarkers, such as tumor mutational burden (TMB), have modest predictive value in HNSCC. Current immunotherapy strategies—broad application of ICB drugs in unselected patients—thus expose most patients to toxicity without benefit at significant cost (\$100,000 per quality-adjusted life year gained)

We performed whole exome sequencing on 133 patients with HNSCC treated with ICB. Additional validation was performed in additional datasets.

Hierarchical clustering of genomic data revealed six molecular subtypes characterized by a wide range of objective response rates and survival after ICB therapy. The prognostic importance of these 6 subtypes was validated in an external cohort. Furthermore, a random forest-based predictive model, using several clinical and genomic features, predicted progression-free survival (PFS), overall survival (OS), and response with greater accuracy than a model based on TMB alone. Recursive partitioning analysis identified three features (based on peripheral laboratory testing and exome sequencing) that classified patients into risk groups with accurate discrimination of PFS and OS.

These findings shed light on the immunogenomic characteristics of HNSCC tumors that drive differential response to ICB and identify a clinical-genomic classifier that outperforms the current clinically approved biomarker of TMB. This validated predictive tool may help with clinical risk stratification in patients with R/M HNSCC for whom ICB is being considered.

P125

MULTI-OMIC PROFILING REVEALS THE NEOPLASTIC AND ENDOGENOUS RESPONSES TO CHECKPOINT BLOCKADE IMMUNOTHERAPIES IN CUTANEOUS T CELL LYMPHOMA

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Mycosis fungoides (MF) and Sezary syndrome (SS) are the two most common subtypes of cutaneous T cell lymphoma (CTCL). Derived from skin homing CD4+ memory T cells, these malignancies can manifest as skin patches, plaques, tumors, and inflammation. The presence of circulating neoplastic T cells in blood and lymph nodes is less common in MF but is a definitive feature of SS. Five-year overall survival rates for patients with advanced MF/SS (stage IIB-IV) range from 0-65%. Cancer immunotherapy holds promise for eliciting durable and potentially curative responses, but little is known about the immunological consequences of giving immune-activating drugs to patients with mature T cell-derived cancers. To gain understanding into the mechanisms of action and inform selection of effective drug combinations for CTCL patients, greater insight is needed into the effects of these therapies in vivo.

Here, we employed a multi-omic systems immunology approach to deeply profile the neoplastic and endogenous immune responses to mono-anti-PD-1 therapy and combination interferon-gamma/anti-PD-1 therapy in patients with advanced MF or SS. To characterize the systemic, local, and cellular response to these therapies, we interrogated longitudinal peripheral blood samples by single-cell mass cytometry, TCR immune repertoire sequencing, and serum proteomics, and we employed high-dimensional proteomic imaging (CODEX) and bulk transcriptomics on longitudinal tumor biopsy samples.

Our results demonstrate that neoplastic T cells have substantially different expression patterns compared to normal T cells. We observed significantly higher levels of metabolic regulators as well as the checkpoint molecule, PD-1, yet we found no evidence of neoplastic cell activation or a phenotypic shift induced by anti-PD-1 therapy. We observed significantly higher expression of PD-L1 on neoplastic T cells from responders compared to non-responders, both pre- and post-treatment. SS patients manifested significantly higher expression of PD-1 on endogenous and neoplastic T cells within tumors, but significantly less overall immune infiltration. SS patients also had significantly higher levels of soluble PD-1 and CXCL13 in serum as compared to non-leukemic MF patients.

Taken together, this study provides insight into the neoplastic and endogenous responses to two immunotherapy regimens in patients with advanced CTCL and can serve as a template for multi-omic analysis of primary clinical cohorts.

P126

INTENSE ANTI-CHOLANGIOCARCINOMA ACTIVITY BY MESOTHELIN-SPECIFIC CAR.CIK LYMPHOCYTES

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No effective therapeutic options are available for advanced and unresectable cholangiocarcinoma (CCA). This unmet clinical need, prompted us to generate a novel and effective cell therapy strategy against CCA redirecting patient-derived cytokine induced killer lymphocytes (CIK), with a chimeric antigen receptor (CAR) against mesothelin (MSLN) that is emerging as relevant CCA target.

Ex vivo expanded CIK are T-NK lymphocytes intrinsically endowed with HLA-independent tumor killing activity. In the last five years, our group demonstrated that CIK may be redirecting with CAR, characterized by double antitumor potentiality, generating an intriguing platform to be exploited against solid tumors.

MSLN-CAR.CIK were generated from patients' PBMC and engineered with second-generation MSLN-CAR including 4-1BB co-stimulation domain. As tumor targets, we employed and characterized a panel of CCA cell lines. CAR expression and extended phenotype of mature CAR-CIK were analyzed by flow cytometry. MSLN-CAR CIK killing ability in 2D models was evaluated at different effector: target ratio (E/T) by flow-cytometry. In order to recapitulate the architectural complexity of CCA, 3D tumor spheroids were developed from different CCA cells bearing a reporter gene (RFP) and co-incubated with effector cells at ratio 2:1. Images were acquired at different time-points using fluorescence microscopy. The tumor recruitment of MSLN-CAR.CIK and infiltration in CCA spheroids were analyzed using confocal microscopy.

We successfully generated MSLN-CAR.CIK from peripheral blood of tumor patients (n=7) with a mean CAR expression of 40%. CAR.CIK expansion rates and immunophenotype were comparable to unmodified controls (NTD.CIK): CD3+CD56+ (45%), CD3+CD8+ (80%) and NKG2D+ (70%). We confirmed as MSLN was highly expressed (>90%) on the membrane of 6/7 CCA cell lines. MSLN-CAR.CIK displayed significantly superior in vitro cytolytic activity (n=12) against CCA as compared with NTD.CIK, even at very low E/T ratios: 80% vs 30% (E/T 2.5:1), 70% vs 20% (E/T 1:2, p< 0.0001), while saving fibroblasts cells lines (MRC-5 cells: 9% vs 20%; E:T 1:1; n=3). The activity of MSLN-CAR.CIK significantly delayed the in vitro re-growth capability of residual CCA cells following the initial treatment as compared with NTD.CIK (p<0.05). The intense tumor killing activity, along with tumor infiltration, by MSLN-CAR.CIK was confirmed also against CCA 3D spheroids resulting significantly superior as compared to unmodified NTD.CIK.

We demonstrated that MSLN-CAR.CIK are effective against CCA in both 2D models and 3D structures, supporting MSLN as a valuable target for advanced CCA. Our findings provide reliable translational rationale to explore cellular immunotherapy with MSLN-CAR.CIK in clinical studies within the challenging field of advanced CCA.

P127

CHIMERIC ANTIGEN RECEPTOR MODIFIED CYTOKINE INDUCED KILLER CELLS AGAINST A TUMOR MICROENVIRONMENT ANTIGEN FOR THE TREATMENT OF SOLID TUMORS AND LYMPHOMAS

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Although chimeric antigen receptor T cells (CAR-T) technology has revolutionized cancer immunotherapy, CAR-T cells for solid tumors are still lacking. We set out to transfer CARCIK-CD19 technology, successfully used to treat B cell acute lymphoblastic leukemia (B-ALL) patients, to new CAR constructs against an extracellular matrix (ECM) molecule overexpressed in different tumor types. This ECM target molecule has been called TMA (Tumor Microenvironment Antigen) since its real identity cannot be revealed at present for patenting reasons.

2 different anti-TMA CAR constructs were generated. These CARs shared the same anti-TMA scFv domain, based on a known anti-TMA monoclonal antibody, but differed in their backbone, one (TMA4) recapitulating the signaling domains present in the anti-CD19 CAR Tisagenlecleucel (Novartis) and the other (TMA5) the CAR structure of CARCIK-CD19 cells developed by our collaborators at the Fondazione Matilde Tettamanti Menotti De Marchi Onlus in Monza. Both CARs were introduced into cytokine induced killer cells (CIK) by transfection together with the Sleeping Beauty transposase for insertion into genomic DNA. CARCIK-TMA cells were tested in vitro to assess their cytotoxic activity, proliferation and cytokine release. In vivo mouse models were set up in Nod-Scid mice subcutaneously infused with MDA-MB-231 cell line and treated with CARCIK-TMA by intravenous infusion.

We have studied TMA expression on putative targets, primary cells and cell lines. Two solid tumor cell lines positive, at different levels, for TMA expression were identified, HT-29 (colon adenocarcinoma cell line) and MDA-MB-231 (breast cancer cell line). MDA-MB-231 cell line express 13.7 times more TMA mRNA than HT-29, normalized on the reference gene GAPDH. In parallel, we have generated two cell lines expressing the TMA domain recognized by our CARs fused to a trans-membrane domain, called here TMA-TM. Stably transfected TMA-TM+ and naturally expressing TMA cell lines were then used to test CARCIK-TMA cells in vitro. The anti-TMA CAR expression and specificity was assessed by flow cytometry using a recombinant TMA protein bound to an histidine tag, the specific antigen binding was verified for both constructs. In vitro functional assays demonstrated that anti-TMA CARCIK cells were cytotoxic against TMA+ targets. In particular, both CARs were cytotoxic against MDA-MB-231 cell line (18.8% mean cytotoxic activity of TMA4 and 40.3% of TMA5 at E:T 3:1), while against HT-29 cells TMA5 was significantly more cytotoxic compared to TMA4 (respectively 31.9% compared to 3.7% mean cytotoxic activity, $p < 0.05$). Anti-TMA CARCIK cells proliferated in co-culture with MDA-MB-231 cell line and also in this case TMA5 proliferation was significantly higher compared to TMA4 (mean proliferation index respectively of 6.6 and 2.7 at 1:1 E:T ratio, $p < 0.05$). Finally, TMA5 in presence of MDA-MB-231 cells secreted significantly more IFN- γ compared to TMA4 (respectively 8.2% compared to 0.9%, $p < 0.05$). Both CARCIK-TMA constructs were tested in vivo. In vivo animal models have been set up with the MDA-MB-231 solid tumor cell line and the high positivity for TMA expression was confirmed by immunohistochemistry. A first experiment infusing three doses of CARCIK-TMA cells was performed and reported the lack of toxicity, a good tumor infiltration by CARCIK-TMA cells but not a relevant anti-tumor activity.

CARCIK-TMA cells were successfully expanded and their in vitro response against the target antigen was confirmed. Between the two constructs, TMA5 reported a higher activity compared to TMA4 at low antigen levels. In vivo mouse models confirmed the antigen specificity of both constructs, that lead to a TMA guided tumor infiltration, but a lack of anti-tumor activity. To improve the CARCIK-TMA anti-tumor activity we are evaluating different strategies, in particular the combination of anti-TMA CAR with a bispecific antibody targeting a specific tumor antigen.

P128

UNDERSTANDING PERK SIGNALING IN TUMOR-ASSOCIATED DENDRITIC CELLS

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Immunotherapeutic strategies aimed at harnessing the anti-cancer potential of T cells have demonstrated impressive effects in treating certain tumor types. However, the response rate to this new approach among ovarian cancer patients remains only marginal. One possible explanation for this phenomenon is the adverse environment engendered by metastatic ovarian tumors, which hampers the protective function of immune cells. Indeed, harsh conditions within ovarian tumors can disrupt the protein-folding capacity of the endoplasmic reticulum (ER) in infiltrating immune cells, resulting in a cellular state known as "ER stress". This stress is typically mitigated by the unfolded protein response (UPR). Nonetheless, relentless activation of the UPR in cancer adversely affects the activity of various immune cell populations within the tumor, including dendritic cells (DCs). We aimed at defining new mechanisms through which the tumor-derived ER stress impairs the function of DCs, with the goal of exploiting this knowledge to develop more effective cancer immunotherapies.

We use both unique genetic mouse models and pharmacologic approaches to characterize the phenotypic consequences of stressed out immune cells residing in murine ovarian tumors.

Recently, our group uncovered that lysophosphatidic acid (LPA), a bioactive lipid that is overproduced in hosts with ovarian cancer, suppresses production of type-I interferon (IFN) by tumor-associated DCs, thereby inhibiting protective anti-tumor immunity. Notably, our current research reveals that activation of the ER stress sensor PERK cooperates with LPA signaling in DCs to induce the overexpression of factors that promote tumor growth and immune escape. Hence, we are currently intercepting PERK signaling and/or LPA sensing in DCs as a novel approach to reprogram intratumoral DC function and elicit potent anti-ovarian cancer immune responses.

Our data raise the intriguing possibility that targeting these pathways in DCs may improve the efficacy of ovarian cancer immunotherapies such as checkpoint blockade or adoptive cellular transfer.

P129

TARGETING PROTEASE-ACTIVATED RECEPTOR 2 TO POTENTIATE THE EFFICACY OF CANCER IMMUNOTHERAPY

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Despite the efficacy of immune checkpoint blockade (ICB), only a minority of patients across cancer types have shown durable clinical benefit. Through a large-scale comparative meta-analysis of predictive transcriptomic biomarkers, we identified protease-activated receptor 2 (PAR2) as a potential mechanism of ICB resistance. PAR2 is a G protein-coupled receptor (GPCR) activated by proteolytic cleavage of its amino terminus by proteases such as trypsin, tryptase, kallikreins and coagulation factors. PAR2 can notably be highly expressed in the tumor microenvironment (TME). In cancer cells, PAR2 promotes proliferation and metastasis, while in immune cells, PAR2 modulates the secretion of cytokines and chemokines, thereby regulating myeloid and T cell function.

Using preclinical mouse models of cancer, we investigated the immunological impact of a potent and selective PAR2 inhibitor, alone and in combination with anti-PD-1 therapy.

Systemic administration of the PAR2 antagonist greatly reduced the accumulation of putative immuno-suppressive myeloid cells and increased infiltration of effector T cells in the TME. Consistent with this, treatment with the PAR2 antagonist increased the efficacy of anti-PD-1 therapy with complete and durable tumor regression observed in preclinical cancer models. On-going studies are further investigating how PAR2 inhibition shapes the transcriptional landscape of tumor-infiltrating immune cells.

Our study reveals the potential of PAR2 as a therapeutic target to potentiate ICB and demonstrates the relevance of a potent and selective small molecule inhibitor targeting PAR2 to improve cancer treatments.

P130

TREG DEPLETION SENSITISES GLIOBLASTOMA TO ANTI-PD-1 BLOCKADE AND SYNERGIZES WITH TIMOR-TARGETING ANTIBODIES THROUGH MYELOID ACTIVATION

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Despite the success of anti-CTLA-4 and anti-PD-1 for melanoma and lung cancer, to date, immunotherapy for glioblastoma (GBM) has been ineffective. Although at a small frequency effector (Teffs) CD8+ and CD4+ T cells are present in GBM tumors, displaying an exhausted phenotype, while regulatory CD4+ T cells (Tregs) express high levels of the IL2 receptor, CD25, suggesting that these cells are actively suppressing the immune response against GBM. The role of regulatory Tregs in glioblastoma and whether these cells can be therapeutically targeted using depleting antibodies that engage activating Fc γ receptors (Fc γ Rs) to promote antibody-dependent cell cytotoxicity (ADCC) or phagocytosis (ADCP) remains unknown. In addition, which cells express Fc γ Rs and whether they can be engaged in GBM have not been previously assessed.

Mouse experiments were performed using an orthotropic glioblastoma mouse model. Changes in the TME were assessed using multicolor flow cytometry and single-cell RNA sequencing, together with genetic and antibody-based techniques that allowed the elimination of different immune populations. Mouse tumors were microdissected using a stereomicroscope to avoid contamination with normal tissues. Finally, human GBM tumors were used to characterize the expression of Fc γ receptors in different immune populations and set up explants to assess the potential of depleting Tregs in human tissues.

Our laboratory has developed the new anti-CD25NIB that depletes Tregs via antibody ADCC by engaging activating Fc γ receptors on macrophages and NK cells whilst preserving IL2 signaling on CD8+ and CD4+ Teffs. The anti-CD25NIB in mouse models of GBM has shown that one dose promoted efficient Treg depletion accompanied by CD8+ and CD4+ Teff activation that results in 50% survival. The effect on survival was dependent on a small subset of proliferating CD8+ Teffs that sense IL-2 following Treg depletion, as their elimination or the blockade of IL-2 completely abrogated the therapeutic activity of this antibody. Single-cell analysis demonstrated that the anti-CD25NIB specifically expanded, proliferating, and effector CD8+ Teff clones instead of progenitor-exhausted or exhausted clones, as shown in the case of PD-1 blockade. As most GBM tumors are resistant to anti-PD-1 treatment, we hypothesized that Treg depletion had the potential to sensitize these tumors to PD-1 blockade due to the different mechanisms of action of the two drugs. The combination between anti-CD25NIB and anti-PD-1 blockade led to a synergistic effect increasing the survival of mice bearing GBM tumors, suggesting a potential for combination due to their different mechanism of action.

The therapeutic effect of the anti-CD25NIB effect was accompanied by an increase in a population of MDM expressing high levels of FcγRIV, which was dependent on IFNγ. The accumulation of MDMs expressing high levels of FcγRIV led to a higher overall density of activating FcγRs in the TME, allowing to combine with other antibodies dependent on FcγR engagement. As EGFRvIII is expressed in the surface of up to 60% of all GBMs, these tumors could be therapeutically targeted with tumor-targeting antibodies that engage FcγRs to promote tumor cell depletion. Coadministration of anti-CD25NIB and an Fc-optimized anti-EGFRvIII antibody led to a synergistic effect with completely eliminating all GBM tumors.

Finally, we set up patient-derived GBM explant to assess the efficacy of the human version of the anti-CD25NIB to eliminate Tregs. Tregs were successfully eliminated following the addition of anti-CD25NIB to the explant and their depletion correlated with the upregulation of Granzyme B on CD8+ T effs.

Our results demonstrate the key role of Tregs in GBM and suggest that the use of Treg-depleting antibodies like the anti-CD25NIB holds promise as therapeutic itself and as a combination substrate to sensitize GBM to checkpoint blockade and make tumor-targeting antibodies more effective.

P131

TALEN®-EDITED ALLOGENEIC INDUCIBLE DUAL CAR T-CELLS ENABLE EFFECTIVE TARGETING OF SOLID TUMORS WHILE MITIGATING OFF-TUMOR TOXICITY

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Adoptive cell therapy based on chimeric antigen receptor (CAR)-engineered T-cells has proven to be lifesaving for many cancer patients. However, its therapeutic efficacy has so far been restricted to only a few malignancies, with solid tumors proving to be especially recalcitrant to efficient therapy. One of the key barriers against CAR T-cell success in solid tumors are cancer-associated fibroblasts (CAFs), that modulate the tumor microenvironment (TME) to inhibit T cell infiltration and induce “T cell dysfunction”. Overexpression of fibroblast activation protein (FAP) in CAFs is in fact associated with poor prognosis in various cancers. Additionally, the sparsity of tumor-specific antigens (TSA) and the low-level expression of CAR-directed tumor-associated antigens (TAA) in normal tissues often results in “on-target off-tumor” cytotoxicity, raising potential safety concerns.

Using our best-in-class TALEN®-based gene editing platform, we present here an innovative CAR-T cell engineering strategy to overcome the challenges of the 'cold' TME. Our allogeneic “Smart CAR T-cells” are designed to express a constitutive TSA-CAR, targeting FAP in solid tumors. Additionally, a second CAR targeting a TAA such as mesothelin is specifically integrated at an activation-inducible locus like PDCD1.

FAP-CAR engagement specifically in the CAF positive solid TME induces expression of the mesothelin-CAR, establishing an AND-gated circuit sensitive to dual antigen sensing. Furthermore, TALEN® and AAV-mediated knock-in of the mesothelin CAR is designed to disrupt the expression of PD-1, a positive regulator of T cell exhaustion. Using comprehensive in vitro and in vivo strategies we demonstrate that TME-restricted co-expression of FAP and ML CAR augments intra-tumor infiltration and persistent anti-tumor cytotoxicity, while limiting “on-target off-tumor” toxicity at bystander sites expressing mesothelin alone.

Our study thus demonstrates the capabilities of TALEN® gene-editing for the design of allogeneic AND-gated Dual CAR T-cells which efficiently target immunotherapy-recalcitrant solid tumors while mitigating potential safety risks, encouraging us to bring them to the clinic.

P132

CHARACTERIZATION AND PROGNOSTIC VALUE OF HLA CLASS I IN ORAL TONGUE SQUAMOUS CELL CARCINOMA

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Oral tongue squamous cell carcinoma (OTSCC) is an aggressive form of cancer with high morbidity and mortality. Human leukocyte antigen (HLA) class I molecules are involved in presenting tumor antigens to T cells, but their expression can be downregulated in cancer cells, allowing them to escape immune surveillance. This study aimed to characterize the expression of HLA class I in OTSCC and evaluate its association with clinical-pathological factors and survival.

The study included 142 treatment-naïve OTSCC patients. HLA class I expression in tumor cells was analysed using immunohistochemistry and reverse transcription quantitative PCR (RT-qPCR). Both intraepithelial and stromal density of CD8+ T cell infiltration was assessed. Clinical and pathological data were collected, and patient survival was followed up for at least five years.

The study found that most OTSCC expressed HLA class I homogeneously. HLA class I homogeneous tumors were significantly associated with a higher number of infiltrating CD8+ T cells, both in the tumor stroma and intratumorally. HLA class I expression alone did not correlate with survival. Interestingly, high intraepithelial CD8+ T cell infiltration was only significantly associated with improved survival in the HLA class I homogeneous tumors.

The findings suggest a role for HLA class I in the pattern and distribution of infiltrating CD8+ T cells. OTSCC often respond poorly to immunotherapies despite their abundant lymphocyte infiltrate. It might be that loss or downregulation of HLA class I in OTSCC with high levels of CD8+ T cells partly explains this phenomenon. High HLA class I expression alone has no prognostic value in our patient cohort, but might be a positive prognostic marker when combined with high levels of infiltrating CD8+ T cells. Further research is needed to elucidate the underlying mechanisms and explore potential immunotherapeutic strategies targeting HLA class I in OTSCC.

P133

DECODING THE MOLECULAR MEDIATORS OF HOST-MICROBE INTERACTIONS IN INFLAMMATORY BOWEL DISEASE

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Humans and their microbiome are intimately connected, and many human-associated microbes are associated with various health and disease states, including inflammatory bowel disease, a strong predisposing factor for colorectal cancer. While there are many associations between host disease states and particular microbes, the potential molecular mechanisms underlying these correlations are not well-characterized. To define the molecular mechanisms enabling these interactions, and explore the variation in these interactions among bacteria, we developed methods to assess bacterial binding to human proteins and a pipeline to identify and characterize the bacterial proteins that mediate binding.

A yeast display screen previously found that a disease-associated member of the microbiome may bind to CD7, a human surface protein expressed on several immune cell types, many present in the gut. Using biochemical reconstitution, we characterized the binding of many strains of this species from diverse human and animal sources to CD7 and found both binding and non-binding strains. Whole-genome sequencing and genomic comparisons among these strains identified genes encoding putative mediators. Using binding assays with recombinant proteins, we identified the bacterial protein that mediates binding to CD7. Recombinant bacterial protein is now being used to elucidate the signaling events induced by bacterial binding to CD7. A similar approach is being applied to several uncharacterized bacteria-human protein interactions, which will define the molecular mediators of key host-microbe interactions and uncover the function of previously uncharacterized bacterial proteins.

P134

PD-L1 CHECKPOINT BLOCKADE PROMOTES REGULATORY T CELL ACTIVITY THAT UNDERLIES THERAPY RESISTANCE

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Immune checkpoint blockade (ICB) through inhibition of PD-1 or PD-L1 has provided a paradigm shift in cancer treatment by unleashing anti-tumor immunity. Although ICB has shown efficacy in multiple types of cancer, the majority of patients do not or only temporarily respond for reasons incompletely understood. Regulatory T cells (Tregs) are an important suppressive cell type that is able to express high levels of PD-1 and could therefore be involved in dictating response to treatment with anti-PD-1/PD-L1 ICB. However, to date, the effect of ICB on Treg function and the relation to treatment resistance has not been fully clarified. Identification and subsequent targeting of this role could overcome resistance to ICB in patients eventually improving immunotherapy efficacy.

To assess the effect of anti-PD-L1 on T-cell subsets (CD8+ T cells, CD4+ T-helper cells and Tregs), we performed spatiotemporal analyses in multiple solid tumor mouse models including the ICB-non-responsive AE17-OVA tumor model using multicolor flowcytometry. We validated the effect on Tregs by performing RNA sequencing and in vitro suppression assays. Using bone marrow chimera experiments with PD-1WT and PD-1KO cells, we could delineate whether the activation of Tregs following ICB was directly mediated via PD-1 on Tregs. To investigate the functional involvement of Tregs in treatment resistant, we depleted Tregs using anti-CD25 or diphtheria toxin (DT) in DEREK mice. To observe whether similar mechanisms were at play in patients, both peripheral blood obtained from mesothelioma and lung cancer patients and single-cell RNA sequencing data on T cells in skin- and lung cancer patients were assessed for the activation of Tregs following ICB in relation to ICB response.

We found that Tregs were primarily activated following anti-PD-L1 ICB in all treatment refractory mouse models evaluated compared to CD8+ T cells and CD4+ Th cells. This was in contrast to treatment-responsive mouse models, where we found a profound activation of CD8+ T cells and only a marginal effect on Tregs. When further dissecting the effect of anti-PD-L1 treatment on Tregs in the AE17-OVA model, we revealed that Tregs increase their immunosuppressive capacity on protein, gene and functional levels following treatment. PD-1 expressed on Tregs appeared to exert a critical role in modulating this phenotype. Treg depletion by using an Fc-optimized anti-CD25 antibody (n=7-10 mice/group) or diphtheria toxin treatment in DEREK mice (n= 7-16 mice/group) sensitized anti-PD-L1 resistant AE17-OVA tumors by delaying tumor growth and prolonged survival compared to both monotherapies. This coincided with strong upregulation of proliferation (assessed by Ki-67) and activation (IFN γ /TNF α /PD-1) by CD8 T cells and CD4-Th cells in peripheral blood. Finally, we showed the clinical importance of Tregs in mediating resistance to ICB as anti-PD-1/PD-L1 ICB induced PD-1+ Treg activation in peripheral blood and tumors following treatment preferentially in non-responding patients compared to responders. As we could observe these effects on Tregs in mice at different immunological sites including tumor-draining lymph nodes (TDLNs), current experiments will reveal whether TDLN-specific depletion of Tregs using DT is capable of driving the responses to ICB we observe. Furthermore, unique data generated in our lab on single-cell sorted, RNA/TCR- and CITE-sequenced T cells including Tregs from matched lung cancer patient (n=20) TDLNs and tumor will reveal how Tregs at the different sites are related to one another.

In conclusion, these data reveal a yet unappreciated role for Tregs underlying anti-PD-1/PD-L1 treatment resistance by direct PD-1+ Treg-activation, thereby providing guidance for identification of

novel therapeutic targets aimed at rewiring Tregs to improve anti-PD-1/PD-L1 efficacy. The most recent follow-up data on this project will also be presented at the conference.

P135

CD8+ T CELLS KILL MHC-I-NEGATIVE TUMORS THROUGH THE NKG2D/NKG2DL AXIS

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The purpose of this study was to characterize an MHC-I- and antigen-independent tumor killing mechanism by CD8+ T cells.

The long-accepted paradigm for adaptive anti-tumor cellular immunity relies on antigen-specific tumor targeting by activated CD8+ T cells. CD8+ T cell cytotoxicity, in turn, is classically believed to depend upon T cell receptor (TCR) recognition of tumor antigens presented exclusively in the context of cell surface major histocompatibility complex I (MHC-I) molecules. Mutations leading to decreased or absent MHC-I expression are therefore purported to constitute a common mechanism by which tumors can evade T cell responses, rendering them immunologically "cold".

Recent pre-clinical and clinical studies, however, have demonstrated somewhat mixed roles for MHC-I in dictating responses to cancer immune-based platforms, such as immune checkpoint blockade (ICB). These mixed findings highlight the need for further investigation into the role of tumor MHC-I, as well as revisiting traditional notions of anti-tumor immunity.

In vitro tumor cytotoxicity studies were conducted with both murine and human cells, while in vivo mouse studies were conducted with orthotopic syngeneic glioma and melanoma models. RNA sequencing data were obtained from both humans and mice.

Here we sought to better evaluate the impact of tumor MHC-I expression on response to ICB. We engineered murine glioma and melanoma lines to lack cell surface MHC-I by knocking out beta-2 microglobulin (B2m), a critical component of MHC-I. Surprisingly, the efficacy of ICB was maintained in vivo against these orthotopic tumor models, despite the absence of MHC-I on tumor cells. Furthermore, survival in treated mice remained dependent on the presence of CD8+ T cells and was independent of NK cells or CD4+ T cells. This cytotoxicity is both antigen- and MHC-agnostic and, instead, is mediated by CD8+ T cell NKG2D engagement of NKG2D ligands on tumor cells. Subsequent tumor cell kill depends on prior TCR activation (albeit even by irrelevant antigen), revealing that adaptive priming can beget subsequent innate killing. This cytotoxicity mechanism is active in vivo in mice, as well as in vitro in human cells, and is required for killing of MHC-negative tumor cells even in tumors with heterogenous MHC-I expression.

In these studies, we demonstrate that CD8+ T cell-dependent immunotherapies can indeed remain effective against tumors uniformly lacking MHC-I. These findings challenge the long-advanced notion that downregulation of MHC-I is a viable means of tumor immune escape, and instead identify the NKG2D/NKG2DL axis as a therapeutic target for enhancing T cell-dependent anti-tumor immunity against MHC loss variants.

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MEK-DEPENDENT METABOLIC CIRCUITS DRIVE TERMINAL CD8+ T-CELL EXHAUSTION

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CD8+ T-cell-mediated clearance of virus-infected cells and tumors is impeded by development of an antigen-driven dysfunctional program known as T-cell 'exhaustion,' characterized by upregulation of inhibitory receptors as well as loss of cytotoxic and proliferative capacity. Recent studies have shown that exhausted T-cells (Texh) are a heterogeneous population of discrete cell states. These include, but are not limited to stem-like 'progenitor' exhausted (Tpex) cells, which upon further antigen stimulation or checkpoint blockade lose their proliferative capacity to differentiate into 'terminally' exhausted (Ttex) cells. Tpex are essential for response to immunotherapy, but no durable treatments for maintaining this pool exist. Our laboratory has previously shown that chronic antigen-dependent impairment of mitochondrial oxidative phosphorylation and accumulation of reactive oxygen species (ROS) drive differentiation of Tpex into Ttex. The mechanism by which persistent TCR stimulation drives metabolic dysfunction remains unclear. In this study, we aimed to understand the mechanism by which chronic antigen promotes mitochondrial dysfunction and loss of self-renewal capacity within the Texh compartment.

We began by leveraging our established in vitro chronic T-cell receptor (TCR) stimulation platform to perform an inhibitor screen to select for TCR proximal signaling intermediates whose inhibition reduced ROS and restored proliferation, and identified MEK as the primary driver of ROS-dependent loss of proliferation during chronic stimulation. We used click chemistry detectable reagents, O-propargyl-puromycin (OPP) and 5-Ethynyl Uridine (5EU), to measure rates of protein translation and transcription respectively. We confirmed our findings in tumor and chronic LCMV infection models.

Characterization of MEK inhibition (MEKi) showed that restoration of proliferative capacity by MEKi was associated with maintenance of a self-renewing Tpex phenotype, leading to a significant increase in TILs in MEKi-treated tumor-bearing mice. Our lab had demonstrated that antioxidant therapy can enhance Tpex population by enabling increased mitochondrial ATP production to support the high metabolic demand of persistent antigen encounter. However, both extracellular flux and stable isotope tracing analysis of T-cells during chronic stimulation showed that MEKi-treated cells had significantly reduced rates of aerobic glycolysis and glucose-derived acetyl-CoA entry into the TCA cycle but equivalent rates of TCA cycle oxidation compared to vehicle-treated cells, suggesting that MEKi might prevent ROS by reducing ATP demand and consequent rates of nutrient uptake. We confirmed this hypothesis by analyzing spent media that showed MEKi-treated Texh cells have reduced uptake of glucose and glutamine. Further, steady-state metabolomics analysis confirmed that MEKi increases ATP/ADP ratios of chronically stimulated T-cells, corroborating our hypothesis that MEKi reduces the metabolic demand of chronic stimulation. Given that protein translation is the highest consumer of ATP in metabolically active cells, we then hypothesized that MEKi might reduce chronic TCR stimulation-driven protein translation. Indeed, RNA sequencing and measurement of protein translation rates using OPP labeling confirmed that MEKi reduced translation. Given that ERK is known to phosphorylate RNA polymerase II (RNAPII) to prime transcription, we hypothesized that MEKi reduces translation rates in chronically stimulated T-cells through regulation of RNAPII. Consistent with this, nascent RNA measurements with 5EU labeling and RNAPII (p-Ser5) Western blots showed MEKi decreased global transcription rates and RNAPII CTD phosphorylation.

Taken together, these results demonstrate that MEK/Erk hyperactivation is the primary driver of increased metabolic demand in Texh and suggest rational strategies to leverage MEKi to balance self-renewal and effector function during prolonged T-cell responses.

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THE ANTI-CORE 1 O-GLYCANS TARGETING MONOCLONAL ANTIBODY NEO-201 RECOGNIZES AND REDUCES THE QUANTITY OF NAÏVE REGULATORY T CELLS IN PBMCS OF CANCER PATIENTS

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NEO-201 is a humanized IgG1 monoclonal antibody which binds to Core 1 and/or extended Core 1 O-glycans expressed by several human solid and blood tumors, as well as neutrophils, but it does not bind to most normal tissues and human immune cell subsets (B cells, CD4+ T cells, CD8+ T cells, NK cells, monocytes). NEO-201 mediates killing of its target cells via antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). In Phase I clinical trial we observed that NEO-201 also binds to circulating regulatory T cells (cTregs) and cause a reduction of the quantity of cTregs in cancer patients with stable disease (SD). One of the reasons of the low response rates and resistance to PD-1/PD-L1 blockade in solid tumors may be due to the activity of Tregs in the tumor microenvironment (TME). The reduction in the percentage of cTregs following NEO-201 treatment supported the rationale of the ongoing phase II clinical trial (Clinical Trial NCT03476681) evaluating the efficiency of the combination of NEO-201 with pembrolizumab in adults with checkpoint inhibitors treatment-resistant solid tumors.

Subjects with recurrent, locally advanced unresectable or metastatic Non-Small Cell Lung Cancer (NSCLC), Cervical Cancer, Head and Neck Squamous Cell Carcinoma (HNSCC), Uterine Carcinoma who have progressed during or after front-line standard of care treatment are being enrolled. To evaluate which subset of Tregs is recognized by NEO-201, PBMCS from 5 cancer patients, enrolled in the ongoing phase II clinical trial, were profiled by flow cytometry for expression of specific Treg markers, including CD3, CD4, CD45RA, Foxp3 and NEO-201. The percentage of Tregs in PBMCS was analysed before starting the treatment and at multiple time points after the first infusion with NEO-201, including C1D15 (14 days of the first infusion), C2D1 (beginning of cycle 2), C3D1 (beginning of cycle 3).

Flow cytometry analysis of PBMCS revealed that NEO-201 recognizes naïve Tregs (nTregs: CD3+/CD4+/CD45RA+/Foxp3low cells) while it does not bind to effector Tregs (eTregs: CD3+/CD4+/CD45RA-/Foxp3high cells). Based on this observation, we compared the percentage of nTregs in the CD3+/CD4+ population from PBMCS in three cancer patients with SD and two cancer patients with progressive disease (PD) pre and post treatment, and we correlated the modulation of levels of circulating nTregs with the clinical response.

Between patients with SD, one patient with cervical cancer showed a reduction of 46.34% (4.76% vs 8.87%) and 40.14% (5.31% vs 8.87%) of nTregs at C2D1 and C3D1 respectively, compared to baseline levels. This patient showed SD after treatment for more than 8 months. Similar pattern was observed in one patient with HNSCC, who showed SD after treatment for more than 8 months. This patient is still receiving treatment. No change in percentage of nTregs has been detected in the patient with NSCLC at C2D1 compared to baseline levels (9.50% vs 9.44%). This patient showed SD for 3 months after treatment.

Conversely, in two patients with PD (both with uterine cancer), the percentage of nTregs increased after the treatment compared to baseline levels. This phenomenon correlates with PD reported at the first re-staging (prior C3D1).

Following TCR stimulation, nTregs proliferate and differentiate into highly suppressive eTregs. Infiltration of TME by eTregs has been correlated with poor prognosis and survival in various types of cancer. Partial decrease of circulating nTregs after treatment with NEO-201 was associated with

stabilization of disease in cancer patients analyzed in this study. These data suggest that depletion of circulating nTregs may prevent the differentiation of nTregs into eTregs and their accumulation in the TME. In this regard, the combination of NEO-201 with pembrolizumab can result in an enhancement of the efficacy of pembrolizumab in cancer patients with checkpoint inhibitors treatment-resistant solid tumors.

P139

TRANSLATIONAL DYSREGULATION RESTRICTS INTRATUMORAL T-CELL EFFECTOR FUNCTION

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Loss of cytotoxic function is a hallmark of CD8+ T-cells within the tumor microenvironment and is associated with a set of genome-wide transcriptional and epigenetic alterations known collectively as T-cell “exhaustion.” However, while CD8+ T-cells rapidly lose effector cytokine production upon tumor infiltration, the precise mechanisms governing loss of cytotoxic protein synthesis within tumors remain unclear. The aim of this study is to determine whether local amino acid insufficiency contributes to T-cell effector dysfunction within tumors.

We began with the observation that several epithelial tumor subtypes whose response to immune checkpoint blockade (ICB) is less than what would be predicted based on tumor mutational burden have a relative overexpression of genes associated with response to amino acid deprivation. In parallel, we noted that CD8+ T-cells exposed to chronic antigen derived from both tumors and persistent viral infections activated genes consisted with an “exhausted” phenotype and lost the ability to produce effector cytokines. However, while T-cells from mice with chronic viral infections progressively downregulated both production of cytotoxic protein and expression of cytotoxic mRNA transcripts, tumor-infiltrating T-cells rapidly lost the ability to produce cytotoxic proteins while accumulating high levels of mRNA transcripts for the same genes, suggesting post-transcriptional mechanisms restricting effector function within tumors.

We therefore asked whether tumor-infiltrating CD8+ T-cells were less able to broadly engage in protein translation. Intraperitoneal administration of O-propargyl-puromycin, a puromycin derivative, to mice bearing either B16-OVA tumors or lymphocytic choriomeningitis virus (LCMV) infection allow in vivo measurements of translation efficiency via flow cytometry after conjugation of fluorophores. To examine local glutamine deprivation in the tumor, we leveraged a novel frameshift-based fluorescent reporter that activates upon glutamine limitation.

The result demonstrated that CD8+ T-cells exhibited diminished translation rates within tumors but not tumor draining lymph nodes and not during chronic viral infections. Next, to determine the mechanism by which protein translation is impaired in tumor-infiltrating T-cells, we measured rates of extracellular amino acid consumption by T-cells during acute or persistent antigen stimulation and found that chronically stimulated T-cells consumed high rates of several amino acids. In parallel, we leveraged a novel qPCR-based strategy to measure codon-specific tRNA charging by amino acids and found that glutamine-tRNA was preferentially uncharged during persistent antigen stimulation, suggesting that tRNAGln uncharging drives intratumoral loss of T-cell effector function; this was confirmed using T-cells transduced with the fluorescent reporter whose expression is triggered by glutamine deprivation, and which was expressed in tumor-infiltrating T-cells but not T-cells from mice with either acute or chronic infections.

Finally, we hypothesized that amino acid deprivation-driven tRNAGln uncharging is partially driven by increased rates of glutamine anaplerosis within the TCA cycle. Accordingly, blocking glutaminolysis with CB-839, a glutaminase inhibitor, was sufficient to partially restore protein translation rates and effector cytokine production by chronically stimulated T-cells under amino acid limitation.

Taken together our results reveal post-transcriptional regulation of effector cytokine translation as a previously unappreciated regulator of intratumoral T-cell function and nominate targeting T-cell amino acid metabolism as a rational strategy to enhance the effectiveness of immunotherapy in cancer treatment.

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HALLMARKS OF IMMUNE CHECKPOINT THERAPY-INDUCED MYOCARDITIS AND MYOSITIS

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Immune checkpoint therapies (ICTs) can induce durable T cell-mediated anti-tumor responses, but also life-threatening immune-related adverse events (irAEs) requiring ICT discontinuation. Myocarditis (inflammation of the cardiac muscle) and myositis (inflammation of the skeletal muscle) are rare irAEs that carry mortality up to 40%. These irAEs often co-occur and present with similar symptoms, suggesting that common mechanisms may contribute to these toxicities. We therefore hypothesized that the mechanisms of ICT-associated myocarditis and myositis are shared.

We enrolled patients with suspected ICT-induced myocarditis and myositis on an IRB-approved laboratory protocol. Clinical diagnosis required: 1) symptoms requiring hospitalization; 2) elevated serum markers (creatinine kinase ≥ 5 x normal, troponin >99 th percentile or detection of autoantibodies for MG); and 3) positive biopsy, electromyography, or cardiac MRI. Patients underwent standard diagnostic testing (including endomyocardial and/or skeletal muscle biopsy) and treatment as clinically indicated. Single cell RNA sequencing (scRNASeq) and immunohistochemistry (IHC) were performed on tissue specimens.

27 patients were enrolled within 96 hours of clinical presentation for suspected immune-related myocarditis and myositis, of whom 20 had evaluable tissue for scRNA-seq. 11 patients had confirmed toxicity (n=10 cardiac muscle; n=6 skeletal muscle; with patient matched samples in n=5). 9 patients with suspected myocarditis and myositis were ruled out due to alternative diagnoses (n=4 cardiac muscle; n=5 skeletal muscle). The most common cancers in this patient cohort were renal cell carcinoma and melanoma. Although all patients received anti-PD-(L)1 based-therapy, the most common ICT received in patients with confirmed irAEs was the combination of anti-CTLA-4 plus anti-PD-(L)1 (n=4, 36%). The median number of cycles of ICT in the confirmed irAEs group was 1 (interquartile range [IQR]: 1-2) and in the control group was 4 (IQR: 3-8). A total of 58,523 cells were analyzed from cardiac and skeletal muscle. scRNA-seq of the affected cardiac tissues revealed enrichment of CD8+ T cells with a cytotoxic phenotype and a population of inflammatory IL-1B+TNF+ myeloid cells. We further identified tissue-resident myeloid cells expressing Fc γ RIIIa, which recognizes IgG antibodies and regulates complement activation. IHC analyses of the affected cardiac tissues identified CD8+ T cells and CD68+ myeloid cells, and the presence of pan-IgG and the complement product C4d, implicating plasma B cells in disease pathogenesis. Consistent with this, half of the patients with confirmed myocarditis had high serum titers of autoantibodies against muscle antigens. scRNA-seq of affected skeletal muscles revealed identical populations of CD8+ T cells and inflammatory myeloid cells as were found in the affected cardiac muscles.

These results nominate immune subpopulations potentially contributing to disease pathogenesis that may inform rational treatments for immune-related myocarditis and myositis. The early onset of myocarditis and myositis following treatment with anti-PD-(L)1 ICTs, which target antigen-experienced T cells, suggests re-invigoration of pre-existing autoimmunity and may inform the discovery of predictive biomarkers in blood for patient selection.

P141

IDENTIFYING MECHANISMS OF RESISTANCE TO HYPOMETHYLATING AGENTS IN A MODEL OF ACUTE MYELOID LEUKEMIA

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Hypomethylating agents (HMAs) are used to treat hematologic malignancies in patients that are unfit for the stress of chemotherapy or bone marrow/hematopoietic stem cell transplantation. High-dose HMA treatment results in direct toxicity to tumor cells, but low dose HMA treatment, such as with 5-Azacytidine (Aza), increases the expression of methylated genes including tumor suppressors, tumor antigens, and machinery for antigen presentation. Currently, clinical trials in patients with myelodysplastic syndromes and myeloid leukemia are evaluating the benefit of combining antigen-specific therapeutic vaccines with HMAs. Identification of genetic or surface protein biomarkers of sensitivity to this combinatorial treatment will be needed to determine which patients will be responsive and which patients are developing resistance to this strategy.

Human leukemia cell lines, HL-60 and Jurkat, along with the mouse acute myeloid leukemia (AML) cell line C1498 were treated with Aza in vitro and expression of genes necessary for antigen presentation were assayed by qPCR. For in vivo studies, C1498 cells transduced with a plasmid for constitutive expression of firefly luciferase (C1498-FLuc) were implanted in syngeneic C57Bl/6 mice via tail vein (80,000 cells/mouse) and allowed to establish until day 4 when treatment was started. Blood smears were used to confirm the successful implantation of C1498-Fluc as well as FLuc positivity by flow cytometry. Survival of implanted mice was carried out until signs of morbidity were observed due to disease progression. Flow cytometry of non-leukemic immune cells was accomplished using specific markers for T-cells and innate myeloid subsets as well as PD-1, PD-L1, and CTLA-4. Fluc positivity was used to gate out C1498 cells.

In vitro, we found that treatment with Aza increases expression of tumor-specific antigens and genes necessary for antigen-presentation in human and mouse AML cell lines. In C1498-implanted immunocompetent mice, a single cycle of three consecutive, daily doses of 5 mg/kg Aza decreased C1498 cells in the blood, spleen, and lungs leading to increased survival, however all Aza-treated mice succumb to disease after only a short extension indicating incomplete clearance of C1498 cells. When treatment is carried out for 8 cycles, overall survival is significantly prolonged and two distinct outcomes are evident. Fifty percent of 8 cycle-treated mice succumb to disease between 5 and 6 weeks after implantation, prior to stopping weekly Aza treatments. The other half survive well beyond the 8-week treatment, up to 5 months post-implant. These data indicate that C1498 cells develop resistance to Aza treatment about 50% of the time whereas the other 50% are highly responsive, mirroring human AML response to Aza treatment. Flow cytometry of normal immune cells from C1498-burdened mice treated with Aza reveal increases in CD4+ and CD8+ T-cells in the blood with a concomitant decrease in Ly6G+ and Ly6C+ myeloid cells; however, increases in surface expression of immune checkpoint molecules PD-1, PD-L1, and CTLA-4 are also observed on these cell types.

Acquired resistance of AML cells to Aza treatment may involve suppression of the immune response through immune checkpoint blockade as evidenced by increased expression of PD-1, PD-L1, and CTLA-4 on non-leukemic immune subsets. Our current studies will further delineate the mechanisms behind Aza resistance in this model to inform ideal combinatorial therapies. We will also determine the efficacy of combining Aza treatment with antigen-specific vaccines and therapeutic antibodies that bind immune checkpoint molecules. The success of these combinatorial strategies might be translated to other poorly immunologic cancers, such as pancreatic cancer, wherein HMA treatment might increase the efficacy of cancer vaccines or other immunotherapies.

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NX-1607, A SMALL MOLECULE INHIBITOR OF CBL-B, IS EFFICACIOUS AS A SINGLE AGENT AND IN COMBINATION WITH RITUXIMAB IN PRECLINICAL MOUSE MODELS OF LYMPHOMA.

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The E3 ubiquitin ligase Casitas B-lineage lymphoma B (CBL-B) is expressed in leukocytes and regulates signaling pathways in T and NK cells, significantly limiting their antitumor effector function. In T cells, CBL-B attenuates activation initiated by TCR engagement in part by mediating the requirement for CD28 co-stimulation, thus setting the threshold for T cell activation. In NK cells, CBL-B functions downstream of TAM receptors and negatively regulates cytokine production and cytotoxicity.

Here, we characterized the antitumor and immune effects of NX-1607, a potent orally bioavailable inhibitor of CBL-B, when used as a single agent in a murine A20 syngeneic B cell lymphoma model and in combination with Rituximab in a Raji cell xenograft model of Non-Hodgkin's Lymphoma (NHL).

We show that NX-1607 treatment of immunocompetent mice bearing subcutaneous A20 B cell lymphoma tumors leads to robust, T-cell dependent, tumor regression. All mice that achieved complete responses (CRs) in response to NX-1607 treatment successfully rejected tumor growth during a subsequent challenge with A20 tumors. Immunophenotyping studies of tumor infiltrating CD8+ T cells from A20 tumor-bearing mice treated with NX-1607 showed higher expression of multiple activation markers (CD25, CD69, PD-1), co-stimulatory markers (4-1BB, GITR, CD226/DNAM-1) and the cytotoxic marker Granzyme B. Profiling of circulating peripheral blood T cells from tumor bearing mice treated with NX-1607 showed an increased percentage of antigen-experienced PD-1+ CD8+ T cells with increased expression of activation markers (e.g., CD69), co-stimulatory markers (e.g., GITR) and the cytotoxic marker Granzyme B.

Moreover, we show in a Raji NHL model of disseminated disease that the combination of daily NX-1607 administration with Rituximab enhanced median overall survival when compared to single agent activity ($p < 0.0001$). The survival benefit provided by NX-1607 was abrogated by depletion of NK cells, which suggests that NX-1607 enhances NK cell-mediated antibody dependent cellular cytotoxicity (ADCC) in vivo.

Collectively, these studies provide insights into the in vivo activity of this novel inhibitor of CBL-B, demonstrating that NX-1607 displays antitumor efficacy in preclinical lymphoma models by enhancing innate and adaptive immune responses. The observed synergistic antitumor effects of NX-1607 in combination with Rituximab support its potential as an adjunctive treatment to enhance antitumor efficacy of antibody therapy for patients with hematopoietic malignancies. NX-1607 is currently being investigated in patients with advanced cancer including solid tumors and malignant lymphoma, including large B cell lymphoma (NCT05107674).

P143

GLOBO H-TARGETED CAR-T CELLS FOR SOLID TUMOR THERAPY

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The chimeric antigen receptor T (CAR-T) cells were genetically engineered to express CARs composed of an antigen-specific scFv antibody, costimulatory domain, and intracellular T cell signaling domain for antigen-specific cytotoxicity. Six CAR-T products approved by US FDA have led to remarkable regression in liquid tumors. However, there are still several hurdles, for example, the immunosuppressive tumor microenvironment, to be encountered for the CAR-T cells in solid tumors.

Development Center for Biotechnology (DCB) has developed CAR-T cells targeting Globo H, a tumor-associated carbohydrate antigen, highly expressed in several cancers.

Our preliminary data suggested that Globo H CAR-T cells showed significant antigen-specific cytotoxicity and cytokine production in Globo H-expressing gastric cancer cells. The combination of Globo H CAR-T cells with anti-PD-1 or PD-L1 antibody overcome the immunosuppressive microenvironment for the enhanced treatment of Globo H-positive solid tumors.

In summary, Globo H CAR-T cells with an anti-PD-1 or PD-L1 antibody can be used for the treatment of Globo H-positive cancers.

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TGFB1 ISOFORM, RATHER THAN TGFB3, IS RESPONSIBLE FOR RESISTANCE TO IMMUNE CHECKPOINT BLOCKADE THERAPY IN MOUSE TUMOR MODELS.

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Cancer immunotherapy holds great promise for improving patient's survival and quality of life. Nevertheless, even in cancer types known to be sensitive to PD-1/PD-L1 blockade, only a fraction of patients achieves complete and durable responses. The analysis of data coming from clinical trials showed how TGF β signaling is often associated with poor cancer immunotherapy outcome and can restrain the effectiveness of immune checkpoint blockade therapy (ICBT). TGF β signaling in tumor-associated fibroblasts (F-TBRS) is particularly associated with poor outcome in the immune-excluded tumor phenotype, in which CD8 T cells accumulate at boundary between tumor and stroma. This information led to a number of clinical trials in which TGF β inhibition is combined with ICBT. The majority of these trials involves pan-TGF β inhibitors and have so far had limited success. This could be due to incomplete TGF β targeting, especially since targeting all three isoforms may be associated with dose-limiting toxicities.

TGF β is a pleiotropic cytokine with complex mechanisms of activation and downstream signaling that are context- and cell type-dependent. TGF β has three isoforms, TGF β 1-3, all of which signal through the same TGFBR1/2 receptor complex, but are heterogeneously expressed by different cell types. TGF β signaling occurs in local tissue niches such as the tumor microenvironment (TME), where the cytokine localizes primarily within the extracellular matrix or at cell surfaces. Multiple cell types including those within the TME can respond to TGF β as evidenced by studies using pan-TGF β inhibitors. In particular, we recently showed that tumor regression induced by anti-pan-TGF β /anti-PD-L1 combination therapy can occur independently of circulating T cells, expands clonally diverse stem cell-like CD8 T cells (TSCL) and activates their progeny within the TME that are ultimately responsible for an IFN γ -dependent anti-tumor response. However, the impact of blocking distinct isoforms of TGF β on key cell compartments in the context of cancer immunotherapy still remains incompletely understood.

Here, we present a thorough analysis of the expression of the three TGF β isoforms in the TME in human tumors and mouse tumor models. To clarify which isoform plays the main role in ICBT resistance, we utilized preclinical tumor models in which we systemically administered monoclonal isoform-specific antibodies to block each of the active TGF β isoforms.

Our analysis shows that TGF β 1 and TGF β 3 are the dominant isoforms in tumors. In bladder cancer patients, TGF β 1 expression is significantly associated with poor outcome, while TGF β 3 expression is associated with poor outcome albeit not significantly. Interestingly, in the same patient population the F-TBRS (that is significantly associated with poor outcome) correlates with TGF β 3, but not TGF β 1 expression. Our preclinical data shows that TGF β 1 is responsible for resistance to ICBT anti-tumor activity in several tumor models: anti-TGF β 1/PD-L1 combination treatment significantly reduced tumor growth compared to aPD-L1 alone. In the EMT6 tumor model TGF β 1 blockade reduced TGF β signaling in the tumor and in the tumor draining lymph nodes, induced TSCL expansion and induces significant IFN γ response in the TME cell populations. Surprisingly, TGF β 3 blockade was detrimental to anti-tumor activity as in combination with anti-PD-L1 did not increase tumor growth control. TGF β 3 blockade resulted in TGF β pathway activation in the tumor (increased SMAD2/3 phosphorylation), loss of TSCL from the TME and reduction of IFN γ activity in all cells of the TME. RNA seq analysis showed that TGF β 1/PD-L1 blockade reduced expression of TGF β 3 in

all cell types analyzed, while TGF β 3/PD-L1 did not, suggesting the existence of a feedback loop between the two isoforms.

Additional experiments will be required to understand how TGF β 1 and TGF β 3 affect TSCL survival and the molecular mechanism behind the two isoforms co-regulation.

P145

ANALYSIS OF ANTITUMOR RESPONSE INDUCED BY SIMVASTATIN AND ANTI-PD1 IN MICE AND HUMAN MELANOMA

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Numerous retrospective clinical data on melanoma and non-small cell lung cancer have recently shown that patients taking the common cholesterol-lowering drug statin, before the onset of the anti-PD1 treatment, have a better progression free survival (PFS) and overall survival (OS). Starting from this evidence, we aim to investigate if statin really increases the efficacy of immune checkpoint inhibitors (ICI) in melanoma and to identify the underlying biologic mechanism.

We analyzed PFS and OS from our cohort of 45 metastatic melanoma patients, of which 11 were taking statin before anti-PD1 treatment for cardiovascular co-morbidity. Next, given the significance of the results, we designed pre-clinical experiments in vivo and tested the clinical efficacy of simvastatin, anti-PD1 or the combination of simvastatin plus anti-PD1 in C57BL/6 mice bearing the B16F1-gp100 melanoma. We measured tumor growth and tumor weight and analyzed by flow-cytometry the tumor-infiltrating immune cells from treated mice. Moreover, we designed specific panels of mAbs to perform high-dimensional (HD)-flow cytometry of PBMCs from the cohort of 45 melanoma patients reported above.

In our melanoma cohort, the median-PFS was 52.9 months in the statin-treated group vs. 8.8 months in the non-statin-treated group (HR 0.39) and the median-OS was not reached vs. 29.3 months (HR 0.41) respectively. 2-years PFS was 50% vs. 10% and 2-years OS 75% vs. 50% in favor of statin-treated patients. Pre-clinical experiments in mice showed that the combinatorial treatment of simvastatin plus anti-PD1 was the only one capable of slowing down tumor growth when compared to control mice. Importantly, this synergistic effect was seen only in mice who were pretreated with simvastatin before tumor challenge, and not in those who started statin concomitant to ICI. Strikingly, FACS analysis of tumor immune infiltrate revealed that intratumoral dendritic cells (DCs) from simvastatin-treated mice displayed a much higher level of MHC-II molecules on their cell membrane. Since statins reduce the intracellular prenylation of small GTPases, such as RhoA/K-Ras and Rab family proteins, which are essential for the regulation of the Pyrin inflammasome, and for the regulation of cytoskeleton organization and vesicular membrane trafficking, we are currently investigating whether these mechanisms are responsible for the increased expression of MHC-II molecules on DCs. As above-reported, we are also performing HD-flow cytometry analyses on basal PBMCs of our statin-treated vs. non-statin-treated melanoma patients to identify immune cell subsets correlating with the positive clinical outcome induced by statins plus ICI.

In conclusion, we detected increased PFS and OS in metastatic melanoma patients taking statin and treated with ICI. In mouse melanoma models, we observed the augmented expression of MHC-II molecules in intratumoral DCs, which paralleled the better control of B16F1-gp100 melanoma growth in mice pretreated with statin and then treated with anti-PD-1 after tumor challenge. These experiments, together with recent published data, would lay the basis to run a prospective randomized clinical trial in order to test whether statin improves patients' responses to ICI.

With a little help from my friends: innate immune cells and stromal cells in the tumor microenvironment

P146

TARGETING CCR2/CCL2 AXIS ATTENUATES TUMOR GROWTH AND IMPROVES RESPONSE TO CHECKPOINT INHIBITOR THERAPY

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The development of checkpoint inhibitor therapies represents a major breakthrough of cancer therapy in the past years. However, many patients do not respond to these therapies. This is partially due to the immune suppressive tumor microenvironment (TME). Infiltration of myeloid derived suppressor cells (MDSCs) and high levels of CCL2 expression in tumors are associated with poor clinical prognosis in many cancer types. MDSCs impair T cell effector functions and contribute to T cell exhaustion. CCR2/CCL2 signaling pathway is critical in promoting the recruitment and accumulation of MDSCs in tumors.

In this study, we utilized CCR2-DTR transgenic mice to selectively deplete CCR2+ cells, thereby targeting MDSCs and tumor associated macrophages. Upon diphtheria toxin treatment, cells expressing high levels of CCR2 undergo apoptosis in transgenic mice. We inoculated CCR2-DTR mice or wild type litter mates subcutaneously with a variety of murine cancer cell lines including MC38 colon carcinoma, MCA205 fibrosarcoma, and LL2 lung carcinoma. All mice were treated with diphtheria toxin (8ng/g body weight) one day after cancer cell inoculation, and subsequently three times a week. We performed longitudinal micro blood samplings (on day 0, 4, 9, 14) on tumor bearing transgenic and wild type mice to evaluate the immune cell composition in blood using flow cytometry. At experimental endpoint, the tumors were resected, and infiltrating immune cells were characterized using flow cytometry and qRT-PCR.

Depletion of CCR2+ cells significantly reduced tumor growth in all three murine cancer models. We observed a marked decrease in the number of circulating monocytic MDSCs (CD11b+ Ly6G-Ly6Chi), and accompanying increase in the number of circulating CD8+ and CD4+ T cells upon CCR2+ cell depletion as early as day 4 after cancer cell inoculation. The increase in circulating T cells was sustained until experimental endpoint. Flow cytometry analysis of the TME indicates a decrease in monocytic MDSCs and tumor associated macrophages, which led to an increase in activated T cells, identified by PD1 and CD137. Combination of CCR2+ cell depletion and checkpoint inhibitor therapy further enhanced T cell responses in both CD4 and CD8 T cell compartments and improves the responsiveness to treatment.

Our findings demonstrated the role of CCR2/CCL2 axis in tumor progression and checkpoint inhibitor therapy resistance. The study provides compelling preclinical evidence supporting further investigation of combining therapeutic strategies targeting CCR2-CCL2 axis with immunotherapies across tumor types.

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PROSTAGLANDIN E2 AND THE TUMOR MICROENVIRONMENT: UNLEASHING THE POTENTIAL OF CANCER IMMUNOTHERAPY THROUGH RATIONAL COMBINATION THERAPIES

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Immunotherapy has dramatically improved the outcome of metastatic melanoma patients, with nearly half of the melanoma patients responding to combinations of immune checkpoint blockade therapy and surviving past 5 years. Nevertheless, there is still an unmet need to develop rational combination therapies for patients that are refractory to immunotherapy or become resistant. In solid tumors, the tumor microenvironment (TME) is a major determinant of anti-tumor immunity and immunotherapy resistance.

We generated mouse models and set out to understand the mediators that lead to an immune-evasive TME and the requirements within a TME for a functional T-cell response.

We found that immunotherapy-resistant tumors produce high levels of prostaglandin E2 (PGE2), an inflammatory lipid previously implicated in immune evasion. We show that genetic perturbation of the PGE2 synthesis pathway in cancer cells re-sensitizes resistant tumors to immunotherapy by re-polarizing the myeloid compartment. Reduction of PGE2 in the TME led to increased number and functionality across all dendritic cell subtypes and other antigen-presenting cells, which in inflammatory hubs stimulated T cells at the tumor site.

While pharmacological modulation with COX2 inhibitors led to increased T-cell infiltration, transient tumor control, and partial remodeling of the TME, a combination of COX2 inhibitors with DC-enhancing agents, was necessary to increase the pro-immunogenic effects exerted by COX2 inhibition, leading to long term immunotherapy response in resistant tumors.

Altogether we show how cancer cell-derived PGE2 inhibits immunotherapy response by creating an immune evasive TME that lacks inflammatory hubs necessary to generate a functional T cell response at the tumor site. Finally, our findings provide rational combination therapies to resensitize resistant tumors to immunotherapy.

P148

JMJD6 INCREASES BREAST CANCER AGGRESSIVENESS BY SUPPORTING M2 MACROPHAGE POLARIZATION

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Over 70% of breast cancers express estrogen receptor alpha (ER α), and estrogen inhibition is the front line adjuvant therapy. Unfortunately, recurrence may occur over time underscoring the need to identify the determinants responsible for acquired resistance. The epigenetic regulator JumonjiC domain-containing protein 6 (JMJD6) has been linked with poor outcome of ER positive breast cancer favoring their aggressiveness through the remodeling of tumor microenvironment. Notably, the role of JMJD6 in breast cancer has so far been investigated only from a cancer cell-intrinsic viewpoint, whereas no data are available to determine the role of JMJD6 expressed by other cells populating the microenvironment.

The mouse PyMT-41C breast cancer cell lines was employed for in vitro and in vivo experiments. Knock-out cells were generated with CRISPR/Cas9 technology. Expression levels of activating transcription factor 3 (ATF3), a transcription factor that has been associated with tamoxifen resistance in MCF7 cells, and glucose-regulated protein 78 (GRP78), a master protein folder regulator, were analyzed by Western blot and Real-Time PCR. Bone marrow-derived macrophages were differentiated towards M1 and M2 polarization. Knock-out macrophages were derived from JMJD6 floxed/LysMCre mice.

The role of JMJD6 was investigated considering its expression in both cancer cells and cells populating the tumor microenvironment. JMJD6 depleted PyMT-41C cells showed increased expression of ATF3 and GRP78. However, at the protein level, only the expression of ATF3 resulted enhanced, suggesting a different post-transcriptional regulation between ATF3 and GRP78.

To evaluate the role of JMJD6 expressed by immune cells, we produced ex vivo bone marrow-derived macrophages from mice JMJD6 $^{flox/flox}$, expressing LysMCre or not, and injected with PyMT-41C cells, and skewed them to M1 and M2 status. We focused on macrophages since they were found regulated by JMJD6 in FACS analysis. Knock-out of JMJD6 reduced M2-like in favor of M1-like polarization. Moreover, as already observed in cancer cells, JMJD6 knock-out increased the expression of ER α also in macrophages.

Our results indicate that JMJD6 affects the sensitivity to hormone treatment, although the final outcome could either be cell type- or species-specific. JMJD6 could regulate the response to endocrine treatment via ATF3, here identified as a new target of JMJD6 transcription regulation, in addition to estrogen receptor. These molecules could also be involved in macrophage polarization supporting the predominance of M2 macrophages and contributing to cancer cell aggressiveness.

P149

ONCOSTATIN M SIGNALLING IS REQUIRED FOR TUMOUR-PROMOTING INFLAMMATION IN THE LIVER AND FOR HEPATOCELLULAR CARCINOMA PROGRESSION

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Hepatocellular carcinoma (HCC) is one of the deadliest cancers and the most common liver tumour. The mechanisms driving HCC progression are very complex and usually HCC is preceded by other liver diseases, which hinders HCC early diagnosis. Indeed, there is a huge need of studies unravelling the mechanistic insights of HCC pre-tumorigenic stages in order to discover novel molecular targets for early diagnosis and treatment. Inflammation is an important contributor to HCC progression. Chronic inflammation is a well-established factor triggering cirrhosis and, later on, HCC. It promotes tissue remodelling and fibrosis, processes primarily mediated by hepatic stellate cells (HSCs) and Kupffer cells (KCs) and, later on, by infiltrated immune cells.

In this context, the inflammatory Oncostatin M signalling pathway may be a promising target. Oncostatin M (OSM) is a cytokine from the Interleukin-6 family that, acting through its receptor OSMR, promotes tumorigenesis, microenvironment remodelling and metastasis in different cancer types. Moreover, OSM/OSMR pathway plays relevant roles in liver development, regeneration and fibrosis, although little is known about its function in HCC onset and progression. Thus, we hypothesised that OSM signalling may contribute to HCC onset and development by affecting not only tumour cells, but also immune and stromal populations of the pre-tumour and tumour microenvironment.

First, we analysed OSM and OSMR expression in human data from different cohorts containing samples from healthy, cirrhotic and HCC patients. Next, we studied HCC formation in mice lacking OSM signalling (OSMR-KO model) compared to controls (OSMR-WT). We chemically induced HCC by using two models with different inflammatory status: low-inflammation Diethylnitrosamine (DEN)-induced model and high-inflammation DEN combined with carbon tetrachloride (DEN+CCl₄) model. Moreover, DEN+CCl₄ model was studied at two different time points of HCC progression to further characterize the dynamics of the tumour microenvironment (by flow cytometry and staining). Samples were analysed visually, histologically and molecularly. Liver functionality was determined by analysing the liver/body ratio weight and Alanine Aminotransferase (ALT) serum levels. Finally, we studied the expression of OSM and OSMR in liver subpopulations (such as hepatocytes, HSCs and KCs) and the correlation between these genes and stromal and immune populations presence in HCC patients samples.

First, we found that OSM and OSMR are overexpressed in cirrhotic/pre-HCC samples, as well as high expression of OSMR is associated with decreased survival in patients' cohorts. This suggested a role of OSM pathway in HCC onset (pre-HCC inflammatory stages) and aggressiveness.

Next, in our animal models, we discovered that the lack of OSMR did not affect inflammation-independent HCC tumorigenesis, while it significantly decreased tumour number and size in the DEN+CCl₄ pro-inflammatory model. In those mice, OSMR depletion alleviated liver inflammation and prevented HCC-associated liver damage. Interestingly, we found that OSM and OSMR were mainly expressed by HSCs and KCs in the liver subpopulations. Moreover, we also discovered that HSCs respond to OSM by upregulating myofibroblast markers such as FAP, LOX or PLOD2, as well as inflammatory cytokines (LIF or IL6). We also characterized by flow cytometry that OSMR depletion remodelled the immune microenvironment in the early stages of HCC development.

In line with all this, in HCC patients' data, we found that OSM and OSMR expression positively

correlates with the infiltration level of fibroblasts, macrophages and certain lymphocytic subpopulations.

Our results strongly support that OSM pathway is a key regulator of HCC-promoting inflammation, and that immune derived OSM mainly activates OSMR expressed by HSCs and KCs to drive HCC progression, suggesting a potential role of OSM/OSMR pathway in HCC at clinical level.

P150

TARGETING REVERSION MUTATIONS IN BRCA1/2 MUTANT CANCERS USING IMMUNOTHERAPY APPROACHES.

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Whilst PARP inhibitors and platinum constitute effective treatments for BRCA1/2 mutant cancers, resistance is a growing problem and is commonly caused by BRCA1/2 reversion mutations; these restore the reading frame of the BRCA1/2 gene, allowing the synthesis of a functional protein. In many cases, BRCA1/2 reversion mutations introduce novel stretches of amino acid sequence into the BRCA1/2 protein, which have the potential to act as tumour specific neoantigens. We aim to determine whether BRCA1/2 reversion-derived neoantigens could be targeted via immunotherapy approaches.

We generated a Brca1 revertant mouse model by using CRISPR-Cas9 to introduce a Brca1 exon 11 frameshift + reversion mutation in the C57BL/6 transformed ovarian cell line, ID8. This model expresses a range of reversion-derived neoantigens predicted to bind the C57BL/6 class I MHC. These binding predictions were confirmed experimentally using in vitro MHC binding assays. The neopeptide with the best MHC class I presentation was then synthesised, loaded onto dendritic cells (DCs) and used to vaccinate C57BL/6 mice. We subsequently assessed immunological response to vaccination in addition to the survival advantage achieved against ovarian cancer development following inoculation with the Brca1 reverted ID8-Brca1-rev cells. In parallel, we also studied the presence of neoantigen-specific T cells in the blood of patients with BRCA1/2 revertant tumors. For this, we predicted HLA class I binding for the peptides encoded by the patients reversion mutations against their matched HLA type, and synthesized the corresponding peptides for in vitro immune assays.

Analysis of the splenocytes of vaccinated mice by IFN- γ ELISPOT assays showed a significant increase in IFN- γ release following exposure to the BRCA1 vaccination neopeptide, thus confirming the successful generation of neoantigen-specific T cells. When challenged with Brca1 reverted ID8-Brca1-rev cells, vaccinated mice showed a significantly delayed tumour or ascites development and prolonged survival, suggesting that vaccination against reversion neoantigens could result in an anti-tumor immune response that enhances survival. Moreover, our patient studies showed that incubation of the patients PBMCs with revertant neopeptides induced a strong increase in IFN- γ release, confirmed by both IFN- γ ELISPOT and ELISA. Reversion neopeptide incubation also elicited T cell cytotoxicity, detected by Granzyme-B ELISA and heightened expression of T cell activation markers, including CD25 and CD137, detected by flow cytometry.

These results show that vaccination of mice against Brca1 reversion-derived neoantigens can successfully generate neoantigen-specific T cells that provide protection against Brca1 revertant tumor development. Moreover, the patient data suggest that BRCA1/2 revertant patients may possess T cells that recognise tumor revertant neoantigens. These patients could therefore potentially respond well to vaccination and/or immune checkpoint therapy.

P151

IDENTIFYING AND TRACKING PERIPHERAL BLOOD T CELLS REACTIVE TO A SINGLE PEPTIDE IN TWO KRAS G12D NSCLC PATIENTS WITH A SINGLE SHARED HLA ALLELE

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Routine next generation sequencing testing for cancer mutations in lung cancer defines clinically relevant molecular subtypes with distinct therapeutic options. However, the immunogenicity of many shared mutations across human leukocyte antigens (HLA) remains unknown. To address this, T cell reactivity against peptides derived from these mutations can be evaluated. We hypothesized that testing for presence of patient cancer-specific, mutation-specific, peptide-specific T cells is feasible with shared cancer mutations and represents an opportunity to monitor treatment response in patients on immune checkpoint inhibitor.

2 patients with KRAS G12D mutant lung cancer with prior clinical response to immune checkpoint inhibitor based therapy were selected and underwent HLA class I typing. They were found to have a single matching HLA allele. Peptides specific to KRAS (G12D) were identified as potentially immunogenic epitopes if they were in silico hits using NetMHCpan 4.0 and MHCflurry 2.0. Functional assays were then performed using these predicted peptides. Peripheral blood mononuclear cells (PBMC) were tested for T cell reactivity before and after standardized durations of co-culture with short peptides of length 9-mer to 12-mer to maximize expansion and detection of peptide reactive T cells. T cell reactivity was evaluated by ELISpot (enzyme-linked immunosorbent spot for IFN- γ) and surface/intracellular markers using flow cytometry.

Patient #1 received immunotherapy and chemotherapy with radiological response. He has good disease control since March 2022 and currently is on immunotherapy maintenance (patient's choice to stop maintenance chemotherapy). Amongst his 6 HLA class I alleles, he was determined to have HLA-C*08:0X. His PBMC were incubated and co-cultured with short peptide pools for at least 11 days. A single peptide X was found to induce T cell reactivity both on ELISpot as well as on flow cytometric evaluation for IFN- γ . The tests were then repeated 1 month and 4 months later for the next 2 time points. Each time, similar T cell reactivity was demonstrable to this single peptide X only. To expand on this finding, Patient #2 with HLA-C*08:0X was identified. He had received 1st line immune checkpoint inhibitor alone with initial response. At the point of progression on immunotherapy alone, he was treated with chemotherapy with immunotherapy as 2nd line treatment from December 2022. Previously, on immunotherapy alone, Patient #2 had demonstrable T cell reactivity against peptide X at time-points corresponding to clinical disease response and control. At the point he had progression of his disease, he had no detectable T cell response to peptide X. 1 month later, after commencement of chemotherapy and immunotherapy combination, he was found to have detectable peptide X-specific T cells in peripheral blood once again. Peptide X-specific T cells remained detectable after 2 cycles of treatment and this time-point corresponded to radiological response to treatment. ELISpot assays for both patients were performed with mutant and wild type peptides and showed specificity for mutant peptide only and not for corresponding wild type peptide, and for mutant 9-mer pool that contained peptide X but not for pools of longer peptide length 10-mer to 12-mer.

We have developed a standardized pipeline for the detection and monitoring of T cells reactive to cancer antigens. We propose that assessing single mutant KRAS G12D peptide T cell reactivity assays is a feasible approach for serial monitoring in patients with KRAS G12D lung cancer, aiming to identify potentially cancer-directed cytotoxic T cells. Our next steps are to validate our findings by expanding immune-monitoring for KRAS specific T cells to additional patients with lung cancer, then to other KRAS-driven cancers.

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DECIPHERING THE IMMUNE MICROENVIRONMENT IN CASTRATION-RESISTANT PROSTATE CANCER (CRPC) THROUGH HYPERPLEX IMMUNOFLUORESCENCE AND AI-ASSISTED IMAGE ANALYSIS: UNVEILING POTENTIAL IMMUNOTHERAPY BIOMARKERS

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Prostate Cancer (PCa) is the most common noncutaneous malignant neoplasm in males in developed countries and remains a significant source of morbidity and mortality. The increasing incidence of prostate cancer and its progression to advanced stages underscores the urgency of finding effective treatment strategies. Chronic inflammation has been implicated as a critical contributor to PCa pathogenesis and progression to advanced metastatic disease, with studies pointing to a complex interplay between inflammation, genetic aberrations, and the tumor microenvironment.

Under chronic inflammation, highly reactive oxygen species secreted by activated phagocytic cells can cause double-strand DNA breaks and increase cell death. This leads to heightened cellular regeneration in the presence of continued DNA damage, elevating the mutation risk. The tumor microenvironment is altered by inflammation-induced angiogenesis and epithelial-mesenchymal transition (EMT), mediated by a range of cytokines produced by inflammatory cells, transforming it into a state that supports tumor cell growth and may enable immune evasion.

This study utilized single-stain CD3 IHC, Hyperplex Immunofluorescence (IF) and AI-assisted image analysis to investigate this complex landscape in castration-resistant prostate cancer (CRPC). In our CD3 IHC screening cohort of 360 hormone-sensitive prostate cancer (HSPC) and 465 CRPC samples, we observed significant inflammation in a substantial subset, challenging the conventional perspective of prostate cancer as a "cold" tumor.

Our analysis using density-based clustering algorithms decoded distinctive spatial patterns of CD3+ cell distribution across the tumor-stroma interface in CRPC, which displayed a significant positive association with patient survival independent of established clinical and biochemical markers of survival.

Additionally, we observed diffuse and nodular patterns of CD3+ inflammation in our screening cohort, with the latter pattern linked to somewhat worse survival outcomes. This led us to hypothesize that these nodules represent immature tertiary lymphoid structures (TLSs), possibly promoting an immunosuppressive environment through the formation of regulatory immune cells, including Myeloid-Derived Suppressor Cells (MDSCs).

Through Hyperplex Immunofluorescence (IF) data, we then charted the immune landscapes of "hot" and "cold" tumors in a smaller CRPC cohort of 42 cases, highlighting distinct patterns of immune response in inflamed tumors. Using infiltration analysis algorithms across the tumor-stroma interface, we identified two distinct types of immune reactions: One was characterized by high, diffuse inflammation with an abundance of CD4, CD8, CD68, and CD20 cells, often accompanied by Myeloid-Derived Suppressor Cells (MDSCs). In contrast, the other was marked by the formation of nodules by B cells, MDSCs, and naïve CD8 cells, suggestive of emerging immature TLSs.

Indeed, further visual evaluation revealed a correlation between mature TLSs and heightened inflammation, marked by a proliferation of CD3, CD4, CD8, and particularly CD20 cells. From these observations, diffuse inflammation and cases with mature TLSs may denote NLRP3 inflammasome activation, while cases with immature TLSs could manifest the Senescence Associated Secretory Phenotype (SASP). This improved understanding of the CRPC immune microenvironment is crucial for refining immunotherapy treatments.

In conclusion, our work illuminates the multifaceted immune microenvironment in CRPC, proposing a novel understanding of its role in disease progression. The identified potential biomarkers provide a promising direction for enhancing the effectiveness of immunotherapy in treating prostate cancer. These insights may help select PCa patients for immune checkpoint inhibition therapy and monitor patient response to therapy, ultimately improving patient outcomes.

P153

CD4+ CD138+ CELLS: A NEW UNCONVENTIONAL T CELL POPULATION IN PANCREATIC DUCTAL ADENOCARCINOMA

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Pancreatic ductal adenocarcinoma (PDAC) is a very aggressive disease characterized by an average 5-year survival rate of about 6%. One of the main features of this cancer is the presence of an immunosuppressive tumor microenvironment (TME) characterized by Th2/M2-type inflammation and by desmoplasia, an accumulation of fibroblasts and extracellular matrix. The aim of our project is to explore in depth the immune contexture of PDAC.

We investigated TME in PDAC by high dimensional flow cytometry analyses involving peripheral blood mononuclear cells, tumor infiltrating lymphocytes and tumor draining lymph nodes. We designed three different panels aimed at investigating B, natural killer, T, myeloid cells and ex-vivo cytokines, transcription factors in treated and chemo naïve patients both with primary and metastatic tumor.

We used different in vitro models in which we mimicked the PDAC TME to characterize the role and the functions of a new cell population emerged from high dimension flow cytometry analyses.

Unsupervised bioinformatic analysis allowed to identify new clusters of very rare unconventional CD4+ T cells expressing the marker CD138. The presence of these cells has been confirmed by another bioinformatic analysis of a published single cell RNA-sequencing data set and by immunofluorescence staining of PDAC tumor samples which revealed their localization in the stoma. In metastatic PDAC patients we found that higher percentages of this cell population significantly correlate with a reduced overall survival.

In literature CD4+ CD138+ T cells were described in mouse model of systemic lupus erythematosus having a role in the activation of autoreactive B cells when exposed to autoantigens.

CD138 has been demonstrated to bind the molecule APRIL; both these proteins are increased in PDAC patients' sera compared to healthy controls and high levels of APRIL correlate with a reduced survival in PDAC.

Our hypothesis is that APRIL is secreted in PDAC TME by M2 macrophages and other immune cells, it is loaded onto dendritic cells (DCs) which in turn transfer this protein to CD4+ CD138+ T cells that could induce B cell help in PDAC worsening the tumor progression.

To test this hypothesis, we performed in vitro experiments conditioning DCs sorted from healthy donors with PDAC sera containing APRIL and CD138 proteins from patients with stage IV disease. We then induced naïve CD4+T cell polarization through a co-culture with conditioned DCs. After 7 days polarized T cells were sorted purified for CD138 expression and cultured with autologous naïve B cells. We found an increment of Th2-type cytokines in presence of T CD138+ cells compared to the CD138- counterpart. Moreover, we found that CD4+ CD138+ exerted B cell help for immunoglobulin production of IgM, IgA and IgG, comprising IgG4 but not IgE.

Using another in vitro model, we found that detectable immunoglobulins were produced only by the B cells which were co-cultured with CD4+ CD138+ T cells polarized with DCs treated with APRIL and CD138 exogenous proteins during their differentiation from monocytes. This leads to understand the importance of the proteins APRIL and CD138 in both antigen presenting and T cell conditioning.

We found a new unconventional population of T cells expressing CD138 as a marker. These cells can perform B cell help activity inducing immunoglobulin production except for IgE. Data from literature depict a controversial role of immunoglobulins in PDAC, in addition RNA analyses of IGH isotype distribution in PDAC surgical samples suggested that most immunoglobulin production tends to correlate with a reduced overall survival for patients. For this reason, we aim to better clarify the function of CD4+ CD138+ T cells and the role of Ig in PDAC, unravelling their potential to become a new target for PDAC therapies.

P154

DEVELOPING ALLOGENEIC CAR-MODIFIED NATURAL KILLER CELLS AS A PLATFORM FOR IMMUNOTHERAPY AGAINST SOLID TUMORS

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Chimeric antigen receptor (CAR)-T cell therapies have proven efficacy in hematological malignancies but are confined to autologous use due to occurrence of potentially life-threatening graft-versus-host disease (GvHD) if transferred into HLA-mismatched patients. Moreover, many tumor antigens recognized by CARs are also expressed in healthy cells, resulting in “off-tumor” toxicity. We aim to overcome these limitations by assessing the efficacy of human natural killer (NK) cells engineered to express “antigen X”-targeted CAR in killing various solid tumor types.

To circumvent the occurrence of GvHD, we use innate immune cells, such as NK cells, as a chassis for CAR engineering. NK cells are not HLA-restricted and have natural cytotoxicity against virus-infected or physiologically stressed cells, such as tumor cells. They express a wide variety of receptors that engage in anti-tumor and anti-viral responses, including NKG2D, the natural cytotoxicity receptors (NCRs: NKp44, NKp46, NKp30) and DNAM-1 (CD226). To achieve “on-tumor” specificity with the avoidance of “off-tumor” toxicity in solid tumors, antigen X was chosen as a CAR target. Antigen X is a membrane-bound protein that is either not normally expressed in most healthy tissues, or shows localized compartmentalization in the healthy tissue in which it is expressed. However, antigen X loses such compartmentalization restriction or is ectopically expressed in some solid tumors, including colorectal tumors. We propose to engineer NK cells to express CAR targeting antigen X by viral transduction and assess their anti-tumor killing capacity against target tumor cell lines by in vitro and in vivo cytotoxicity assays. We will also investigate the pre-clinical anti-tumor efficacy and safety of the CAR construct introduced into NK compared with ab T cells as a chassis for CAR engineering.

“Antigen X”-targeted CAR-NK cells efficaciously killed “antigen X”-expressing colorectal tumor cells in both CAR-dependent and independent manner in a bioluminescence imaging-based in vitro cytotoxicity assay. Importantly, the anti-tumor cytotoxic effects of “antigen X”-targeted CAR-NK cells were significantly increased compared to non-CAR counterparts. Such an increase was not observed when “antigen X” non-expressing tumor cells were used for the cocultures, suggesting that the CAR is indeed recognizing its specific target antigen. Similarly, CAR-T cells exhibited increased cytotoxicity against “antigen X”-expressing colorectal tumor cells, but not the “antigen X” non-expressing cells, compared to non-CAR T cells. Work is ongoing to directly compare the anti-tumor cytotoxicity of “antigen X”-targeted CAR-NK with that of their T cell counterparts using in vitro and in vivo assays.

Our findings can be potentially exploited to enable wider therapeutic application of allogeneic NK cells bearing alternative CARs that harbour NK-specific endodomains which confer optimal NK function with the advantage of minimal/no toxicity to solid tumor patients.

P155

THE POTENTIAL OF TUMOR ORGANOID AND HSPC DERIVED NK CELLS TO UNCOVER PERSONALIZED IMMUNE ESCAPE MECHANISMS

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NK cells are well known for their capability to exert cytotoxicity and kill cancer cells. While numerous advances have been reported in the field of NK cell tumor biology and led to the development of, for example, CAR NK cells or novel inhibitory antibodies, the big breakthrough moment is yet to come. The heterogeneity of a person's NK cell population, their potential dysfunction in cancer patients, and various known, as well as unknown, tumor escape mechanisms, seem to slow down the progress of developing clinically successful NK cell therapies. In addition, for many years our knowledge has been built on experiments using cancer cell lines and NK cell lines, such as NK-92. It is likely that the breadth of NK cell driven tumor immune responses has not been fully captured and requires a model system closer to the patient.

Here, we use patient derived tumor organoids (PDTOs) and pairs of pre and post ICB treated PDTOs as a personalized model system. While we utilize NK cells derived from autologous peripheral blood mononuclear cells (PBMCs) to represent a patient's NK cell tumor reactivity, we foresee superiority in the use of hematopoietic stem and progenitor cell (HSPC) derived NK cells for the future of immunotherapies. Such NK cell products may outperform a patient's own NK cells or could facilitate better tumor infiltration.

Hence, we evaluated both stem cell derived and autologous NK cell reactivity towards tumor organoids, connected tumor specificity with relevant inhibiting and activating ligands, narrowed down on the effect of IFN gamma and present how ICB treatment may lead to escape mechanisms from NK cell recognition.

PDTOs were established from patients with NSCLC and CRC tumors at various time points of their ICB treatment regimes. Organoids and PBMCs were derived from material of the ITO and NICHE study and approved by the medical Ethical Committee of the Netherlands Cancer Institute–Antoni van Leeuwenhoek hospital. NK cells were isolated from PBMCs or derived from HSPC from donor blood (Dolstra et al., 2017). After cytokine stimulation, NK cells were co-cultured with organoids. Depending on the assay, tumor organoids were pre-stimulated with IFN gamma. NK cell reactivity was measured by flow cytometry and killing assays. Tumor organoids were analyzed by flow cytometry of surface maker and RNA sequencing.

We are presenting an approach to capture tumor specific NK cell reactivity on a personalized level and validate the use of PDTOs and a clinically relevant NK cell product as a model system to facilitate advances of the field. Comparing tumor responses of autologous NK cells to stem cell derived NK cells, indicates how tumor reactivity is driven by NK cells as well as tumor cells, hinting towards possibilities how to interfere with either side. Phenotyping of a panel of tumor organoids, eliciting a range of NK cell reactivities, underlines the importance of certain activating and inhibiting ligands and correlates novel factors to decreased NK cell recognition. In line with recent in vivo screens (Dubrot et al., 2022), our approach captures the inhibiting effect of IFN gamma on NK cell activation. Lastly, we present decreased NK cell reactivity towards tumor organoids from post ICB treated tumors compared to pre-treatment. Using transcriptomics data coupled with surface expression profiles, we narrow down possible mechanism how tumors become invisible to NK cells.

Our work presents a timely relevant approach to understand NK cell tumor reactivity on a personalized and elevated level. Our results are in line with recent findings and, in addition, present novel tumor escape mechanisms. Particularly interesting is the decrease in NK cell reactivity towards post ICB treatment PDOs and we present initial findings hinting towards potential mechanisms. Overall, we hope to inspire others and to use our findings to accelerate the progress of NK cell therapies.

P156

TETHERED IL-15-IL15R AUGMENTS ANTITUMOR ACTIVITY OF CD19 CART CELLS BUT DISPLAYS LONG-TERM TOXICITY IN IMMUNOCOMPETENT MICE.

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Adoptive cell therapy with T cells genetically modified to express chimeric antigen receptors (CAR-T) is one of the most promising advanced therapies for cancer treatment, especially in some hematological tumors. However, clinical results demonstrate that more than 40% of patients with B-cell malignancies relapse after this treatment. Because of its pro-survival and proliferative properties, IL-15 was proposed for the 4th generation of CAR-T cells to improve their persistence. In this work, we have studied the antitumor activity and potential toxicity of CD19 CART cells expressing a tethered IL15-IL15R protein in the BALB/c immunocompetent murine model challenged with A20 tumor cells.

Conventional anti-murine CD19 CAR-T cells and CD19 IL15-IL15R CAR-T cells were prepared by retrovirus infection of activated T cells. CART were characterized in vitro by measuring CART expression levels, IFN- γ production, cell proliferation and lytic activity in response to antigen stimulation. In vivo activity was analyzed by measuring the CD19 B cell depletion capacity under different regimens of lymphodepletion (TBI of 1 and 4 Gy). Transcriptomic profile of CART cells was analyzed by RNAseq. Toxicity of CART cell therapy was analyzed by measuring body weight, biochemical parameters, TCR clonality, mice survival and immunohistochemistry of different tissues. Antitumor efficacy of CART cells was measured in BALB/c mice challenged with A20 tumor cells.

Conventional anti-murine CD19 CAR-T cells showed low persistence and poor efficacy in immunocompetent mice treated with mild lymphodepletion regimens (Total body irradiation (TBI) of 1 Gy). The efficacy was improved when mice were previously treated with a high TBI regimen (4 Gy). The murine IL15-IL15R fusion protein enhances the persistence and in vivo efficacy of CD19 CAR-T cells, as they are able to eradicate established A20 B cell lymphoma. However, CD19 IL15-IL15R CAR-T displays important long-term toxicities, with marked splenomegaly, weight loss, ALT elevations, and significant inflammatory findings in some tissues. Mice survival is highly compromised after CD19 IL15-IL15R CAR-T cell transfer, particularly if a high TBI regimen is applied before CAR-T cell transfer.

Tethered IL-15-IL15R augments the antitumor activity of CD19 CAR-T cells but displays long-term toxicity in immunocompetent mice. Inducible systems to regulate IL15-IL15R expression should be considered to control this toxicity.

P157

LIPOSOMAL FORMULATION OF TOLL-LIKE RECEPTOR AGONISTS AS IMMUNOTHERAPY OF CANCER

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Immunotherapy is currently the most promising approach to cancer treatment. Among the most successful are checkpoint inhibitors, chimeric antigen receptor T-cell therapy (CAR-T), and monoclonal antibodies. However, only a fraction of cancer patients benefit from these therapies, and new generations of immunotherapies are needed. One of the strategies is macrophage-targeted therapies including the reprogramming of immunosuppressive macrophages into antitumoral macrophages. Our idea is to reprogram macrophages with immunostimulatory compounds that activate the Toll-like receptor (TLR). Different TLR ligands have been shown to be effective in inducing an antitumor immune response, but their systemic administration is limited due to toxicity. To address this problem, we have developed a liposomal formulation of TLR agonists to improve safety and targeting.

The aim of this study was to evaluate the therapeutic potential of liposomal formulations of TLR agonists in human in vitro settings. Our main goal was to investigate the direct effect of TLR agonists in the co-culture of macrophages and cancer cells.

In all experiments, human monocyte-derived macrophages (hMDMs) were differentiated from CD14⁺ monocytes in the presence of macrophage colony-stimulating factor (M-CSF). hMDMs were plated with cancer cells (PANC-1, MDA-MB-231, and SKOV-3), stimulated after attachment with TLR agonists (soluble or liposomal), and co-cultured for 3 days. Cells were then harvested and the number of cancer cell was counted using flow cytometry. TNF- α in the medium was measured by ELISA. Nitrite (a metabolite of nitric oxide) was detected using the Griess Reagent. Metabolic activity of cells in monocultures was evaluated by MTT assay.

First, we investigated the antitumor activity of 11 ligands of different TLRs. We measured the number of PANC-1 cancer cells in co-culture with hMDMs after treatment with TLR agonists. We observed a reduced number of cancer cells after stimulation with 9 out of 11 agonists and we have chosen lipopolysaccharide (LPS, TLR4 agonist), Resiquimod (R848, TLR7/8 agonist), and ODN 2006 (TLR9 agonist) as the most promising. Next, we validated selected agonists in the co-culture with other cancer cell lines: SKOV-3 and MDA-MB-231. We confirmed a decreased number of cancer cells in co-culture with hMDMs after addition of LPS, R848, or ODN. Soluble LPS, R848, or ODN did not decrease the metabolic activity of cancer cells in monoculture as measured by MTT assay, suggesting the essential role of macrophages as mediators of anticancer effect. In the next step we used liposomes containing R848 and observed a similar effect as for soluble R848, proving that the liposomal formulation does not reduce the activity of R848. Furthermore, we investigated the effectiveness of hMDMs polarization and its mechanism of action. We were unable to detect nitrite values in the medium after TLR agonist stimulation. Conversely, a large increase in TNF- α release was observed after R848 stimulation and a modest increase after LPS stimulation, however, ODN stimulation did not affect TNF- α release, indicating different mechanisms of activity of the tested TLR agonists on macrophages.

In conclusion, we have demonstrated potent antitumor activity of TLR agonists: of LPS, R848, and ODN. We successfully produced liposomes containing R848, which exhibited antitumor effect on co-cultures of human macrophages and cancer cells. In the near future, we will investigate the antitumor activity of liposomal LPS, liposomal ODN, and their combinations. We will evaluate the potential of macrophage-mediated enhancement of antitumor immune response including T cell activation. We

will investigate the synergism of liposomes containing TLR agonists with other immunotherapy modalities on human cells.

P158

MULTILAYERED CD4+ T-CELL IMMUNITY TO CUTANEOUS MELANOMA

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There is a rapidly growing interest in the contributions of CD4+ T cells to cancer immunity. This has been fuelled by the observation that CD4+ T cells can elicit strong antitumoral responses in murine models, as well as by a recent surge in transcriptional analyses of CD4+ T cells in human tumors which have implied an important role for cytotoxic CD4+ T cells in cancer. The high degree of functional heterogeneity and plasticity amongst CD4+ T cell states have rendered the interpretation of their antitumoral functions a major challenge.

Our research investigates CD4+ T-cell responses using a recently-developed orthotopic melanoma model that uniquely recapitulates immune-dependent outcomes seen in patients, including tumor outgrowth, spontaneous metastasis, long-term persistence of microscopic lesions and complete tumour eradication. Through single-cell RNA sequencing analysis and intravital two-photon microscopy, we performed in-depth spatial and phenotypic characterization of tumor-infiltrating CD4+ T cells. We used genetic approaches to identify key mechanisms involved in the antitumoral CD4+ T cell response.

In this model the adoptive transfer of naive or activated antigen-specific CD4+ T cells demonstrated remarkable protection against tumor development. In addition to a classical “helper” function, CD4+ T cells acted as peripheral effector cells and orchestrated antitumoral immunity from within the tumour microenvironment (TME). Intravital imaging revealed intimate tripartite interactions between melanoma cells, tumor debris-laden antigen-presenting cells and CD4+ T cells. Furthermore, we showed that CD4+ T cells could directly bind to MHC II+ melanoma cells as well as professional antigen-presenting cells and examined how the source of MHC II could influence the ensuing CD4+ T cell response. We identified that CD4+ T cells directly suppressed tumor outgrowth by employing TNF α and Fas ligand, rather than perforin-mediated cytotoxicity. In addition, IFN γ was critical for protection, acting both via induction of nitric oxide production in myeloid cells and directly on melanoma cells.

Our work provides a comprehensive characterisation of the complex and context-dependent roles of MHC II and CD4+ T cells in the TME which have important implications for the modulation of this axis in cancer immunotherapies.

P159

PEPTIDES AND PEPTIDOMIMETICS BLOCKING TNF/TNFR2 COMPLEX AS A NEW WAY OF TREATING OVARIAN CANCER

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One of the most fatal gynecological cancers is ovarian cancer. Among all diagnosed woman nearly 65% dies, which places this cancer in fifth place in terms of mortality. Ovarian cancer remains highly fatal due to difficult early diagnosis and poor availability of effective therapy. An effective way to treat this type of cancer is by blocking immune checkpoints. Immune checkpoints are known as regulators of the natural immune response. So far, antibodies have been used to block the formation of such ligand-receptor complexes. Such large molecules, however, are characterized by low bioavailability, high costs and cause many side effects. The alternative to antibodies can be smaller molecules such as peptides or peptidomimetics. TNFR2 receptor is known to be highly expressed on the ovarian cancer cell surface and regulatory T cells (Tregs) surface. TNF-TNFR2 complex formation leads to decreased immune response, thereby further development of cancer. Inhibition of the TNFR2 receptor causes the activation of the apoptotic path of cancer cell and stops the expansion of suppressive Tregs. Action on these two approaches can contribute to restraining the development of cancer and become an effective future therapy. The undertaken research focuses on peptides that may block the formation of the TNF-TNFR2 complex.

The design of those peptides is based on the binding sides of the natural ligand for the TNFR2 receptor i.e. TNF. In our work, we will show preliminary results of studies on the design and chemical synthesis of peptides potentially inhibiting TNF-TNFR2 interactions. The peptides were synthesized by solid phase synthesis, purified and identified using liquid chromatography. The Inhibitory properties will be assessed by competitive ELISA test.

Studies have shown that peptides based on the TNF protein binding site interact with the selected receptor. Peptides containing disulfide bridges proved to have the best inhibition properties. This indicates the high relevance of the beta-sheet structure, which is present in the stucture of the TNF protein.

Rational design is promising approach to find new therapeutical molecules. This promising method can predict potential binding properties and validate them experimentally.

P160

BLOCKADE OF SIGLEC-9 ON MACROPHAGES RESCUES T CELL ANTITUMOR ACTIVITY AGAINST TRIPLE-NEGATIVE BREAST CANCER

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Triple-negative breast cancer (TNBC) is an aggressive tumor with limited treatment options. TNBC is characterised by a significant presence of infiltrating myeloid cells, which are associated with poor clinical outcomes. Tumour-associated macrophages (TAMs) play a variety of roles in the tumor microenvironment (TME); promoting angiogenesis, inflammation and immune suppression. To date, checkpoint receptor blockade has resulted in limited efficacy in TNBC, highlighting the need of new therapeutic approaches that can overcome the features of cold tumors. In this context, the family of Siglecs (sialic acid binding immunoglobulin-type lectins) is a group of immunomodulatory receptors that are emerging as promising targets. In particular, Siglec-9 signaling on TAMs drives their polarization toward a tumor-promoting phenotype. Previous studies have demonstrated the contribution of Siglec-9 to tumor progression. These results led to the development of anti-Siglec-9 antibodies that block the interaction of Siglec-9 with its cognate ligands.

In this study, we have explored the expression and function of Siglec-9 and its mouse ortholog Siglec-E in TNBC, with a focus on the impact on T cell cytotoxic activity against tumor cells.

To study the effect of Siglec-9 on the interaction between key cellular components of the TME, we developed a co-culture assay comprising: Siglec-9+ primary macrophages, a TNBC cell line (MDA-MB-231) engineered to express the human model antigen CD19, and anti-CD19 CAR-T-cells. In addition, we set an orthotopic mouse model based on TNBC EO771 cell line in order to characterise the infiltrating macrophages by flow cytometry and bulk RNAseq.

We observed that blockade of Siglec-9 with an existing monoclonal antibody (mAbA) enhanced CAR-T cytotoxicity in vitro in the presence of macrophages. Furthermore, the orthotopic TNBC mouse model demonstrated the presence macrophages expressing Siglec-E in the TME. These Siglec-E+ macrophages present a unique set of features associated with the hallmarks of immunosuppression.

In conclusion, our data show that Siglec-9 is expressed by TAMs in the tumor microenvironment of TNBC and acts to suppress antigen-specific T cell responses, resulting in an attractive emerging therapeutic target.

P161

ENHANCED IMMUNOSTIMULATORY AND ANTI-TUMOR ACTIVITY OF CPG 7909 ENCAPSULATED WITHIN ACM POLYMERSOMES

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We encapsulated CpG 7909, a B class oligodeoxynucleotide (ODN), within a synthetic Artificial Cell Membrane (ACM) polymer-based nanocarrier for targeted delivery to human antigen presenting cells (APCs), including plasmacytoid dendritic cells (pDCs) and B cells that express Toll-like receptor 9 (TLR9).

We comprehensively evaluated the effect of incubating human peripheral blood mononuclear cells (PBMCs) with ACM-CpG 7909 in a dose-response study to ascertain its potential as an innate immune modulator for cancer therapy. To demonstrate anti-tumor efficacy, we co-administered ACM-CpG 7909 intra-muscularly (IM) or intra-tumorally (IT) in a CT26 syngeneic mouse model resistant to anti-PD-1 treatment.

Production of multiple cytokines by human PBMCs was significantly increased by ACM-CpG 7909. For example, IFN α levels rose up to ~500 folds compared to the ~20-fold increase by free CpG 7909. Moreover, the dose-response curve presented as an inverted-U that resembled the profile induced by the type C ODN CpG 2395, suggesting that encapsulation led to a structural alteration that evoked a class C-like response. At low concentrations, ACM-CpG 7909 exceeded the ability of free CpG 7909 to stimulate the production of IFN α , IL-6, IFN γ , G-CSF, IL-10 and IL-1RA. In addition, we examined the activation status of various immune cell types present in PBMCs. ACM-CpG 7909 triggered the dose-dependent, gradual upregulation of CD69 on B cells and of CD86 and CD80 on pDCs, effects that closely resembled the activity profile of CpG 2395. Activation of monocytes, CD8+ T cells, CD4+ T cells, NKT cells and CD56bright NK cells by CpG 7909 was also significantly enhanced by ACM encapsulation. In clinical studies, CpG ODN overcame anti-PD-1 resistance in advanced melanoma (Ribas et al., Cancer Discov., 2021). In our CT26 tumor mouse model, we demonstrated that anti-PD-1 therapy alone was insufficient to control tumor growth whereas co-administration of ACM-CpG 7909 by the IM or IT route led to potent tumor suppression. This effect was rapidly lost with the cessation of IM treatment but maintained after IT treatment.

Cumulatively, this work showed that ACM encapsulation robustly increased the immunostimulatory effect of CpG 7909. Combining anti-PD-1 with ACM-CpG 7909 significantly suppressed CT26 tumor growth and this effect was enhanced by IT administration of ACM-CpG 7909. A recently completed phase 1 clinical trial (ClinicalTrials.gov identifier: NCT05385991) of ACM-CpG 7909 as an adjuvant for a SARS-CoV-2 booster vaccine (ACM-001) showed that it was well tolerated and induced good efficacy signals. An investigator-initiated dose-escalation/expansion trial to examine the safety and efficacy of ACM-CpG 7909 in patients with advanced/metastatic solid tumors is planned at the National Cancer Center Singapore.

P162

PHOSPHATIDYLSERINE AS A TUMOR TARGET FOR CAR-T CELL THERAPY IN SOLID TUMORS.

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One of the main challenges for the translation of CAR-T cell-based therapies to the treatment of solid tumors is the identification of specific antigens on the tumor cell membrane. In this work, we focused on phosphatidylserine (PS), a phospholipid normally present on the inner surface of the cell membrane, which is externalized during apoptosis acting as an “eat-me” signal for macrophages allowing their silent removal. PS externalization is highly deregulated in cancer cells and tumor microenvironment, and, thus, we evaluated if it could be considered as a tumor antigen for CAR-T cell designs based on AnnexinV, a protein with high affinity for PS.

PS expression in tumor cell lines, lymphocytes, CART cells, and tumor tissues was measured by flow cytometry. A CAR including AnnexinV as a receptor to target phosphatidylserine was designed to generate AnxaV CART cells. A chimeric fusion protein consisting of AnnexinV linked to EDA protein (Extra Domain A from fibronectin) was produced in E coli and used as a linker to redirect the antigen specificity of an EDA CART. CART cells were characterized in vitro by measuring CART expression levels, IFN-g production, cell proliferation, and lytic activity in response to antigen stimulation. Antitumor activity of CART cells was measured in vivo in a murine model of teratocarcinoma based on the injection of the F9 tumor cell line.

We demonstrated that PS is widely exposed in many tumor cell lines and tumor tissues. AnxaV CAR-T cells recognized PS on the surface of tumor cells. However, PS is also exposed in T lymphocytes after activation, which triggered a fratricide effect in AnxaV CAR-T cells. This could be bypassed by using an inducible AnxaV CAR. As an alternative, we prepared the bi-functional protein EDA-AnxaV to redirect the antigen specificity of EDA CAR-T cells to PS-expressing tumors. We found that EDA CAR-T cells can kill PS-expressing tumor cells in the presence of the adaptor EDA-AnxaV in vitro, showing a trend to improve the antitumor activity of EDA CAR-T therapy in vivo in the F9 teratocarcinoma tumor model.

PS could be considered as a potential tumor antigen for CAR-T cell-based therapies when the fratricide effect on CAR-T cells is bypassed by inducible CAR constructs or by using bi-functional adaptor proteins.

P163

CD11B EXPRESSION IDENTIFIES A UNIQUE SUBTYPE OF ANTIGEN EXPERIENCED TUMOR-INFILTRATING CD8+ T CELLS IN SOLID TUMORS

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Tumor-infiltrating lymphocytes (TILs) include heterogeneous CD8+ T cell subsets that play a critical role in antitumor immunity. These subsets encompass bystander, tumor-reactive and terminally exhausted T cell subpopulations, each exhibiting unique functions. Identifying functional tumor-reactive T cells holds direct applications for developing effective personalized immunotherapies. In this study, we evaluated the potential of B2 integrin family members as biomarkers of tumor-reactive CD8+ T cells in orthotopic tumor models and human endometrial cancer.

To investigate the expression kinetics of B2 integrin members, we employed flow cytometry and qPCR to analyze primary mouse and human CD8+ T cells at various timepoints following polyclonal and antigen-specific activation. For the examination of integrin expression in tumor-infiltrating lymphocytes (TILs), we established two orthotopic mouse models of breast cancer (EO771) and melanoma (YUMMER1.7). Additionally, we assessed integrin expression in ex vivo expanded TILs derived from endometrial cancer samples. FACS sorting was performed to isolate CD11b+CD8+ and CD11b-CD8+ T cells for bulk RNAseq analysis.

We observed that LFA-1 is constitutively expressed in naïve T cells, whereas CD11b is upregulated at a later timepoint after activation. CD11b expression is induced at RNA and protein levels in both human and mouse CD8+ T cells after 96 hours of activation, peaking at 10 days and persisting on the cell surface for an extended period.

To assess the potential of CD11b as a marker of distinct tumor-reactive T cells, we first confirmed its expression on murine CD8+ TILs obtained from breast and melanoma orthotopic tumor models. Additionally, we validated the expression of CD11b on ex vivo expanded TILs isolated from patients with endometrial cancer. CD11b+CD8+ cells significantly accumulate over time during tumor progression. Of note, this population was predominantly localized within the tumor microenvironment rather than noninvolved tissues.

We combined flow cytometry and bulk RNAseq analysis to gain comprehensive insights into the role of CD11b+CD8+ T cells. Our data suggest that CD11b+CD8+ T cells present a distinctive phenotype with increased cytotoxic potential, based on higher levels of proinflammatory cytokines and cytotoxic molecules.

In summary, we identified CD11b expression in distinct antigen-experienced CD8+ T cells. The potential of CD11b as a biomarker for functional tumor-reactive CD8+ T cells and its application in TCR-based immunotherapies warrants further exploration.

P164

PERIPHERAL KIR2DL2/DL3+NK AND HELIOS+TREGS PREDICTS NIVOLUMAB RESPONSE IN METASTATIC RENAL CANCER PATIENTS.

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Despite encouraging results, in metastatic renal cancer (mRCC) nivolumab response is not as wide as expected. The ReVoluTion clinical trial (NCT03891485) is a longitudinal, prospective, observational trial with the aim to identify biomarkers predictive of nivolumab efficacy in peripheral Tregs and NKs phenotype and function.

The study included 69 patients with mRCC starting from the 2nd line of nivolumab treatment and 57 patients were evaluable. Heparinized peripheral blood samples (24 mL) were collected before starting nivolumab (T0), at 1 month (T1), 3 months (T3), 6 months (T6) and 12 months (T12) of treatment. In addition, peripheral blood was collected from 62 healthy donors (HD). Peripheral blood mononuclear cells (PBMCs) were isolated from blood of mRCC patients and HD by Ficoll-Hypaque density gradient. Tregs and NK phenotype and function were evaluated at time 0 and at 1-3-6-12 months post nivolumab by flow cytometry. CFSE-T-effector proliferation-Tregs dependent and CD107a externalization toward K562 cells as NKs function were evaluated.

Compared to HD, the mRCC patients had similar frequency of peripheral NK cells (CD3-CD56+) and their subpopulations NKdim and NKbright but lower activating receptors NKp44 ($p < 0.001$) and higher NKp46 and NKp30 ($p < 0.001$ and $p < 0.01$) frequency. In addition, the NKs inhibiting receptors, KIR2DL1, KIR2DL2/DL3 and PD1 were significantly increased in mRCC patients as compared to HD ($p < 0.001$, $p < 0.05$ and $p < 0.001$ respectively). As for function, NKs derived-mRCC patients exhibited lower CD107a externalization than HD ($p < 0.05$). Frequency of Tregs (CD4+CD25+CD127lowFoxp3+) and relative naïve, effector and not suppressive subsets, was higher in peripheral blood of mRCC patients as compared to HD ($p < 0.01$). According to disease assessment post three months of therapy, the patients were identified as responders (R: 33/55) and non-responders (NR: 22/55). Interestingly, NR patients at baseline presented significantly higher frequency of CD3+ T cells ($p < 0.05$) and lower frequencies of PD-1+Tregs ($p < 0.05$), HELIOS+Tregs ($p < 0.01$) and KIR2DL2/DL3+NK expressing ($p < 0.001$). The logistic regression methods identified CD3+ ($p = 0.029$), KIR2DL2/DL3+NK ($p = 0.047$) and HELIOS+Tregs ($p = 0.025$) as independent predictors of nivolumab responsiveness. Through receiver operating characteristic curve (ROC) method, the cut-off values that best discriminate R from NR patients were identified. HELIOS+Treg and KIR2DL2/DL3+NK demonstrated a good performance in term of area under the curve (AUC > 0.7, $p < 0.05$). Moreover, mRCC patients that at time 0 presented HELIOS+Tregs >34.3% were significantly associated with longer progression-free survival (PFS) (3.0 vs 22.0 months, $p = 0.0029$) while mRCC patients that at time 0 presented KIR2DL2/DL3+NK >35.3% displayed longer overall survival (OS) (15.0 vs 33.0 months, $p = 0.035$). Moreover, mRCC patients with low HELIOS+Tregs and low KIR2DL2/DL3+NK display the shortest PFS as compared to the other combinations.

In conclusion, the peripheral frequencies of HELIOS+Treg and KIR2DL2/DL3+NK at time 0 may predict response to nivolumab treatment.

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DEVELOPMENT OF ⁶⁸GA-LABELED R54-DENDRIMER: A NEW PET PROBE TARGETING CANCER/TUMOR MICROENVIRONMENT CXCR4.

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CXCR4 is a suitable target for molecular imaging and therapeutics. The recently developed CXCR4 antagonist, peptide R54, was the backbone for a PET tracer [⁶⁸Ga]-NOTA-AMBHA-R54 (or [⁶⁸Ga]Ga-R54) (PCT/EP2020/087792) able to discriminate CXCR4-positive tumors in preclinical models. [⁶⁸Ga]Ga-R54 is CXCR4 high selective, displays rapid renal excretion and good image contrast. To improve imaging sensitivity and tracer uptake in tumor/tumor microenvironment, a novel nanosystem based on an amphiphilic dendrimer was developed. Dendrimers are a family of highly branched, monodispersed, synthetic macromolecules with abundant surface functional groups and generation-dependent sizes, molecular weights, and architecture. Dendrimers physical size and structure impact on in vivo excretion and circulation time aimed to improve enhanced permeability and retention (EPR) effect in tumors.

Dibenzocyclooctyne (DBCO) and hydrophobic alkyl chains (C18) were added to the amino terminus of R54 thus forming the dendrimer monomer (C18-DBCO-R54). The R54-functionalized Polyamidoamine (PAMAM)/Lysine dendrimers were prepared through solid-phase peptide synthesis (SPPS) method. Different mole ratio of C18-NOTA (hydrophobic alkyl chain with NOTA terminals) and C18-DBCO-R54, 1:0.05; 1:0.1; 1:0.2 and 1:0.4 realized four different formulations of self-assembling supramolecular dendrimer nanosystem (SSDNs) named F1, F2, F3 and F4. Competitive ¹²⁵I-CXCL12 binding was conducted on human T cell leukemia cell line CCRF-CEM in the presence of F1, F2, F3 and F4 to assess binding affinity. Saturation binding experiments were performed with ⁶⁸Ga-labeled SSDNs using CCRF-CEM cells.

Surprisingly, the monomeric unit (C18-DBCO-R54) displayed 10-fold increased affinity for CXCR4 as compared to peptide R54 (IC₅₀: 1.4±0.3 vs 14.5±3.5 nM, respectively). R54-Dendrimers displayed nanomolar CXCR4 affinity with F1 and F2 the best compounds (F1: IC₅₀=20±3.7; F2: IC₅₀=21.5±3.0; F3: IC₅₀=39.6±3.3; F4: IC₅₀=38.6±0.8). As expected the empty- F5 and F6 dendrimers did not specifically bind CXCR4 on CEM cells. Preliminary results obtained by saturation binding experiments showed high affinity for [⁶⁸Ga]Ga-F1 with K_d= 7.2 nM and [⁶⁸Ga]Ga-F3 with K_d= 35.17 nM, while [⁶⁸Ga]Ga-F2 and [⁶⁸Ga]Ga-F4 showed less affinity with K_d= 85.1 nM and K_d= 294.3 nM respectively.

In conclusion we showed that the R54-functionalized Polyamidoamine (PAMAM)/Lysine dendrimers specifically target CXCR4 on human cancer cells. In vivo studies with [⁶⁸Ga]Ga-F1 and [⁶⁸Ga]Ga-F3 are ongoing on CHO-CXCR4 xenograft. R54 dendrimers may provide the way for combinational anticancer therapies along with the in vivo imaging of the targeted CXCR4-overexpressing tumor.

P166

DECIPHERING HOW IMMUNE-STEM CELL CROSSTALK SHAPES CANCER PROGRESSION

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Squamous cell carcinoma (SCC) is one of the most common malignancy world-wide, affecting lung, head & neck (HN), esophagus and skin. Due to its high recurrence rate of aggressive tumors and poor prognosis of metastatic SCC, it remains life-threatening. The increased risk of SCC occurrence observed in immunocompromised individuals highlight the importance of immune system in SCC progression. Moreover, high resistance to current therapies underscores the importance of alternative approaches. Central to this goal is to improve our understanding of how these tumor-initiating (stem) cells (tSCs) progress from benign to invasive state and become therapy-resistant. Murine skin is ideal for tackling these important issues in physiological context. As epithelial stem cells (EpSCs) acquire oncogenic mutations, they transition from a hyperproliferative, benign state (papilloma) to aggressive, metastatic, and therapy-resistant SCCs. However, changes occurring along the path to malignancy and SCC invasion remains elusive.

In skin, the native EpSCs express SOX9, which is required for SC maintenance and wound repair. Upon acquiring oncogenic mutations, EpSCs progress along a journey from benign to invasive state, maintaining their SOX9 levels. Intriguingly, SOX2, yet another TF belonging to the same SOX family, starts to be expressed by tSCs heterogeneously in the SCC stage. Notably, SOX2 expression correlates with poor prognosis, likely marking the transition to invasive state. Given that previous studies have clearly demonstrated that both SOX2 and SOX9 are required for SCC formation, and SOX2+ tSCs have greater resistance to chemo- and immuno-therapies, SOX2+ tSCs are likely to be the cells that will need to be targeted to prevent cancer relapse. Hence, a prerequisite to developing new therapeutics is to understand how SOX2 differs from SOX9 in regulating chromatin and transcriptome dynamics of tumor progression and how SOX2+ tSCs shapes their surrounding tumor microenvironment to evade immune attack.

To unravel the mechanisms of SOX TF dynamics and the crosstalk between SOX2+ tSCs and immune cells within tumor microenvironment (TME) during malignant progression, I use multitude of techniques. By using high throughput strategies to probe chromatin accessibility, histone modifications and TF binding, I am deciphering how the chromatin and transcriptomic landscape changes in tSCs as they transition from non-invasive to invasive state. Additionally, I use iterative imaging technics to map spatiotemporal dynamics of SOX TF+ tSCs and their immune TME. And finally, I would like to elucidate the crosstalk between SOX2, TME, and immune evasion.

Altogether, understanding the molecular roots of malignancy with respect to SOX TFs that govern chromatin and transcriptome dynamics of tumorigenesis and how Sox2+ tSCs might alter the immune TME for immune evasion will open the doors for new therapeutic approaches and is likely to have broad implications in cancer biology.

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CIRCULATING IMMATURE NEUTROPHILS EARLY DETECT HYPERPROGRESSIVE DISEASE UPON FIRST LINE PD1/PDL1 INHIBITORS (ICI) IN NON-SMALL CELL LUNG CANCER PATIENTS SELECTING BEST CANDIDATES FOR PLATINUM-BASED CHEMOTHERAPY AND ICI COMBINATIONS.

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Hyperprogressive disease (HPD) has been described in \approx 14-25% of pretreated non-small cell lung cancer (NSCLC) patients upon single-agent PD-1/PD-L1 inhibitors (ICI) and has not been reported upon platinum-based chemotherapy (PCT) and ICI combinations. So far, no predictive biomarkers are available for HPD early detection.

NSCLC patients treated with 1st line single-agent ICI or PCT-ICI were assessed for HPD and circulating neutrophils. HPD was defined as delta tumor growth rate (TGR) $>50\%$ and/or TGR ratio ≥ 2 . Circulating low density neutrophils (LDNs) were assessed by flow cytometry on peripheral blood mononuclear cells (PBMCs). LDNs were defined as CD66b+CD15+ cells among CD11b+ PBMCs and immature subtypes as CD10- LDNs. The LDNs predictive role was assessed by penalized model-based tests.

144 NSCLC patients were included: 75 treated with single-agent ICI, 69 with PCT-ICI. In the SA-ICI cohort, HPD occurred in 8 (11%) patients, while progressive disease (PD) and response or stable disease (PR/SD) occurred in 33 (44%) and 34 (45%) of patients respectively. Immature circulating CD10- LDNs were significantly higher in baseline blood samples of HPD patients [median: 39.3, interquartile range (IQR): 28.7] compared to PD [median: 7.4, IQR: 14.9, $p < 0.01$] or PR/SD patients [median: 3.7, IQR: 12.6, $p < 0.01$]. Circulating CD10- LDNs were associated with HPD [odds ratio (OR): 1.17, 95% CI: 1.06; 1.29], with a good prediction capability [cross-validated AUC 0.97 (95%CI: 0.94;1.00)]. A 30.5% cut-off value for CD10- LDNs circulating neutrophils was identified by Youden index to discriminate HPD from others. In the PCT-ICI cohort, 14 patients had circulating CD10- LDNs $\geq 30.5\%$, being at high risk of HPD. However, no HPD was observed in PCT-ICI cohort and dynamic LDNs evaluation in high HPD risk patients showed 52.3% (IQR: 28.4) median reduction in CD10- LDNs upon PCT-ICI, versus only 8.9% (IQR: 34.6) reduction in HPD patients upon single-agent ICI, suggesting that PCT prevents HPD by reducing selectively immature LDNs.

Baseline circulating immature neutrophils characterize HPD upon 1st line single-agent ICI and a 30.5% cut-off of immature neutrophils could select NSCLC patients to be addressed to PCT-ICI combinations.

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MONITORING OF MYELOID CELL DISTRIBUTION BY HUMAN SIRPα NANOBODY PET IMAGING IN A HUMAN CD47/SIRPα RECEPTOR KNOCK-IN TUMOR MOUSE MODEL

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Tumor-infiltrating myeloid cells have an opposing impact on cancer development, progression, and eradication of various tumor entities dependent on their polarization. Upregulation of the signal-regulatory protein α (SIRP α) by myeloid cells, especially macrophages, is associated with poorer survival in several cancer types, suggesting SIRP α as a promising target for both monitoring and targeting tumor-infiltrating macrophages. Thus, we generated a set of human SIRP α -specific nanobodies (hSIRP α Nbs), characterized by small size (15kDa), high target affinity, and favorable tissue penetration, and evaluated them for binding specificities, affinities, and their potential to block the SIRP α -CD47 interaction.

For non-invasive PET imaging, we identified a therapeutically inert hSIRP α Nb and analyzed its applicability for visualization of myeloid cells in a newly developed hSIRP α knock-in (KI) mouse tumor model. The hSIRP α Nb was conjugated with the chelator NODAGA, radiolabeled with copper-64 (⁶⁴Cu), and its radiochemical purity was assessed by HPLC. The immunoreactive fraction of ⁶⁴Cu-hSIRP α Nb was tested in vitro using HT1080 cells transiently expressing hSIRP α . For in vivo evaluation, MC38 hCD47 KI tumor-bearing hSIRP α KI mice were either injected with ⁶⁴Cu-hSIRP α or a non-binding ⁶⁴Cu-control Nb. Additionally, wild-type (wt) mice were injected with ⁶⁴Cu-hSIRP α Nb as a second control. Ten-minute static PET scans were performed 5 min, 90 min, 3 h, and 6 h after tracer injection.

Radiolabeling yielded high radiochemical purities of $\geq 95\%$ for both tracers and ⁶⁴Cu-hSIRP α Nb showed an immunoreactive fraction of $\sim 82\%$. PET imaging revealed a fast ⁶⁴Cu-hSIRP α Nb accumulation in the tumor of hSIRP α KI mice that remained high over 6 h, whereas a fast clearance was obtained in the tumors and the blood of both control groups. Quantitative analysis 3 h after tracer injection demonstrated a significantly higher ⁶⁴Cu-hSIRP α Nb accumulation in tumors of hSIRP α KI mice (1.89 ± 0.09 %ID/cc) compared to ⁶⁴Cu-control Nb (0.57 ± 0.05 %ID/cc) injected and wt mice (0.60 ± 0.05 %ID/cc). Additionally, we observed a significantly higher ⁶⁴Cu-hSIRP α Nb uptake in myeloid cell-enriched organs like the spleen, liver, salivary glands, and bone as well as in the blood of hSIRP α mice. No differences in ⁶⁴Cu-hSIRP α Nb uptake were observed between the three experimental groups in the kidneys and muscle tissue.

In summary, we demonstrate the applicability of the novel ⁶⁴Cu-hSIRP α Nb for visualizing the distribution of SIRP α cells by non-invasive in vivo PET imaging. Our findings highlight the potential of hSIRP α Nb PET for monitoring cancer immunotherapy-induced changes in myeloid cell distribution patterns in the tumor and primary and secondary lymphatic organs and most importantly to uncover treatment success or failure in cancer patients.

P169

ROLE OF EICOSANOIDS IN INTESTINAL INFLAMMATION AND EPITHELIAL CELL EXTRUSION

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Epithelial-derived cancers, or carcinomas, are the most common type of malignancy. The intestinal epithelium is a critical barrier site for immune tolerance and host protection. Intestinal epithelial cells play a crucial role in homeostasis and the initiation of immune responses. Aberrant epithelial cell extrusion is commonly observed during infection, inflammatory bowel disease (IBD), and colorectal cancer. Defining the pathway of cell extrusion will provide greater insight into the role of extrusion in various states of inflammation in the intestine. The inflammasome is a multiprotein complex formed after the recognition of intracellular infection or danger signals, notably, inflammasome activation leads to cell extrusion and eicosanoid release. We hypothesized that eicosanoids act as a signal for extrusion following inflammasome activation in intestinal epithelial cells.

Murine intestinal organoids were grown on transwells and treated with FlaTox, for intracellular delivery of flagellin, to activate the NAIP/NLRC4 inflammasome and stimulate extrusion. Transwells were also treated with a variety of inhibitors of eicosanoid synthases and receptors. Using this model, cells were stained with propidium iodide (PI), a dye that is excluded from healthy cells, and we created 3D images of the monolayers using structured illumination microscopy. These images were quantified to determine the percent of extruded cells. Live confocal microscopy was also conducted to confirm the inhibition of extrusion observed in transwell experiments.

We observed that inhibition of both leukotrienes and prostaglandins simultaneously was required to block extrusion, though differences were found between organoids derived from the small and large intestines.

Our findings suggest that either both a leukotriene and a prostaglandin are required for extrusion to proceed or, more likely, that the pathway is highly redundant underscoring the importance of extrusion in the intestinal epithelium. We also reveal important differences in eicosanoid signaling between the small and large intestines. Interestingly, Mesalazine, a therapy for ulcerative colitis, is believed to inhibit eicosanoid synthesis. This study will help us unravel the link between IBD progression to colorectal cancer and eicosanoid signaling.

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T CELL PRIMING NICHE ARE COMPROMISED IN THE TUMOUR DRAINING LYMPH NODE

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Conventional dendritic cells (cDC) play key roles in directing anti-tumour T cell immune responses with increased levels of cDC1 in the TME being positively prognostic and correlating with response to immunotherapy in several tumours. cDC, however, have other key roles driving T cell priming in the tumour draining lymph node (LN). Several studies have shown that this initial T cell priming step is sub-optimal in the context of cancer and that interventions including both PD-1 blockade and therapeutic vaccination can improve this leading to patient benefit. Despite their key role in initiating anti-tumour T cell responses there remain many open questions as to how the two main lineages of cDC, cDC1 and cDC2, cooperate to drive effective T cell immunity. Further complexity is introduced by the fact that both subsets comprise both migratory DCs (mDCs), which carry antigen from the TME to the LN, and resident DCs (rDCs), which receive antigen from these cells, each behaving differently based on their origin. All of these cell types have been implicated in T cell priming but the lack of robust tools to manipulate individual subsets means many details remain unresolved. Here, we aim to thoroughly define the roles of cDC subsets during priming and to investigate the cause of sub-optimal T cell priming in the cancer setting.

We have developed a highly multiplexed staining panel which allows for the identification of these four cDC subsets by confocal microscopy, and combined this with markers for other key cells in the LN including neutrophils, monocytes and T cell subsets. A machine learning approach allowed robust segmentation of the densely packed tissue, and flow cytometry approaches coupled with linear discriminant analysis were used to assign cellular identities. This allowed us to define the spatial organisation and interactions occurring within the LN throughout a response at a single-cell level. Mice were either infected with influenza as a model driving robust T cell immunity, or injected i.v. with B16F10 melanoma cells to model a tumour response in the same draining LN.

Spatial analysis revealed that during influenza infection mDCs relocate from the marginal zone of the LN to infiltrate deeply into the T cell zone, where they closely associate with activated CD4 and CD8 T cells. Interestingly, despite the general view that cDC2 are most important for CD4 T cell priming it was cDC1 which most closely associated with both activated CD4 and CD8 T cells. rDC also relocated upon viral challenge moving into the T cell zone agreeing with previous literature suggesting this occurred during type 1 immune challenges. When this was compared to changes occurring in response to tumour development, it was clear that there were significant differences in the organisation of both cDC and T cell subsets; indeed, the relocation of cDCs is significantly reduced in the tumour draining lymph nodes, along with a significantly lower association with activated T cells. To investigate whether this was a consequence of LN alterations we injected B16F10 subcutaneously in mice and allowed them to develop and then vaccinated with IFA/OVA in the flank to the contralateral or ipsilateral draining node. This showed that immune responses were capable of forming in the tumour draining node suggesting that cDC behaviour may be directed by signals received in the TME.

Here, we show that T cell priming niches are compromised in the tumour draining lymph node, with a lack of interaction between DCs and T cells that is associated with reduced T cell activation. Further spatial analysis is undergoing to identify specific interaction partners of cDC subsets during anti-viral and anti-tumour immune priming. By deeply characterising these niches and identifying any key interactions lacking in the tumour draining lymph node, we aim to define specific deficits in T cell priming in the cancer setting, which would help direct effective anti-tumour immunotherapy.

P171

AN EXPANDED META-ANALYSIS OF TUMOR AND T CELL-INTRINSIC MECHANISMS OF SENSITIZATION AND RESISTANCE TO CHECKPOINT INHIBITION

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Though cancer is heterogeneous, all tumors exhibit characteristic hallmarks, including immune evasion. Immunotherapies, like checkpoint inhibition (CPI), attempt to remove barriers to immune-recognition of tumors and require major histocompatibility complex (MHC) presentation and T-cell recognition of tumor antigen. We previously identified TMB and clonal TMB as the best pan-cancer biomarkers of CPI response, though over half of CPI responders have low TMB and there is an urgent need for improved biomarkers of response.

To this end, we performed an extensive literature review of over 500 papers to identify recently published biomarkers of CPI response and resistance. Next, we developed a reproducible, end-to-end, and open source snakemake pipeline to process whole exome and whole transcriptome sequencing and call CPI biomarkers obtained from our literature review.

We assembled a checkpoint inhibitor treated cohort of over 3000 patients spanning 10 tumor types, 25 studies, and both monotherapy and combination therapy treatments. New tumor types now include gastric, glioblastoma, prostate, esophageal, and breast; and tumor types with increased representation include melanoma, renal cell, colorectal, head and neck, and non-small cell lung cancers. We collated and harmonized available whole-exome and transcriptomic data to validate our previously identified biomarkers of response, namely clonal TMB, total TMB, and CXCL9 expression both pan-cancer and within distinct tumor types. We plan to evaluate expression and co-occurrence of new and emerging checkpoint molecules, especially those observed in cell types other than T cells, such as macrophages and monocytes. Finally, we plan to create an updated multivariable model to combine relevant biomarkers and evaluate predictive value with TMB alone, and the original CPI1000+ model.

Findings from our study will help improve understanding of the biological mechanisms influencing response to CPI treatment and improve identification of patients with most potential to respond to immunotherapy.

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COMBINATION THERAPY WITH STAT3 AND TGF- β INHIBITORS TO REPROGRAM IMMUNOSUPPRESSIVE MACROPHAGES IN LUNG AND PANCREATIC CANCERS

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A high number of solid tumors are still nowadays considered “uncurable cancers”. The standard of care for these patients comprises surgery, radiotherapy and chemotherapy in combination with targeted therapies towards extracellular targets (i.e. EGFR) and immunotherapies (i.e. anti-PD1). These new treatments have resulted in outstanding clinical responses in some cancer types. Unfortunately, these encouraging results are only obtained in less than 20% of lung cancer patients and worst results have been achieved in pancreatic cancers. An opportunity exists in targeting simultaneously important biological pathways in cancers, i.e. STAT3 and TGF- β , whose abnormal activation in cancer cells and tumor-associated macrophages (TAMs) has been associated with enhanced tumor growth, immunosuppression and metastasis. In cancer cells, aberrant activation of STAT3 and TGF- β pathways (for instance by IL-6/IL-10 and TGF- β , respectively), is reported to promote cell survival, inhibit apoptosis and promote metastasis through the process of epithelial to mesenchymal transition (EMT), while in TAMs, it has been linked to their polarization to an immunosuppressive, pro-tumoral M2 phenotype. Therefore, these pathways are promising targets for cancer therapy.

In this study, we evaluated the effect of a treatment combining stattic, a STAT3 inhibitor, with Galunisertib, a TGF- β inhibitor, in lung and pancreatic cancer models. Stattic and galunisertib treatments were evaluated in vitro for toxicity and immunomodulatory activity, using primary human and murine M-CSF-differentiated macrophages. Cytotoxic activity of treated macrophages towards cancer cells was evaluated with an in vitro functional assay by flow cytometry. For in vivo experiments, the CMT167 and 393P lung cancer models and K8484 pancreatic cancer models were used; tumor-infiltrating leukocytes were evaluated by flow cytometry and multispectral immunophenotyping analysis.

In vitro, galunisertib showed a clear inhibition of the TGF- β -induced EMT process in CMT167 cells, evidenced by an increase in epithelial markers and a decrease of the TGF- β -induced mesenchymal markers. Macrophages treated with stattic and/or galunisertib showed a reduction in expression of M2 phenotype markers and an increased cytotoxic activity towards cancer cells. In vivo, the intratumoral synergistic combination of stattic and galunisertib significantly decreased tumor growth in lung and pancreatic cancer immunocompetent murine models. Experiments performed in IFN- γ KO mice and immune deficient mice (Nude and NSG) revealed that a fully functional immune system is crucial for the response to the treatment. Analysis of the tumor microenvironment showed a decrease in STAT3 and SMAD2 phosphorylation and an increased cytotoxic T cells activity. Moreover, combination of stattic+galunisertib with the immunostimulatory molecule resiquimod further enhanced the efficacy of the treatment, even leading to tumor eradication in some cases.

Our results shed light on the potential of STAT3 and TGF- β inhibitors for TAMs-targeted immunotherapy and long-lasting tumor-remission in lung and pancreatic cancers. Further investigations are needed to clarify the immunological aspects and to assess their efficacy in orthotopic models of lung cancer, as well as in other type of solid tumors.

P173

NLRP9 INFLAMMASOMES DRIVE HOMOLOGOUS RECOMBINATION-DEFICIENT TUMOR GROWTH AND IMMUNE SUPPRESSION

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Homologous recombination repair deficiency (HRD) is a major cause of cancer susceptibility. While inherent genomic instability in HRD cells likely contributes to carcinogenesis, there are few recurrent mutations other than the near-universal loss of p53 that could explain cancer predisposition. Chronic inflammation is a risk factor for cancer and elevated inflammatory signaling occurs constitutively in HRD cells due to high levels of chromosome segregation errors. Moreover, chromosome instability correlates with an immune suppressive microenvironment and poor response to immune therapy across cancer types 6, suggesting that a nonpermissive tumor microenvironment for T-cell mediated cytotoxicity exists in HRD and other aneuploid tumors.

Here, we created a novel syngeneic murine HRD high grade serous ovarian cancer model to identify mediators of elevated inflammatory signaling that impact the tumor immune microenvironment. We also draw upon a collection of HRD ovarian cancer biopsy specimen.

NLRP9 inflammasome activation occurs in human and mouse HRD cancers. While genetic ablation of NLRP9 increased HRD cancer cell proliferation in vitro commensurate with reduced pyroptotic cell death, its loss strongly attenuated cancer progression in vivo. NLRP9 deficiency resulted in a reduction in tumor associated macrophage (TAM) infiltration and changes in their polarization. Moreover, macrophage, but not T cell, depletion eliminated tumor growth specifically in HRD ovarian cancers that lacked NLRP9 inflammasome activity. NLRP9 deficiency was also characterized by increased CD8+ T-cell infiltration and sensitivity to immune therapy with aPD1/aCTLA4 that yielded curative responses and survival in treated mice.

These results implicate NLRP9 inflammasome activation in HRD cancer progression and resistance to T-cell dependent therapies. Targeting the inflammasome may represent a therapeutic vulnerability for established cancers and the early interception of premalignant lesions in BRCA1/2 mutation carrying individuals.

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THERAPEUTIC POTENTIAL OF LIPOSOMAL TLR LIGANDS AS AN ANTICANCER DRUG.

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Apart from cancer cells, tumors also contain various types of host cells, mostly immune cells. The presence of immune system cells in the tumor microenvironment may shape the therapeutic response. The lack of response to some immunotherapies is in many cases associated with the immunosuppressive effect of immune system cells, in particular macrophages, monocytes and dendritic cells. Immunoactivation and reprogramming of these myeloid cells may enable an effective immune response against the tumor. Immunoactivation and reprogramming of immune cells present in the tumor microenvironment can take place through the action of immunomodulators, such as ligands of TLRs (Toll-like receptors) present outside and inside macrophages. Triggering TLRs with appropriate ligands polarize macrophages in the pro-inflammatory direction. What results in production of large amount of inflammatory cytokines e.g. TNF α (and metabolites e.g. nitric oxide (NO)). These effect could induce antitumor immune response, however Systemic administration of TLRs ligands is harmful and may cause systemic inflammation or even brain swelling. Therefore, loading TLRs ligands into liposomes that could seems to be a good solution. Liposomes are a good tool for delivering various types of particles to tumors, which is confirmed by many studies. In addition, liposomes can protect the cargo from decomposition too early in the body, which increases the chance of the functional drug reaching its target, i.e. the tumor.

In our experiments we used flow cytometry, qPCR, Griess Assay. Moreover in in vitro experiments we used mouse bone marrow-derived macrophages (BMDM) and mouse breast cancer cells (EMT6 and 4T1).

First, we selected TLRs ligands with the highest antitumor activity, we made screen of TLRs ligands specific for all TLRs. We selected resiquimod (R848), a ligand for TR7/8 and LPS – a ligand for TLR4 as the most potent in inhibitors of cancer cells grow in the coculture with mouse bone marrow-derived macrophages (BMDM). Importantly, R848 or LPS had no inhibitory capacity on cancer cells when used in the absence of BMDM.

Using co-culture of BMDM with mouse breast cancer cells (EMT6 and 4T1) we confirmed that even the small doses of R848 and LPS significantly induce the level of NO produced by macrophages, with a significant decrease in the number of viable cancer cells in co-culture. What is more, experiments conducted using liposomal R848 gave a very similar effect, confirming anticancer activity of TLR ligands in the liposomal formulation.

All our results indicate a strong therapeutic potential of liposomal TLR ligands as an anticancer drug.

P175

DISCOVERY OF WTX-330, A CLINICAL STAGE CONDITIONALLY ACTIVATED IL-12 INDUKINE THERAPEUTIC WITH POTENT ANTITUMOR ACTIVITY IN MURINE SYNGENEIC TUMOR MODELS, INCLUDING THOSE RESISTANT TO CHECKPOINT BLOCKADE

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Cytokine therapy could become a pillar of cancer immunotherapy given its potential to activate the immune system and promote antitumor activity. However, many cytokine therapies are limited in their antitumor effectiveness in patients due to dose limiting toxicities. IL-12 is a pleotropic inflammatory cytokine with broad stimulatory effects on immune cells and robust antitumor activity in syngeneic murine tumor models, making it an attractive target for cancer immunotherapy. Unfortunately, systemic administration of recombinant human IL-12 causes severe toxicity in patients. With the goal of improving the therapeutic index for IL-12 therapy, we have engineered an IL-12 prodrug referred to as an INDUKINE molecule that is dosed systemically but designed to be selectively activated in tumors.

Mice bearing MC38, CT26, B16F10, and EMT-6 syngeneic tumors were treated with surrogate mWTX-330 (a chimeric IL-12 containing INDUKINE molecule), and tumor growth and body weight were monitored over time. Tumor tissues were harvested at various timepoints and analyzed by flow cytometry, NanoString GeoMx Digital Spatial Profiling (DSP), and high-plex immunofluorescence to characterize the mechanisms associated with response.

mWTX-330 is comprised of a half-life extension domain and an inactivation domain tethered to chimeric IL-12 by tumor protease-sensitive linkers. These linkers were chosen for their selective processing by a majority of human tumors in vitro as well as mouse tumors in vitro and in vivo. The linkers have been shown in preclinical studies to remain stable in normal human tissues including serum. Systemic administration of mWTX-330 in mice was well tolerated, resulted in robust antitumor immunity in multiple tumor models, including those resistant to checkpoint blockade, and preferentially activated tumor-infiltrating immune cells versus peripheral cells. Antitumor activity was dependent on in vivo processing of the protease cleavable linkers and required CD8+ T cells for full efficacy, although NK and CD4+ T cells also contributed to tumor growth inhibition. Within the tumor, mWTX-330 increased the frequency of cross-presenting dendritic cells (DCs), activated natural killer (NK) cells, skewed conventional CD4+ T cells toward a T helper 1 (TH1) phenotype, drove regulatory T cells (Treg) fragility, and increased the frequency of polyfunctional CD8+ T cells. mWTX-330 treatment also increased the clonality of tumor-infiltrating T cells by expanding underrepresented T-cell receptor (TCR) clones, drove CD8+ T and NK cells towards increased mitochondrial respiration and fitness, and decreased the frequency of TOX+ exhausted CD8+ T cells within the tumor.

Based on the strong biological rational and preclinical studies of surrogate mWTX-330, we created WTX-330, a fully human IL-12 INDUKINE molecule that is stable in human serum and is reliably and selectively processed by human tumor samples in vitro. A first-in-human clinical trial to evaluate the safety profile, pharmacokinetics, and preliminary antitumor activity of WTX-330 in patients with relapsed/refractory advanced or metastatic solid tumors or non-Hodgkin lymphoma is now underway (NCT05678998).

P176

INTERPLAY OF CAIX AND CCR2-CCL2 AXIS IN NEUROBLASTOMA XENOGRRAFT MODEL

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Despite significant advancements in immunotherapy for solid tumors, a considerable number of cancer patients still do not respond to treatment. Neuroblastoma is the most common cancer in infants that accounts for 15% of cancer mortality in children. Hypoxia impacts malignant progression of neuroblastoma. A limited understanding of the interactions between tumors and immune cells in hypoxic tumor niche hinders the development of effective therapies that target tumor hypoxia in neuroblastoma. The carbonic anhydrase IX (CAIX) is a key enzyme helping cancer cells survive in tumor hypoxia, by maintaining the physiological pH in cancer cells and acidifying tumor microenvironment. It leads to suppressed tumor immune responses and treatment resistance. CCR2-CCL2 axis is another hypoxia driven important mediator of tumor microenvironment. Both CCR2-CCL2 and CAIX are well established tumorigenic signals in neuroblastoma tumors.

Our laboratory has developed a novel, highly selective CAIX inhibitor. It shows exceptional sensitivity and specificity toward CAIX with the picomolar binding constant. SKNAS is well established human neuroblastoma cell line that is not MYCN amplified. CAIX expression in SKNAS cell line was performed by WB and flow cytometry. The functional activity of lead CAIX inhibitor in SKNAS cells was confirmed by measuring the rise in extracellular pH directly inside a hypoxic chamber. CCL2 secretion and its response to CAIX blockade was tested by performing transwell assay under hypoxic conditions. The treatment efficacy of combination of our lead CAIX inhibitor with CCR2 blockade was tested in SKNAS xenograft model in Nude mice.

SKNAS cell line has a high CAIX expression in tumor hypoxia as compared to normoxia. The functional activity of lead CAIX inhibitor in SKNAS cells was confirmed by measuring the rise in extracellular pH directly inside a hypoxic chamber. It significantly ($p < 0.05$) reduced hypoxia-induced acidification of SKNAS cells at 50-200nM, while the effect on cells in normoxia was not evident. Under hypoxic conditions, the addition of healthy donor peripheral blood mononuclear cells (PBMCs) to upper chamber of the transwell system in which SKNAS cells were grown in the lower chamber, increased CCL2 secretion in both- tumor cells and PBMCs. Importantly, CCL2 secretion was diminished by the addition of 200nM of the lead CAIX inhibitor. We then tested CAIX inhibition and CCR2 inhibition efficacy *in vivo* using SKNAS xenograft model. We compared tumor growth in mice who received combination treatment to mice who received single agent treatment versus controls. We observed an additive effect of CAIX inhibition with CCR2 blockade in diminishing tumor growth. Although treatment with single CCR2 blockade did not show efficacy, CAIX inhibitor as a single agent showed decreased tumor growth trend. Interestingly, tumor flow cytometry analysis from harvested SKNAS tumors showed an increase in VEGFR and CCR2 expression in tumors treated with the combination as compared to controls. It is likely a result of a compensatory signaling. In addition, expression of CD44, a transmembrane glycoprotein regulating cell growth, differentiation and motility, role of which in neuroblastoma is controversial, was decreased in tumors that were treated with combination as compared to other treatment cohorts.

Our data shows that there is an interplay between CAIX and CCL2 in SKNAS neuroblastoma xenograft model. Inhibiting both pathways has an additive effect. Further studies are needed to better decipher signaling mechanisms involved.

P177

TUMOR MACROPHAGE IMMUNITY REGULATION BY MITOCHONDRIAL STRESS RESPONSE MEDIATOR LONP1

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The lack of effective therapies targeting immunosuppressive tumor infiltrating myeloid cells, or the abundance of tumor associated macrophages (TAM) represents a critical barrier to successful and persistent antitumor immunity and treatments, especially immunotherapy.

Control mice (LonP1F/F) and mice with conditional deletion of LonP1 in myeloid cells (LonP1F/F;LysMCre) were subcutaneously injected with melanoma cell lines B16F10 or SM1 into flanks. Subsequently, tumor macrophages were assessed using flow cytometry, western blot, qPCR, and functional assays.

Melanoma tumors inoculated into LonP1F/F;LysMCre mice showed a significant increase in TAMs, infiltrating T cells, and activated CD4+ T cells. These LonP1 knockout (LonP1KO) TAMs are a recently infiltrated, immature subset (MHC-II+Ly6C+) and have increased expression of antigen presentation associated markers (MHC-I, MHC-II, CD40, CD80, and CD86). Current work is investigating the ability of LonP1KO TAMs to present endogenous antigen to T cells or whether an antigen-independent mechanism is directing the increased T cell infiltration and activation in LonP1F/F;LysMCre tumors. Furthermore, LonP1KO TAMs show increased iNOS and decreased Arg1; ongoing experiments are investigating the intrinsic ability of LonP1 and mitochondrial fitness to regulate TAM programming away from an immunosuppressive phenotype. Lastly, we are testing a newly synthesized small molecule LonP1 inhibitor as a potential therapeutic to target macrophage immunity.

Targeting tumor macrophage LonP1 represents a novel and promising target to remodel the immune landscape in tumor beds. Additionally, ablation of LonP1 in macrophages improves their immunostimulatory capacity and could boost current immunotherapy modalities.

P178

UL144- AND CD160-DERIVED PEPTIDES AS INHIBITORS OF THE BTLA/HVEM COMPLEX FORMATION

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BTLA (B and T lymphocyte attenuator) and HVEM (herpes virus entry mediator) belong to inhibitory ICPs (immune checkpoints). Upregulated expression of BTLA and HVEM is associated with unfavourable prognoses for patients with hepatocellular carcinoma, melanoma, colorectal cancer, gastric cancer and many other types of cancer. It was stated that the BTLA/HVEM complex also plays an important role in protecting the organism against autoimmunity and could have a substantial role in autoimmune diseases such as systemic lupus erythematosus. Thereupon compounds able to modulate this protein complex formation have been proposed as a new target in cancer and autoimmune diseases treatment.

BTLA also binds to UL144, encoded by the human cytomegalovirus, while HVEM interacts with CD160. As evidenced by recent studies, both UL144 and CD160 are able to inhibit the BTLA/HVEM complex formation and activate immune response. Based on the crystal structure of the BTLA/UL144 (PDB code: 6NYP) and CD160/HVEM (PDB code: 6NG3) complexes and results obtained from molecular mechanics/generalized Born surface area analysis performed for them, we designed and synthesized UL144- and CD160-derived peptides and checked their ability to inhibit the BTLA/HVEM binding. Disulfide bonds were introduced to the amino acid sequences of some peptides, which could enable them to adopt the β -hairpin structure similar to the appropriate fragments in UL144 or CD160. The peptides were synthesized by solid-phase peptide synthesis, purified by reversed-phase high-performance liquid chromatography, then subjected to one or two steps of oxidation and purified again. The ability to disrupt protein complex formation was verified using an enzyme-linked immunosorbent assay.

The results showed that UL-144 and CD160-derived peptides are able to inhibit the BTLA/HVEM complex formation. The peptides stabilized with disulfide bonds have better inhibitory properties than their linear analogues. It indicates that the presence of disulfide bonds in peptides' structure is crucial for their inhibitory potential.

Fragments of UL144 and CD160 are promising candidates for inhibition of the BTLA/HVEM complex. Further research will show the potential of using peptides as a new way for immune checkpoint blockade therapy.

P179

IMMUNE-BASED BIOMARKER ACCURATELY PREDICTS RESPONSE TO IMIQUIMOD IMMUNOTHERAPY IN CERVICAL HIGH-GRADE SQUAMOUS INTRAEPITHELIAL LESIONS

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Topical imiquimod is a non-invasive alternative to a Large Loop Excision of the Transformation Zone (LLETZ) in the treatment of cervical high-grade squamous lesions (cHSIL), and is effective in approximately 60% of primary cHSIL. Prediction of therapy responses upon imiquimod could increase therapy efficacy, prevent unnecessary side effects and aid in patient selection and counselling.

In 35 primary cHSIL and 10 recurrent cHSIL patients treated with imiquimod, biopsies before and after 10 weeks of imiquimod treatment were analyzed by two multispectral seven-color immunofluorescence panels for T cell (CD3, CD8, PD-1, Tbet, FoxP3, TIM3, DAPI) and myeloid cell (CD68, CD14, CD33, CD163, CD11c, PDL1, DAPI) composition to study the immune composition in relation to imiquimod response.

The immune microenvironment of complete responders (CR) prior to imiquimod is characterized by a strong and coordinated infiltration by T helper cells (activated PD1+/type 1 Tbet+), M1-like macrophages (CD68+CD163-) and dendritic cells (CD11c+). The lesions of non-responders (NR) displayed a high infiltration of CD3+FOXP3+ regulatory T cells. Based on the pre-existing immune composition differences a quantitative simplified approach using one color immunohistochemical biomarker, the CHSIL Immune Biomarker for Imiquimod (CIBI), was developed which can be automatically and unbiasedly quantified and has an excellent predictive capacity for complete response to therapy (ROC AUC 0.95, $p < 0.0001$) with a sensitivity of 95.2% and specificity of 81.8% scores. In our cohort, this CIBI biomarker accurately predicted complete response in 20/21 patients (positive predictive value 95.2%), and no response in 10/11 patients (negative predictive value 90.9%). In recurrent CHSIL responses to imiquimod are worse compared to primary cHSIL lesions which is correlated to an increased number of regulatory T cells, myeloid derived suppressor cells and less Tbet+ infiltrating T cells indicating that the pre-existing local immune microenvironment is essential for therapy responses.

In conclusion, a pre-existing coordinated local immune response, fostering an imiquimod-mediated increase in local T cell infiltration is associated with the capacity of cHSIL patients to respond to imiquimod. The CIBI immunohistochemical biomarker has strong potential to select cHSIL patients with a high likelihood to experience a complete response to imiquimod immunotherapy and is now validated in the PRedICT-TOPIC trial.

P180**IMPACT OF TERTIARY LYMPHOID STRUCTURES ON THE TUMOR-SPECIFIC T CELL LANDSCAPE AND RESPONSE TO PD-1 BLOCKADE IN NON-SMALL CELL LUNG CANCER**

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While PD-1 blockade has become a prominent pillar of anti-cancer therapy in lung cancer, it is still poorly understood how this treatment induces T cell reinvigoration in human cancer tissue. Although CD8 T cell infiltration has been acknowledged as an important factor for therapy response, the recent advent of single cell technologies has unveiled the heterogeneity in T cell states present within and across tumors. Moreover, the spatial organization of immune cells in the tumor, with some tumors forming coordinated multicellular immune niches, has been identified as an additional factor for therapy success. In particular, the presence of tertiary lymphoid structures (TLS) has been associated with better clinical outcome upon PD-1 blockade, especially in lung cancer. However, it remains unclear whether TLS immunologically contribute to response and how their presence affects the CD8 T cell landscape. By combining ex vivo technologies with high-dimensional transcriptional and functional profiling of cancer tissues, we here investigated how TLS impact T cell specificity, state, and capacity for reinvigoration upon immune checkpoint blockade in human lung cancer.

To understand whether TLS harbor a distinct T cell repertoire as compared to the tumor parenchyma, we performed TCR-beta sequencing of microdissected TLS and tumor bed regions from non-small cell lung cancer (NSCLC) samples. This analysis revealed that the majority of expanded TCRs was shared between TLS and tumor regions, with many TCRs occurring in multiple regions, suggesting that TLS likely do not expand a distinct T cell repertoire as compared to the tumor parenchyma. To assess whether these shared TCRs display specific cell states, we performed paired single-cell RNA/TCR-seq of T cells from matched tumor suspensions. We found that the vast majority of shared expanded TCRs were CD8+ and displayed a dysfunctional phenotype, indicative of tumor reactivity. Interestingly, individual cells within a clonotype showed distinct dysfunctional states. To assess whether this difference in dysfunctional states could be driven by the spatial context of the T cells, we employed the patient-derived tumor fragment (PDTF) ex vivo technology developed by our lab. PDTFs correspond to 1mm³ sized individual tumor areas maintaining the composition and architecture of the tumor microenvironment. By allocating PDTFs to either TLS or tumor origin, we profiled T cell infiltrates in these individual regions by single-cell RNA/TCR-seq. Strikingly, we found that the expanded, likely tumor-specific T cell clones were biased towards early dysfunctional, precursor-like (TCF7+) and transitional (GZMK+) states in TLS regions. Vice versa, in tumor bed regions, we observed an increase in T cells with a resident memory (ZNF683+) phenotype relative to the TLS. In contrast, late-dysfunctional (CXCL13+) states were more variably distributed across regions, but also showed enrichment in the tumor bed in some patients.

To determine whether TLS and tumor-dwelling T cells differ in their capacity for reinvigoration, we treated PDTFs with PD-1 blockade ex vivo. As previously reported, individual PDTFs from anti-PD-1 responsive tumors showed heterogeneity in immune activation. Strikingly, we observed that anti-PD-1 strongly elicited transcriptional rewiring of CXCL13+ dysfunctional T cells to adopt an activated and cytotoxic phenotype. Additionally, we observed different effects of PD-1 blockade on more precursor-like states in TLS and cells with tissue resident phenotypes in tumor regions.

Collectively, these findings suggest a model in which the level of dysfunction is driven by the tissue context and where TLS may serve as a reservoir of precursor-like cells. Upon reinvigoration, T cells residing in TLS or tumor regions may acquire distinct effector functions, thereby contributing differently to response upon PD-1 blockade.

P181

AN ENZYMATIC SYSTEM FOR PRECISE CELL TARGETING

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The ability to precisely identify subsets of cells using a ligand against a surface marker and deliver a label or payload—i.e., target—is fundamental in both research and clinical settings. Techniques like antibody staining are fundamental to immunology and immunotherapy research but can fail to identify rare, important cell populations or distinguish those demarcated by low-copy number markers. While targeted therapies like antibody-drug conjugates show clinical promise, toxicity often remains a significant concern. We are designing a new type of sensitive, precise, and bio-compatible targeting system that is based around a modified biotin ligase. When targeted to a particular cell type by a covalently-linked antibody (or other ligand), this enzyme iteratively attaches biotin molecules to surrounding lysine residues, which are ubiquitously present at the cell surface as part of various membrane proteins. This approach takes advantage of enzymes' inherently catalytic nature to achieve built-in signal amplification and introduces additional parameters—substrate affinity, enzyme kinetics—that could be adjusted to optimize activity.

We recombinantly expressed and isolated ultraID, a previously reported truncation variant of biotin ligase from *A. aeolicus*, as a fusion protein with the SpyCatcher system to allow for straightforward, rapid conjugation to a variety of targeting ligands. These targeting moieties included nanobodies produced in-house as well as commercial antibodies to which the SpyTag peptide was conjugated using standard bioconjugation techniques. Various cell types, including monocytic, T cell, melanoma and breast cancer cell lines as well as primary murine T cells, were treated with the targeted ultraID, ATP and biotin (substrates of the biotin ligase, typically at 1 mM and 50 μ M respectively) and the efficiency of biotin labeling was assessed via flow cytometry.

Cells treated with targeted ultraID, ATP and biotin were labeled in a target-dependent manner, i.e. only cells expressing the relevant marker had their membrane proteins biotinylated. This selectivity was observed in isolation as well as in admixed populations of cells, even when the target cells represented only ~5% of the population. Furthermore, directly comparing the sensitivity of our enzymatic system with conventional antibody staining indicated an order of magnitude gain in sensitivity. We evaluated the impact of different treatment conditions (time, concentration, temperature), identifying optimal reaction conditions and determining that significant activity is preserved at convenient timescales and temperatures (RT and \leq 1h). We also demonstrate targeted labeling of a tumor cell line at ATP concentrations in the 50-200 μ M range, which corresponds to the extracellular ATP concentration in the TME. We anticipate that this could provide an additional layer of specificity for tumor cells and tumor resident/infiltrating immune cells.

We have applied this approach to a range of targets in a variety of cell types, using both nanobodies and commercially-available antibodies. We are currently testing how effectively this system can improve the detection of challenging subsets within populations of murine TILs. We are also evaluating whether this strategy can be used to increase the sensitivity of antibody-drug conjugates targeting key breast cancer markers like Her2 and Trop2. We anticipate creating activatable versions of this system that take advantage of local environment-specific features or incorporate a two-marker requirement for greater selectivity. This approach is designed with modularity in mind, with the ligand and cargo both readily exchanged to target different subsets of immune or tumor cells for a range of applications, from detection and tracking to selective ablation to delivering nucleic acids for gene therapy.

P182

ALTERATIONS IN THE IMMUNE MICROENVIRONMENT OF ENDOMETRIOSIS AND ENDOMETRIOSIS ASSOCIATED OVARIAN CARCINOMA: A LONGITUDINAL COHORT STUDY

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Chronic inflammation in ovarian endometriosis may be key in the development of endometriosis associated ovarian carcinoma (EAOC). However, the exact influence of the immune microenvironment in the malignant transformation of endometriosis remains unknown, hindering the development of optimal screening tools and promising therapeutics. This study aimed to identify local immune alterations in endometriosis and EAOC for future in depth investigations.

In this unique longitudinal cohort of endometriosis patients, 5 tissue groups were defined. Of these, 3 study groups contained tissue from patients who had endometriosis at least one year prior to EAOC. These groups included endometriosis prior to carcinoma (group 1), clear cell carcinoma or endometrioid carcinoma (2) and endometriosis tissue obtained during tumor debulking surgery (3). The 2 control groups consisted of endometriosis tissue from women without ovarian carcinoma (4) and uterine adnexal tissue from healthy individuals (5). RNA was isolated from paraffin embedded tissue samples and gene expression profiles were compared between the groups with the Nanostring IO360 panel.

In endometriosis compared to adnexa, several immune related pathway scores were increased, with positive enrichment of the dataset of genes involved in costimulatory signaling and the lymphoid and myeloid compartment. In contrast, in endometriosis lower levels were observed than in adnexal tissue of genes involved in DNA damage repair and epigenetic regulation.

Comparison between carcinoma and endometriosis revealed that carcinoma had higher expression of genes involved in apoptosis, autophagy and hypoxia. This increase was also seen in all of the investigated stromal pathways, with the exception of genes involved in matrix remodeling and metastasis, which were significantly lower in carcinoma. The results for the immune related pathways did not differ significantly between endometriosis and carcinoma samples. In addition, higher expression levels were seen in carcinoma for genes of the signaling pathways JAK-STAT, MAPK, NF- κ B, NOTCH and PI3K-Akt and decreased expression was observed in carcinoma for genes of the Hedgehog, TGF- β and Wnt signaling pathways.

Comparing the histological carcinoma subgroups revealed positive enrichment in endometrioid carcinoma of the gene sets involved in antigen presentation, costimulatory signaling and the lymphoid compartment. In contrast, the clear cell carcinomas were significantly enriched for genes involved in angiogenesis and matrix remodeling and metastasis.

Most of the results from the endometriosis subgroups showed overlap. Between control endometriosis tissue and preceding endometriosis, no significant differences were seen in the expression levels of the investigated genes. However, the preceding endometriosis samples had significantly lower expression scores for genes involved in autophagy and Hedgehog, MAPK and Notch signaling.

Compared to preceding endometriosis, concurrent endometriosis had significantly upregulated levels of FOSL1 and NFATC2 and higher scores for genes involved in angiogenesis and PI3K-Akt, TGF- β and Wnt signaling.

Endometriosis is an inflammatory disease and chronic inflammation in endometriosis may be key in the development of EAOC. Endometriosis itself does not display activation of cancer associated

pathways as seen in EAOB and a gradual change was seen in many of the inflammatory genes from healthy adnexal tissue to endometriosis and later to EAOB. The similarities between the endometriotic subgroups suggest that the endometriotic immune microenvironment remains largely the same in patients with or without carcinoma.

P183

HIERARCHICAL SINGLE CELL TYPING THROUGH LOCAL CLASSIFIERS AT TAXONOMY BRANCHING POINTS

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Cell types are conceptually organized in hierarchies which are often used to manually annotate single cell RNA sequencing (scRNAseq) data, a laborious task requiring extensive domain expertise. The taxonomy underlying annotations is constantly changing as our biological understanding continues to improve. While (semi)supervised learning of classifiers can partly automate this process, they are generally inflexible to taxonomy modifications often requiring full retraining with access to primary data. Moreover, their ability to generalize often remains unclear due to the absence of independent external validation data annotated with identical taxonomies. Here, we present scFlorist, a modularized hierarchical cell type classification method where parts of the hierarchical classification can be independently trained and updated to allow for progressive learning of hierarchical cell type annotations and knowledge synthesis across centers without access to primary data. Using a custom cell type classifier testing resource of 6 consistently and independently annotated peripheral blood mononuclear cell datasets we show that scFlorist outcompetes state of the art methods.

scFlorist uses an ensemble of local classifiers at branching points in the taxonomy to modularize cell type classification. Local predictions are used to partition the data into subsets and apply progressively more specific classifiers, mimicking approaches used for manual cell typing. Each local classifier can be continuously updated and exchanged allowing with flexibility in model choice. This allows local classifiers to be trained at different centers with distinct domain expertise and combined. To counter error propagation along the hierarchy, scFlorist uses a thresholding method which allows it to learn from both granular and less granular cell type annotations. Moreover, we implemented distribution free uncertainty quantification and show that scFlorist can correctly detect new cell types as well as cell types with insufficient quality for more granular annotations.

To test the generalization of scFlorist's predictions, we generated a cell type classifier testing resource. We consistently and independently (without dependencies through integration or batch correction) re-annotated 6 PBMC datasets. This resource spans 1.4 million peripheral blood leukocytes and 175 total donors in health, infectious diseases and cancer.

We trained scFlorist on five training datasets with 6-fold cross validation and tested it on the remaining dataset using different local classification models. A fully connected neural network on log-transformed median library size + pseudocount (log1p) normalized data showed the best performance for all metrics (hierarchical F1-scores, hierarchical recall, and hierarchical precision). scFlorist also outcompeted state of the art models treeArches, scPoli, scANVI, and CellTypist in these metrics. In particular, scFlorist achieved better discrimination of $\gamma\delta$ T cells from other T cells, NKT cells from NK cells/ILCs and T cells, as well as memory CD8+ T cells from non-memory populations. We also show that scFlorist can learn new information in a continual manner by introducing data sequentially rather than at all at once, which enables the model to be trained in a federated manner without access to primary data. Exchanging local classifiers trained on other data or altering local model architectures can further increase performance.

scFlorist enables highly accurate cell typing while providing flexibility to modify underlying taxonomies. By exchanging or progressively incorporating new training data into local classifiers researchers can cooperatively synthesize their biological insights without need for retraining. Our PBMC classifier testing resource can be used both to evaluate the performance of future models as

well as enable biological discovery without the need for creating dependencies by batch correction or data integration.

P184**DECIPHERING THERAPY- AND RESPONSE-MEDIATED MECHANISMS OF CANCER IMMUNOTHERAPIES BY CD69-IMMUNOPET**

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Cancer immunotherapies (CIT) aiming to enhance the antitumoral immune response are essential treatment options across various tumor types. However, due to inhomogeneous response rates there is a crucial need for reliable and broadly applicable biomarkers to predict therapy responses and resistance mechanisms at early time points. Thus, we investigated the applicability of immunoPET (PET imaging using radiolabeled antibodies) of the early activation antigen CD69 expressed on immune cells in a syngeneic breast cancer mouse model after CIT-administration to differentiate responding from non-responding tumors and performed mass cytometry (CyTOF) studies of the tumor immune cell infiltrate.

α CD69 monoclonal antibody (mAb) was conjugated with the chelator NODAGA and radiolabeled with copper-64 (^{64}Cu - α CD69). Immunoreactivity and specific binding of ^{64}Cu - α CD69 were determined in vitro by using CD69 overexpressing cell lines and immune cells. For in vivo studies, C57BL/6N mice were inoculated orthotopically with the MMTV-PyMT derived cell line S2WTP3. Tumor-bearing mice received twice weekly either a α PD-1 monotherapy, a combined CIT (α PD-1 + α 4-1BB) or isotype control mAbs. 24 h after therapy initiation, ^{64}Cu - α CD69 was injected intravenously and PET/MRI scans were conducted 24 h and 48 h afterwards. Tumor growth rates of <1.5 (tumor volume d10/baseline d0) were considered as CIT responsive. For high dimensional immune cell phenotyping tumors were analyzed by CyTOF after the second PET scan (72 h after onset of CIT).

^{64}Cu - α CD69 yielded a high radiochemical purity of $>95\%$ and immunoreactive fractions of $>70\%$. The α PD-1 (2/5 responders) and the α PD-1 + α 4-1BB (3/5 responders) treated groups revealed a significant tumor growth inhibition compared to the isotype treated control group. Independent of mono or combined CIT, CD69-PET of tumors identified 100% of the responding mice 72 h after CIT (8.81% injected dose per cubic centimeter (%ID/cc) ± 1.1 SD) compared to non-responding (6.83 ± 0.77 %ID/cc) and control mice (7.13 ± 0.59 %ID/cc). After validation of CD69-PET as a reliable early response assessment tool, we conducted CyTOF analyses of the tumor immune cell infiltrate after CD69-PET with unsupervised clustering algorithms. Both α PD-1 + α 4-1BB and α PD-1 treated groups exhibited a response-independent significant increase in myeloid cells, particularly of Ly6Chi CX3CR1mid macrophages. α PD-1 + α 4-1BB treated tumors revealed a significant decrease of NK cells not related to the response, but showed other response-dependent changes: Ly6Chi monocytes of responders increased significantly ($7.07\% \pm 2.44$ SEM; control: $0.6 \pm 0.13\%$), whereas neutrophil granulocytes expanded in non-responders ($11.26 \pm 1.78\%$; control: $0.82 \pm 0.2\%$). Within the lymphoid cell subsets, CD4+ T cells increased in both CIT groups and α PD-1 + α 4-1BB non-responders revealed a significant increase of B cells. The analysis of T cell activation and exhaustion markers of CD4+ effector memory T cells exhibited an upregulation of VISTA, LAG-3 and OX40 in responders and non-responders, while responders of both CIT groups showed a significant elevation of the activation marker ICOS ($12.17 \pm 1.37\%$) compared to non-responders ($8.05 \pm 0.58\%$) and controls ($3.42 \pm 0.4\%$). Thus, CyTOF analysis revealed a higher frequency of activated effector memory CD4+ T cells in the TME of CIT responsive mice.

In conclusion, immunoPET of CD69 proved as an early therapy response stratification tool and enabled non-invasive in vivo monitoring of the antitumoral immune responses with great promise for

clinical guidance of cancer immunotherapies. In addition, the combination of CD69-immunoPET with subsequent high dimensional single cell analysis of the tumor infiltrate revealed distinct changes of the immune cell composition as well as activation and resistance marker expression patterns, indicating response-dependent changes that could be clearly distinguished from other unrelated CIT induced effects.

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EFFECTIVE GENERATION AND ANTI-TUMOR ACTIVITY OF CANINE CAR.CIK AGAINST SPONTANEOUS SARCOMAS FROM PET DOGS: ESTABLISHING BASES FOR NOVEL IMMUNOCOMPETENT LARGE ANIMAL MODELS OF CAR-IMMUNOTHERAPY

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Cellular immunotherapy with lymphocytes redirected by Chimeric Antigen Receptors (CARs) has provided impressive clinical responses in hematologic malignancies. Its extension to solid tumors holds great promise but it is currently challenging and in high need for reliable translational models. The commonly employed immunodeficient murine models may lack crucial functional elements of the tumor immune microenvironment that are critically important in the perspective of exploring both efficacy and toxicity of CAR-therapies.

The immunocompetent canine model is endowed with high molecular and immunological homology with humans. Treatments in pet dogs, spontaneously developing sarcomas, can help bridging the gap between reliable experimental platforms and clinical studies.

In the last decade our group demonstrated the effective generation, CAR-engineering and preclinical anti-tumor activity of Cytokine-Induced Killer lymphocytes (CIK) against human sarcomas. CIK are ex-vivo expanded T-NK lymphocytes endowed with MHC-independent tumor killing potential.

CSPG4 and B7-H3 are relevant CAR-targets as they are intensely expressed by multiple solid tumors, including sarcomas, characterized by limited presence in healthy tissues and associated with tumorigenesis and tumor aggressiveness.

Purpose of this work is to explore a preclinical canine cellular immunotherapy model, evaluating the generation and functional activity of canine CAR.CIK against sarcomas spontaneously occurred in pet dogs.

We exploited a canine-derived platform based on CIK lymphocytes, sarcoma cell cultures and 3D spheroids.

Canine sarcoma samples were obtained from surgical specimens and the tumor tissue was processed by mechanical and enzymatic dissociation to derive sarcoma cell cultures (osteosarcoma n=7, fibrosarcoma n=1).

CAR.CIK were generated from canine PBMC (n=3) engineered with a 2nd-generation anti-CSPG4.CAR or anti-B7-H3.CAR, including the 4-1BB co-stimulatory domain.

We refined activation, transduction and expansion methods to successfully generate highly functional canine CSPG4-specific and B7-H3-specific CAR.CIK lymphocytes.

We successfully generated and redirected canine CIK by anti-CSPG4 and anti-B7-H3 CARs. Mean expression of CARs by CIK was 37%±2. The expansion rate (10-20 fold) and the immunophenotype (mean CD3=89%±9; CD8=65%±11; CD4=41%±9; CD5=77%±6; NKp46=75%±8; n=5) of CAR.CIK were comparable with paired unmodified controls (NTD.CIK). Both CAR-targets were intensely and diffusely expressed by canine sarcoma cells (CSPG4: 82%±9 n=2; B7-H3: 55%±8 n=6).

CAR.CIK efficiently killed canine sarcomas in vitro compared with NTD.CIK, even at low effector/target (E/T) ratios (CSPG4-CAR.CIK: 40% vs 11%; B7-H3-CAR.CIK: 46% vs 6%; E:T 1:1; n=6, p<0.0001), while sparing canine normal cells (MDCK cell line: 13% vs 0%; E:T 1:4; n=2).

To investigate and monitoring CAR.CIK killing kinetics we developed 3D sarcoma spheroids that

better mimics solid tumor architecture. CAR.CIK showed active infiltration and killing activity against 3D canine sarcoma spheroids (B7-H3-CAR.CIK: 54% vs 7%; E:T 2:1; n=2).

We successfully generated canine CAR.CIK lymphocytes, applying a generation protocol that closely retraces the procedures adopted in human studies.

We confirmed that canine CAR.CIK are highly effective and specific against canine sarcomas, underscoring CSPG4 and B7-H3 as valuable and relevant CAR targets even in this model. Our findings provide a highly feasible and scientifically relevant model, along with a reliable rationale to support clinical studies with CAR.CIK within immunocompetent pet dogs affected by incurable sarcomas.

P186

ELUCIDATING MOLECULAR MECHANISMS OF NK CELL DESENSITIZATION AND EXHAUSTION

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Natural killer (NK) cells exhibit powerful antitumor effector functions and have great therapeutic potential. NK cells can eliminate tumor cells that evade control by CD8⁺ T cells including cells that lose expression of some or all self-MHC I molecules. However, while NK cells initially recognize and kill MHC I-deficient tumor cells, persistent exposure to the MHC I-deficient environment gradually desensitizes NK cells, rendering them incapable of controlling tumors. The molecular basis of NK cell desensitization is not understood mechanistically, and represents one of the most important unresolved issues in NK cell biology.

We aimed to develop an understanding of molecular modulators of NK cell desensitization. We systematically evaluated NK cells desensitized in MHC I-deficient tumors, as well as in diverse tumor-free conditions including residency in B2m^{-/-} mice, absence of MHC I-specific inhibitory receptors, ionomycin treatment, and persistent stimulation with plate-bound antibodies targeting activating receptors.

Transcriptomic profiling comparing functional and desensitized NK cells from these settings revealed several genes that are commonly dysregulated across multiple comparisons. These genes included protein tyrosine phosphatases and regulators of intracellular signaling pathways and actin cytoskeleton network. Knockdown or ectopic expression of some of these genes in NK cells in vitro altered their capacity to degranulate and/or produce cytokines, supporting the power of our approach in discovering genes with roles in modulating NK cell function.

Validating the roles of these genes through in vivo tumor models will elucidate approaches to engineer NK cells that can kill tumors more efficiently for adoptive cell therapy of cancer.

P187

UTILIZING A NOVEL CD137 (4-1BB) AGONIST FOR CANCER IMMUNOTHERAPY AGAINST TOBACCO-CARCINOGEN-INDUCED LUNG CANCER

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Immunotherapies utilizing checkpoint inhibitors has shown remarkable success in the treatment of various cancers. In addition to immune checkpoint inhibitors, immune co-stimulation has been also explored as a strategy to enhance the immune response against cancer. Co-stimulatory receptors play a crucial role in T cell activation and proliferation. One such receptor is CD137, also known as 4-1BB. CD137 generates a potent co-stimulatory signal that promotes T cell proliferation, survival, and effector function when it interacts with its natural ligand, 4-1BBL (4-1BB ligand). This interaction increases the antitumor activity of T cells and helps in overcoming immunosuppression within the tumor microenvironment.

A/J female mice were used as a preclinical model due to their susceptibility to tobacco-carcinogen-induced lung cancer. The mice were treated with 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), the most carcinogenic nicotine derivative to induce lung tumors. NNK was administered at a weekly dose of 25 mg/kg for eight weeks. SA-4-1BBL protein was generated by fusion of murine 4-1BBL and streptavidin (SA-4-1BBL) to obtain the co-stimulatory activity in soluble form. The SA-4-1BBL molecule was utilized as a single agent to investigate immunotherapeutic effectiveness against carcinogen-induced lung cancer. 100 µg of SA-4-1BBL was subcutaneously injected at weeks six and eight of NNK injections. To investigate the involvement of several immune cell types in the immunomodulatory effects of SA-4-1BBL, CD4+, CD8+ T cell, and NK cell depletion was performed. Animals were euthanized and their lungs and lung draining lymph nodes were harvested at the end of the study (18 weeks). Two trained investigators were evaluated the whole lung for macroscopic tumor nodules using a dissecting microscope. The lung and lung-draining lymph nodes collected, processed and subsequently stained for deep immune phenotyping. For histological analyses of microscopic tumor nodules, lung tissues were fixed in formalin and paraffin embedded. Tissue sections were taken, and stained with H&E (hematoxylin and eosin) staining to evaluate microscopic tumor nodules formation. To show the proliferation in the microscopic tumor nodule areas, proliferation cell nuclear antigen (PCNA) antibody was used for immunofluorescence staining. Student's t-tests and one-way ANOVA were used to determine significant differences between groups. A two-way ANOVA was used to assess the body weight changes between different treatment groups. All p-values ≤ 0.05 were considered statistically significant.

Treatment with SA-4-1BBL resulted in significantly fewer macroscopic and microscopic tumor numbers than the control group (NNK+ vehicle). Microscopic tumor nodules had higher PCNA (proliferating cell nuclear antigen) expression. Animals treated with SA-4-1BBL showed significantly smaller tumor nodules than control group. Depletion of CD4+, CD8+ T cells and NK cells diminished the antitumoral effect of SA-4-1BBL. SA-4-1BBL treatment significantly increased CD4+ central memory and naïve T cell numbers in the lung as well as lung draining lymph nodes.

Taken together, these findings suggest that SA-4-1BBL represents a novel approach for harnessing the immune system's antitumor potential and altering the tumor microenvironment. These data further support to clinical approaches for immunotherapy using SA-4-1BBL, to boost the immune system to recognize and eliminate cancer cells.

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A NOVEL COMBINED IMMUNOTHERAPY TO IMPROVE THERAPEUTIC RESPONSES IN SYMPTOMATIC BRAIN METASTASIS BASED ON TARGETING A SUBPOPULATION OF IMMUNOSUPPRESSIVE ASTROCYTES

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The diagnosis of brain metastasis involves high morbidity and mortality. Recently, immune checkpoint blockade antibodies (ICB) have shown clinical benefits mainly when applied to asymptomatic brain metastasis patients. However, variability is broad and responses to this therapy drop considerably when treating clinically relevant disease. Although potentially involved in the lack of response to immunotherapy, corticoids do not seem to fully explain this situation. Thus, it is currently unknown how to effectively target symptomatic brain metastases with immunotherapy. We previously reported a clinically relevant protumoral program driven by STAT3 activation in a subpopulation of reactive astrocytes during advanced stages of the disease. By further exploiting astrocyte heterogeneity, we have found a potential strategy to improve the number of responders to immunotherapy in symptomatic brain metastasis.

Specifically, we have developed a comprehensive strategy including genetic and pharmacologic approaches to define a novel immunosuppressive axis involving astrocyte-secreted TIMP1 signaling on CD63+ CD8+ T cells.

Based on these findings we developed a combined immunotherapy to boost the systemic activation of T cells with ICB while blocking local TIMP1-dependent immunosuppression in various preclinical models. Furthermore, the detection of TIMP1 in the CSF provides a biomarker to select patients for this therapeutic approach. By doing so, we envision it would be possible to select the patients who would benefit the most from the combined immunotherapy. Even more, our data using Patient Derived Organotypic Cultures from fresh brain metastasis neurosurgeries confirmed that our therapeutic strategy is valid for symptomatic brain metastases from any primary source.

In conclusion, our study has identified a novel combined immunotherapy that could be especially relevant for symptomatic brain metastases. Our findings have emerged from a strong scientific rationale whereby a specific subpopulation of astrocytes is shown to activate the local immunosuppressive environment for brain-infiltrating CD8+ T cells.

P189

MYELOID DIRECTED IMMUNOTHERAPY REINVIGORATES CYTOTOXIC T LYMPHOCYTES TO CONTROL NEUROBLASTOMA

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Neuroblastoma is the most common extracranial solid tumor in children. This embryonal neuroendocrine tumor is immunologically “cold” with a paucity of infiltrating immune cells and blunted response to immunotherapy. Neuroblastoma cells exist in two differentiation states known as mesenchymal and adrenergic. The mesenchymal and adrenergic lineages have disparate immunologic properties making this tumor subtype difficult to treat with existing immunotherapies. Mesenchymal cells express major histocompatibility complex class I (MHC-I) and tumors dominated by this cell type are infiltrated with cytotoxic T lymphocyte infiltrates (CTL). Conversely, adrenergic tumors have minimal MHC-I expression with minimal infiltrating CTLs. Therefore, identifying immunotherapy agents capable of targeting both lineages for immune-mediated destruction is a critical unmet need in pediatric immune oncology. Agonistic anti-CD40 antibodies are an exciting therapy currently in clinical trials for immunologically “cold” adult malignancies. Anti-CD40 binds myeloid cells (macrophages, dendritic cells) stimulating these cells to become tumoricidal and secondarily activate anti-tumor CTLs. We posit agonistic anti-CD40 will reshape the immunologic microenvironment of neuroblastoma resulting in the destruction of these tumors.

Using an immune-competent (9464D) preclinical neuroblastoma model we demonstrated agonistic anti-CD40 activates neuroblastoma-specific CTLs leading to control of neuroblastoma tumors. We generated a 9464D cell line that expresses chicken ovalbumin (9464D-OVA) as a surrogate tumor antigen. This model allows the response of neuroblastoma-specific CTLs to be tracked.

Analysis of individual CTLs post-immune therapy identified changes in the activation and differentiation of neuroblastoma-specific CTLs post-therapy. As neuroblastoma is a malignancy of early childhood ongoing studies are investigating the role of development on CTL function, differentiation, and response to immune therapy. Our initial studies identified the neuroblastoma-specific CTLs from neonates more robustly control neuroblastoma tumors than those from later stages of development. Using this model, we will identify the developmental features governing anti-neuroblastoma immunity.

In summary, we demonstrate agonistic anti-CD40 is an effective immune therapy agent activating CTL responses using a preclinical model of pediatric cancer neuroblastoma. As this is a disease of early childhood and CTL function is controlled by development, understanding the impacts of age on immune therapy response is essential for extending this therapy into clinical practice.

P190

TISSUE-SPECIFIC DETERMINANTS OF NK CELL RESPONSES

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Natural killer (NK) cells are circulating innate lymphocytes that are poised to respond rapidly upon encounter with cancer or virally-infected cells as well as proinflammatory cytokines. During their development, NK cells become epigenetically poised to respond rapidly and broadly to immunological stimuli via germline-encoded NK cell receptors (NKR) and cytokine receptors. Moreover, NK cells circulate systemically, conferring 'innate' immune surveillance as they transit through both lymphoid and non-lymphoid tissues. This lifestyle of NK cells contrasts with that of naïve T and B cells, which generally rely on secondary lymphoid organs such as the lymph nodes and spleen for their initial activation.

In addition to their critical role in early immune responses, it is now appreciated that NK cells can also take on features of adaptive lymphocytes such as antigen-specific clonal expansion and long-lived memory responses. These processes have been best characterized following cytomegalovirus (CMV) infection in mouse and human where NK cell receptor-mediated recognition of viral-encoded peptides gives rise to CMV-specific adaptive NK cells. However, it is still not fully understood how additional signals, including different tissue microenvironments, collectively program these adaptive NK cells.

We utilized the model of mouse cytomegalovirus (MCMV) to study robust NK cell activation and generation of adaptive NK cell responses. Adoptive transfer of Ly49H+ NK cells into Klrk8-/- recipient mice permits the longitudinal tracking of antigen-experienced adaptive NK cells that undergo clonal expansion and differentiation following MCMV infection. TNFR2-deficient mice (The Jackson Laboratory) were acquired to study the role of TNF- α signaling in NK cell activation and differentiation. Flow cytometry and RNA-sequencing analyses were used throughout the study to investigate tissue-specific NK cell responses.

Although CMV infection is systemic and anti-MCMV NK cells exhibit cytokine-induced activation across multiple tissue sites of infection, we surprisingly found that MCMV-specific NK cell activation and expansion occurs predominantly in the spleen and that these splenic NK cells up-regulated canonical and non-canonical NF- κ B components to a greater extent. We further identified TNF- α signaling as a critical regulator of both innate and adaptive NK cell responses through engagement of distinct downstream signaling arms of TNFR2. Notably, TNFR2-deficient NK cells exhibited hyper-activation and ultimately failed to undergo robust proliferation in the spleen.

In prototypical immune responses, innate lymphocytes, unlike adaptive lymphocytes, are thought to respond rapidly at initial sites of infection. Here we show evidence that NK cell responses are optimally generated in the spleen during viral infection. These findings highlight the central role of the spleen in facilitating adaptive NK cell responses, which may provide insight into how we can better generate NK cell immunity across diverse settings, including antitumor responses. Moreover, we found that TNF- α regulates both the innate and adaptive processes in NK cells, including during MCMV infection and following ex vivo stimulation, suggesting that TNF- α may be a core regulator of NK cell activity. Altogether our work uncovers the key role of TNF- α in the balancing of the innate and adaptive processes of NK cells and catalyzes further investigation into optimizing TNF activity to promote beneficial NK cell immunity.

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TUMOR CELL TYPE AND ANATOMICAL SITE-SPECIFIC PD-L1 EXPRESSION AND FUNCTION IN TUMOR IMMUNE EVASION

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PD-(L)1-based immune checkpoint inhibitor (ICI) immunotherapy is believed to work by blocking PD-L1 binding to PD-1 expressed on activated cytotoxic T lymphocytes (CTLs) to re-activate PD-L1-inhibited CTLs to suppress tumor. scRNA-seq shows that PD-L1 is mainly expressed in tumor cells (tPD-L1) and subsets of myeloid cells (mPD-L1) in human cancer patients and tumor-bearing mice. The function of cell type-specific PD-L1 has been a hotly debated topic. In tumor-bearing mice, although a study shows that tPD-L1 is sufficient to promote tumor immune evasion, other studies determine that mPD-L1 plays the dominant role in limiting T cell anti-tumor response to promote tumor immune evasion. However, tPD-L1 level is the strongest predictor of a better overall survival of squamous cell carcinoma after ICI immunotherapy. In addition, breast cancer patients with PD-L1-positive tumors had an increased overall survival after ICI immunotherapy. Because the tumor-bearing mouse models are subcutaneous tumors whereas the overall survival likely arises from coordinated suppression of metastases in cancer patients, the discrepancy between tumor-bearing mice and human cancer patients thus may likely be due to the different tumor anatomical locations.

We therefore aimed at testing the hypothesis that tPD-L1 function is cell-type- and tumor anatomical site-dependent. A tumor-specific CTL and tumor cell co-culture model, colon, breast, and melanoma primary and experimental lung metastasis mouse models were used to determine tPD-L1 function. scRNA-seq was used to determine tPD-L1 functional pathways in the cellular and molecular levels. Human patient datasets were used to validate findings in mouse models.

We determined that tPD-L1 does not shield tumor cells from tumor-specific CTL-mediated lytic activity when only tumor cells and CTLs are in the microenvironment in co-cultures in vitro. PD-L1 deficiency only in tumor cells has no significant effect on primary tumor growth in colon, breast, and melanoma subcutaneous tumor-bearing mice. However, deleting tPD-L1 decreases lung metastasis in a CTL-dependent manner in tumor-bearing mice. Depletion of myeloid cells impairs tPD-L1 promotion of tumor lung metastasis in mice. Myeloid cell-specific PD-L1 (mPD-1) deletion in mice also impaired tPD-L1 function in tumor immune evasion. scRNA-seq reveals that tPD-L1 engages mPD-1 to activate SHP2 to antagonize the type I interferon (IFN-I) and STAT1 pathway to repress Cxcl9 and impair CTL recruitment to lung metastases. Human cancer patient response to PD-1 blockade immunotherapy correlates with IFN-I response in myeloid cells.

Our findings determine that tPD-L1 engages mPD-1 to activate SHP2 to suppress the IFN-I-STAT1-CXCL9 pathway to impair CTL tumor recruitment in lung metastasis.

P192

MECHANISMS OF SARCOMA RESPONSE TO RADIATION AND IMMUNOTHERAPY

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Activating the immune system has the potential to improve tumor response to radiation therapy (RT). Using an autochthonous mouse model of soft tissue sarcoma (STS), we observed that intratumoral injection of the TLR9 agonist CpG-ODN enhanced tumor growth delay after a single fraction of 20 Gy. We hypothesize that radiotherapy induces immunogenic cell death that releases tumor antigens, and CpG ODN activates myeloid cells, which together lead to increased tumor antigen cross-presentation and activation of cytotoxic T cells.

We generated autochthonous STS in 129/SvJ or Rag2yc DKO mice by intramuscular injection of adenovirus expressing Cas9 and a sgRNA targeting p53 into the gastrocnemius muscle, followed by intramuscular injection of the carcinogen 3-methylcholanthrene. After tumor development, mice were randomized to receive 0 or 20 Gy (Day 0) and vehicle control or CpG-ODN (Day 3 and Day 10) when tumors reached 70-150 mm³. For intratumoral immune microenvironment analysis, once tumors reached 300 mm³, mice were randomized to receive 0 or 20 Gy (Day 0) and vehicle control or CpG-ODN (Day 3). Tumors were harvested on Day 6 for bulk RNA seq, single-cell RNA seq, and mass cytometry analysis to study the mechanism by which CpG-ODN enhanced tumor response to radiotherapy.

In vivo tumor growth delay studies demonstrated that CpG-ODN + RT combination treatment significantly delayed tumor growth rate compared to other groups, and this treatment effect was negated with lymphocytes (Rag2yc DKO) or CD8 depletion. Bulk RNA seq, single-cell RNA seq, and mass cytometry showed that, compared to unirradiated, CpG-ODN alone, and RT alone tumors, autochthonous sarcomas treated with a combination of 20 Gy and CpG-ODN have a significantly upregulated CD8 T cell population. The increased CD8 T cell population expresses markers associated with activation and proliferation, such as Granzyme B, Ki-67, and interferon- γ . The combination treatment also promoted antigen presentation by myeloid cells through the upregulation of MHC-I, MHC-II, and CD80. TCR clonality expansion analysis suggests that there is preferential tumor-antigen-specific T-cell expansion. Bulk RNA seq and single-cell RNA seq also demonstrated downregulation of the TGF- β signaling pathway in CpG-ODN + RT treated sarcomas compared to RT alone, a known pathway to be upregulated after radiotherapy that contributes to the suppression of anti-tumor immunity.

In a primary mouse model of STS, RT combined with CpG-ODN significantly delays tumor growth compared to RT or CpG-ODN alone, which is associated with the upregulation of activated CD8 T cells. The positive treatment outcome data collected in a mouse model that mimics the co-evolution of the immune system and cancer cells in humans provide a strong rationale for conducting clinical trials in soft tissue sarcoma patients using CpG-ODN + RT.

P193

CYTOSKELETAL REMODELING OF LEUKEMIA CELLS IS DRIVEN BY NFAT2 AND REGULATES SUSCEPTIBILITY TO PERFORIN-MEDIATED KILLING

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Cytoskeletal remodeling is crucial for migration, infiltration and metastasis and overall tumor aggressiveness. In addition, cytoskeleton remodeling protects from cell membrane damage, e.g. in case of cytotoxic lymphocytes from content of their own cytotoxic granules, but also by preventing perforin/granzyme-induced lysis in case of tumor cells. Antibody dependent cellular cytotoxicity (ADCC) a major effector mechanism by which Rituximab that is routinely used for treatment of chronic lymphocytic leukemia (CLL) mediates lysis of tumor cells and thus therapeutic efficacy. We previously demonstrated that in CLL patients loss of the transcription factor NFAT2 (NFATc1) correlates with an aggressive disease course. NFAT2 further is involved in regulating cytoskeleton reorganization of immune cells. Here we investigated the role of NFAT2 with regard to susceptibility of CLL cells to perforin-induced lysis.

To investigate whether and how loss of NFAT2 affects ADCC in CLL, we generated a CRISPR/Cas9 based NFAT2 knockout (KO) in MEC-1 CLL cells.

Cytotoxicity assays revealed profoundly higher resistance of NFAT2 KO CLL cells to NK cell killing compared to scrambled control (SCR) CLL cells, without affecting the level of recognition as revealed by analyses of NK cell activation, degranulation and IFN γ release. Treating MEC-1 cells with NK cell isolates containing cytotoxic granule content revealed increased resistance of NFAT2 KO CLL cells to membrane permeabilization compared to SCR control cells, with perforin blockade equalizing susceptibility to membrane permeabilization, thereby confirming the involvement of NFAT2 in maintaining membrane integrity.

Next, we investigated the role of NFAT2 in the E μ -TCL1 mouse model for CLL by inducing a conditional B cell specific NFAT2 knockout. We previously demonstrated that TCL1 NFAT2 / mice show strongly diminished overall survival compared to TCL1 NFAT2^{+/+} mice, proving the role of NFAT2 for CLL aggressiveness (Maerklin et al, NatCommun, 2017). Using this model, we show that CLL cells of TCL1 NFAT2 / mice are less susceptible to perforin lysis compared to that of TCL1 NFAT2^{+/+} mice. When we classified CLL patients into NFAT2-low and NFAT2-high cases according to the NFAT2 transcript level, CLL cells of NFAT2-low patients showed higher resistance to perforin-mediated lysis and less perforin binding in the cell membrane, confirming our findings obtained with MEC-1 and murine CLL cells.

Finally, inhibition of CDC42, a key regulator of actin cytoskeleton remodeling, with ZCL278 resensitized MEC-1 NFAT2 KO cells to perforin mediated lysis. Similar effects were observed with the tubulin cytoskeleton stabilizing agent Paclitaxel, indicating that cytoskeletal remodeling is crucial for perforin resistance.

Together, our results demonstrate that loss of NFAT2 allows CLL cells to evade NK cell effector function. This holds true for both, constitutive cytotoxicity and therapeutically induced ADCC and is due to lowered susceptibility to perforin-mediated membrane permeabilization, which can be overcome by inhibiting cytoskeletal rearrangement. Thus, NFAT2 loss facilitates resistance of CLL cells to immunotherapeutic treatment modalities.

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IMMUNO-REACTIVITY PROFILING OF DNA MISMATCH REPAIR PROTEINS IN COLORECTAL CANCER; AN INDICATION FOR IMPROVED CHARACTERIZATION AND PATIENT MANAGEMENT IN A DEVELOPING COUNTRY

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Defects in the DNA mismatch repair (MMR) proteins, result in a phenotype called microsatellite instability (MSI), occurring in approximately 20% of colorectal cancer (CRC) cases. This study was aimed to evaluate the expression of mismatch repair proteins in colorectal cancer in order to determine the frequency of abnormal mismatch repair protein expression in CRC cases in Nnamdi Azikiwe University Teaching Hospital Nnewi, Nigeria.

In this study, 55 formalin fixed, paraffin wax embedded tissue blocks of colorectal carcinoma subjects and 39 non colorectal carcinoma tissues from 2012 to 2017 were used. The selected blocks were sectioned and stained with Haematoxylin and Eosin staining techniques. MLH1 and MSH2, the most commonly altered MMR genes, protein expression were evaluated using immunohistochemistry (Avidin-biotin complex method). MLH1 and MSH2 expressions in the tissue were scored based on proportion and intensity of immune- labelling using semi-quantitative method.

The average age of CRC in this study was 58.3 years and the highest number of cases was seen in the 5th and 6th decades of life. There was a significant statistical association between age and MSI-status ($p=0.032$). There was slight male predominance of colorectal carcinoma with a male: female ratio of 1.2:1. There was no statistical association between gender of the subjects and MSI- status ($p=0.248$). Fifteen (27.3%) of the colorectal carcinoma subjects in this study were found to be MSI-positive; 86.7% (13/15) showed loss of protein expression of MLH1 and 13.3% (2/15) showed loss of protein expression of MSH2. The markers showed positive and negative predictive values of 27.27% and 100% respectively, high sensitivity of 100% and specificity of 49.5%. Colorectal carcinoma occurred more in colon and majority of the histological types were adenocarcinoma making up 89.09% of all the tumours while mucinous carcinomas was (10.9%). Well differentiated adenocarcinoma (52.7%) was the commonest colorectal carcinoma and there were no relationship between mismatch repair proteins with anatomical sites.

A significant number of colorectal tumours were deficient in mismatch repair proteins and recognition of MSI can help in the management and prevention of colorectal cancer

P195

COMMON METABOLIC ADAPTATIONS EMPOWER CD8 T CELL TISSUE RESIDENCY AND ANTITUMOR IMMUNITY

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Lodged in tissues throughout the body, tissue-resident memory CD8 T cells (TRM) are key constituents of the memory T cell arsenal offering robust, long-term protection from reinfections. Effective anti-tumor immunity requires sustained T cell function in tissues, and tumor infiltrating lymphocytes (TIL) with characteristics of TRM maintain enhanced effector functions, predict responses to immunotherapy, and accompany better prognoses. The metabolic adaptations required for T cells to transition from an itinerant lifestyle in circulation to tissue residency in response to infections or malignancies are not well understood. We hypothesized that an improved understanding of the metabolic programs underlying tissue residency could inform new approaches to empower T cell responses in tissues and solid tumors.

To define the basis for the metabolic reprogramming supporting TRM differentiation, survival, and function, we leveraged in vivo functional genomics, untargeted metabolomics, and transcriptomics of virus-specific memory CD8 T cell populations. In addition to genetic perturbations, we employed dietary and pharmacological approaches to investigate lipid import, sensing, and utilization by TRM and TIL.

We found that memory CD8 T cells deployed a range of adaptations to tissue residency including a marked upregulation of a Srebp2-dependent production of non-steroidal metabolites, such as ubiquinone, derived from the mevalonate/cholesterol pathway. This metabolic adaptation was most pronounced in the small intestine (SI), where TRM interface with dietary cholesterol and maintain a heightened state of activation, and was shared by functional TIL in diverse tumor types in mice and humans. Enforcing ubiquinone synthesis through Fdft1 deletion or Pdss2 overexpression promoted mitochondrial respiration, memory formation upon acute viral infection, and enhanced antitumor immunity. In addition, we found that pharmacological inhibition of Fdft1 with a fungal-derived metabolite, zaragozic acid, is able to promote increased tumor control in mouse models of colorectal cancer and melanoma in a CD8-dependent manner as a single agent or in combination with anti-PD1 treatment.

In sum, through a systematic exploration of TRM metabolism, we reveal how these programs can be strategically coopted to potentiate CD8 T cell memory formation in the context of acute infections and power CD8 T cell function in the context of tumors.

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CD8 CIS-TARGETING OF IL-2 REWIRES TUMOR-SPECIFIC T CELL IMMUNITY IN HUMAN CANCER TISSUES WITH POOR SENSITIVITY TO PD-1 BLOCKADE

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Immune checkpoint blockade (ICB) has revolutionized cancer treatment and can lead to complete and durable responses in some cancer patients. Nevertheless, response rates largely vary between cancer types and a significant proportion of patients still does not experience (durable) therapeutic benefit from this approach. Therefore, a key challenge in the field is to identify alternative therapeutic strategies that effectively activate anti-tumor immunity, particularly in cancers with poor sensitivity to ICB. To this end, model systems are required that preserve the human tumor microenvironment outside of the patient and allow to perturb immune activity within this intricate ecosystem.

We recently developed the patient-derived tumor fragment (PDTF) platform that allows the short-term ex vivo culture of 3D fragments of human tumor tissue in the absence or presence of immunotherapy. After culture, treatment-induced immune activation can be visualized by multiple readouts. We previously demonstrated that PDTFs display immune responses to ex vivo PD-1 blockade that replicate the clinical response of the same patient. Notably, we found, in line with clinical observations, that, while immune infiltration is increased in tumors responding to ICB, there is also a considerable portion of immune-infiltrated tumors that remains unresponsive to ICB. Importantly, these T cells often display a dysfunctional state, suggesting the presence of a tumor-reactive T cell pool that potentially could be leveraged for immunotherapy. This observation had prompted us to explore whether alternative stimuli can induce reactivation of tumor-specific T cells in these non-responsive tumors. To modulate antitumor immunity in a controlled way, we here exploited an interleukin-2 variant cis-targeted to CD8 T cells (CD8-IL2v), which can specifically and potently activate this subset.

Treating PDTFs from different cancer types including anti-PD-1 resistant ovarian, breast and renal cancers, with CD8-IL2v, we found that the expression of proliferation and cytotoxic markers was induced in tumor-resident CD8+ T cells in most tumors. In contrast, a proinflammatory cytokine and chemokine response could only be detected in a fraction of tumors. Perturbation studies revealed that TCR signaling is imperative for the downstream immune response following CD8-IL2v treatment. To identify the underlying mechanisms, we performed single cell RNA/TCR-seq of PDTFs which showed that upon CD8-IL2v tumor-resident T cells acquire two separate transcriptional activation programs, of which one is dependent and one independent of TCR signaling. Importantly, the TCR-dependent activation program seemed to be largely restricted to the dysfunctional T cell pool, while TCR-independent changes were also observed in memory subsets. This was further supported by TCR expansion and sharing patterns.

Collectively, our results are compatible with a model in which IL2 can 'arm' T cells with effector capacity, preparing them to undergo full functional reactivation as soon as they encounter their target antigen. Intriguingly, we found that CD8-IL2v can induce responses in anti-PD-1 resistant tumors, suggesting that at least some anti-PD-1 resistant tumors may harbor a tumor-specific T cell pool that either cannot be reinvigorated by PD-1 blockade alone or is distinct from the one susceptible to reprogramming by PD-1 blockade.

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DUAL TARGETING OF INNATE AND ADAPTIVE IMMUNE CHECKPOINTS WITH A PD L1/SIRPA BISPECIFIC MACROPHAGE ENGAGER (BIME) FOR THE TREATMENT OF SOLID TUMORS

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Tumor-associated macrophages are major component of immune cells in the tumor micro-environment (TME) that express an array of effector molecules leading to the inhibition of anti-tumor immune responses. Signal regulatory protein α (SIRP α) is a myeloid-lineage inhibitory receptor that restricts phagocytosis through engagement of its ligand CD47 expressed on tumors and normal tissues. Compared to anti-CD47 therapeutics, targeting myeloid-restricted SIRP α may provide a differential pharmacokinetic, safety, and efficacy profile. Here, we report the construction of a SIRP α antagonist-based bispecific macrophage engager (BiME) called ES019, which uses PD-L1 antibody as a tumor associated antigen (TAA) targeting arm and also as a tool to relieve the inhibition of T cell. The PD-L1/SIRP α bispecific macrophage engager aims to promote macrophage phagocytosis against PDL1-expressing tumor cells, which, together with PD-L1 blockade, will eventually lead to an activation of T cell adaptive immunity resulting in further tumor cell killing.

Through Elpiscience proprietary BiME platform, we have generated a panel of single domain antibody (sdAb) based anti-PDL1/SIRP α bispecific antibodies, including different orientations, ratios, and IgG isotypes of anti-PDL1 arm and anti-SIRP α arm. These bispecific antibodies were evaluated for PDL1, SIRP family homologue binding, PD1-PDL1 and CD47-SIRP α blocking properties by ELISA and FACS. In vitro function activity was determined by phagocytosis assay using human monocyte derived macrophage and mouse bone marrow derived macrophage. In vivo anti-tumor efficacy was tested in a syngeneic tumor model with hSIRP α knock-in mice. The pharmacokinetic (PK) and safety profile were assessed in hSIRP α knock-in mice or cynomolgus monkeys. Stress tests were carried out to evaluate the developability of bispecific antibodies.

In this study, we demonstrated that the anti-PDL1/SIRP α bispecific antibody ES019 binds to PD-L1-expressing tumor cell and macrophage simultaneously; it effectively inhibited CD47-SIRP α signal and triggered strong macrophage phagocytosis, which eventually leading to the switch of immune-suppressive M2 macrophages into inflammatory M1 macrophages. In vitro activity of ES019 showed potent macrophage and T cell activation for PD-L1-expressing tumor cell killing in the presence of peripheral blood mononuclear cells, but without nonspecific killing to PDL1 negative cells. Remarkably, ES019 showed almost 100% tumor growth inhibition by in vivo SIRP α knock-in syngeneic models. In summary, we demonstrated that the anti-PDL1/SIRP α bispecific antibody ES019 exhibited super anti-cancer effects, evidenced by potent phagocytosis in vitro and almost complete tumor regression in vivo.

Based our bispecific macrophage engager (BiME) platform, we have developed a PD-L1/SIRP α bispecific antibody that is capable of activating macrophages and T cells to kill cancer cells with the potential to overcome the limitations of traditional anti-PD1 therapies. The anti-PDL1/SIRP α bispecific antibody, designed for tumor cell and immune cell dual targeting, demonstrated significantly enhanced tumor therapeutic efficacy and specificity versus monotherapies.

P198

CONTROL OF RHABDOID TUMORS BY POLY(I:C) IS MEDIATED BY A GLOBAL REMODELING OF THE TUMOR MICROENVIRONMENT

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Rhabdoid tumors (RT) are aggressive tumors of infancy characterized by the loss of SMARCB1, a core component of the SWI/SNF chromatin-remodeling complex, but with no other recurrent mutations and a strikingly low mutational burden. Given the genetic similarity with self, RTs have been historically assumed to be invisible to the immune system and, consequently, to represent poor candidates for immunotherapy. However, we recently demonstrated that RTs have epigenetic mechanisms of tumor immunogenicity and that both human and mouse RTs: i) are highly infiltrated by clonally expanded CD8+ T and myeloid cells; ii) bear activated CD8+ T cell subpopulations expressing druggable inhibitory checkpoints, such as PD-1, Tim-3 and LAG-3; and iii) that blockade of the PD-1/PDL-1 pathway induce the regression of established RTs in mice, yet some tumors escape or are resistant to treatment. Thus, there is a need for improved immunotherapies.

Deep immune characterization of human and mouse rhabdoid tumor microenvironment (TME) indicated that tumor-associated macrophages (TAMs) constitute the most abundant cell subpopulation. Depletion of macrophages using CD64-hDTR mice, delayed tumor growth, highlighting their negative impact on tumor control. Based on the observed high expression of TLR3 in both human and mice myeloid cells infiltrating RT samples, we treated RT-bearing mice by intratumoral administration of poly(I:C), a synthetic dsRNA and a TLR3 ligand, which induced a significant delay of tumor growth. To better understand the underlying mechanism of action, we studied the effect of poly(I:C) on T and myeloid cells from the TME by FACS, scRNAseq, and immunofluorescence.

We observed that poly(I:C) decreased the number of protumoral macrophages, promoted tumor infiltration by neutrophils and CD8+ T cells, and induced the selective expression of iNOS in peritumoral regulatory myeloid cells (mReg). Of note, chemical inhibition of iNOS abrogated the previously observed recruitment of neutrophil and CD8+T cells. Additionally, poly(I:C) administration was associated with direct the activation of macrophages and cDC1, both expressing TLR3, with the latter accumulating in draining lymph nodes. Finally, poly(I:C) favored the accumulation of progenitor exhausted CD8+ T cells (TPEX) in the TME. Given that this CD8+ T cell subpopulation is associated to a better response to anti-PD-1, we evaluated a combinatory treatment of poly(I:C) with anti-PD1, which as expected induced an impressive complete tumor rejection with full memory against tumor rechallenge.

Our findings indicate that modulation of the TME by poly(I:C) inhibits RT resistance to anti-PD1 treatment. This combination represents a promising immunotherapy approach for clinical translation.

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HYALURONAN IS AN EFFECTIVE IMMUNOLOGICAL ADJUVANT FOR THE CREATION OF PROTEIN-BASED VACCINES AGAINST HER2/NEU-EXPRESSING BREAST CANCERS

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Background. Vaccines for the prevention or treatment of infectious diseases are among the greatest medical advances of the 20th century, but the transposition of this knowledge to cancer yielded to unsatisfactory results. This highlighted the necessity of validating or ameliorating new vaccination platforms to be more effective in breaking the tolerance against self-antigens. In this regard, we have recently demonstrated that the simple conjugation of protein Ags to hyaluronan (HA) fragments of 200 kDa leads to robust and long-lasting antigen-specific immune responses, in the absence of additional adjuvants. Notably, high Ab titers were detected in different mouse strains and in both infant and aged mice, suggesting that HA-based vaccines might be particularly advantageous for poorly responding subjects such as elderly, children and cancer patients.

Hypothesis and aim. One of the leading causes of death among women is breast cancer (BC). Overexpression of the human epidermal growth factor receptor 2 (HER2/neu) occurs in 15-30% of cases, and is considered an adverse prognostic factor. Several are the HER2 target therapies available, even though generally well tolerated, such strategies present different drawbacks especially frequent onset of resistance/tolerance to therapy. Therefore, 20% of patients with HER2+ early BC experience relapse and develop advanced disease. In this regard, anti-HER2 vaccines could be employed in a therapeutic setting even in combination with other therapies, and exploited also in an adjuvant setting to prevent the development of metastases or to target early stages of disease. On this ground, willing to exploit the potentiality of our HA-based vaccination approach in the context of a real disease model, HA was conjugated to the extracellular domain of rat HER2 (rHER2) to be validated in both the preventive and therapeutic vaccination settings.

HA fragments were chemically modified introducing aldehyde groups and conjugated to the extracellular domain of rHER2/neu. Female BALB/c or transgenic BALB-neuT mice were immunized with the resulting bioconjugate, rHER2 alone or emulsified with alum as controls, and humoral and cellular antigen-specific immune responses were fully characterized. Vaccinated BALB/c mice were challenged and re-challenged with rHER2/neu-overexpressing TUBO cells to assess the protective or therapeutic activity of HA vaccination strategy. To understand the mechanisms underlying the elicited antitumor responses, *in vivo* cell-depletion experiments were performed.

HA performed efficiently as robust and long-lasting humoral (IgG1, IgG2a, and IgG2b) and cellular responses were detected using very low antigen doses and number of boosters. Outstandingly, at 1-year post-vaccination, anti-rHER2/neu specific antibodies were still detectable, maintaining their effector functions (inhibition of cell proliferation, CDC, ADCC, trogocytosis). HA-induced Th1/Th2 responses turned out effective in both the prophylactic (100% mice survived) and therapeutic (tumor regression in 15% of mice) settings, and effectively broke tolerance against rHER2/neu, remarkably delaying spontaneous tumor appearance and growth in BALB-neuT mice. Notably, *in vivo* depletion studies evidenced that both humoral and cellular responses are mandatory for the success of HA-based anticancer vaccination, but, strikingly, CD8+ T cells seemed to play only a marginal role.

Overall, HA combines the unique immunomodulatory features of a TLR agonist with the tolerability of a fully natural polymer, proving to be a promising adjuvant for the creation of effective and safer cancer vaccines, showing high potential for a rapid clinical translation. Combinatorial treatments may

enable to successfully eradicate poor-responsive tumours, as well as to overcome tumour-mediated immunosuppression and immune escape mechanisms. Therefore, the therapeutic potential of HA-based vaccination will be assessed in combination treatments.

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SINGLE-CELL RNA ANALYSIS REVEAL EFFECTOR-LIKE CD8+CAR-T CELL SUBPOPULATIONS ASSOCIATED WITH RESPONSE IN LYMPHOMA PATIENTS

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CD19-targeting CAR T cell therapy has shown remarkable success against various B cell malignancies but there is still room for improvement. Amongst lymphoma patients, there are still patients that do not respond to CAR-T cell therapy and patients that initially respond but later relapse. In order to improve long-term responses amongst lymphoma patients, mechanisms underlying initial response and tumor relapses must be addressed. It has recently become evident that the composition and cell-intrinsic functions of individual CAR-T infusion products are important for clinical response.

The clinical trial (EudraCT 2016-004043-36; NCT03068416) was a phase II, open-label, one-armed, single center trial. The study included 28 patients of which 24 patients received CAR-T treatment. Treated patients had diffuse large B-cell lymphoma (DLBCL) (n=21), indolent B-cell lymphoma (n=2) (one follicular and one marginal zone lymphoma) or ALL (n=1). Clinical parameters contributing to response was evaluated as well as an in detail evaluation of the CAR-T cell infusion product using single-cell RNA sequencing and multi-color flow cytometry.

In our study, we have investigated the clinical response to third generation anti-CD19 CAR T cells in 23 patients with B cell lymphoma. In addition, we evaluated the CAR T cell infusion products using targeted single-cell RNA sequencing and multi-color flow cytometry to find potential T cell subsets and cell-intrinsic functions associating with clinical response.

Patients were divided into responders (CR + PR) and non-responders (SD + PD), based on the radiological response at the 1 month follow up after CAR-T cell treatment, and their individual CAR-T infusion products were compared. Single cell RNA sequencing of CAR-T cells revealed that patients who responded to CAR-T cell therapy had an enrichment of effector CD8+CAR-T cells in their CAR-T infusion products. Importantly, the cells associated with response displayed high polyfunctionality and cytotoxic and cytokine secretion signatures and a low dysfunction signature. In contrast, cells from the infusion products of non-responders were less polyfunctional and expressed an elevated T cell dysfunction gene signature.

Flow cytometry analysis results concurred with the single cell RNA data. Unstimulated effector (TE, CD45RA+CCR7-) CD8+CAR-T cells expressed higher levels of chemokines and cytotoxic molecules in responders than in non-responders. Importantly, after exposure to CD19+ tumor cells, a higher proportion of TE CAR-Ts produced the effector molecules IFN- γ and TNF- α in responders compared to non-responders. These results suggest that the TE in the infusion product of responding patients react better to tumor stimulation compared to those of non-responding patients.

In summary, we identified a subset of effector CD8+CAR-T cells, using single-cell RNA expression analysis of individual CAR-T cell infusion products, correlating with clinical response. This can be

used as a guidance in the CAR T cell production processes, to obtain CAR T cells with the desired characteristics.

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NFAT1 AND NFAT2 ARE MAJOR REGULATORS IN NK CELL IMMUNOSURVEILLANCE

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NK cells are lymphoid components of innate immunity and play an important role in tumor immunosurveillance. One of the major transcriptional regulators in lymphoid cells is NFAT (Nuclear Factor of Activated T Cells), controlling lymphocyte development and activity. However, while the role of NFAT signaling is well defined in T cells, the cytotoxic lymphocytes of adaptive immunity, surprisingly little is known regarding the relevance of this transcription factor family in NK cells as effector cells of innate immunity. Available data indicate that NFAT activity is dispensable for development of NK cells, whereas effects of the immunosuppressive drugs cyclosporin A and tacrolimus that inhibit calcineurin and consecutively NFAT implicate an involvement of the NFAT family in NK cell function.

We here employed different genetic mouse models and functional analyses to unravel the role of NFAT1 (NFATc2) and NFAT2 (NFATc1) in NK cell reactivity.

In vitro, knockdown (KO) of NFAT1 (NFATc2) or NFAT2 (NFATc1) enhanced NK cell degranulation and resulted in increased production of granzyme B and perforin upon stimulation of activating receptors like NK1.1 or Nkp46 or upon co-culture with different leukemia and solid tumor cells. In line, cytotoxicity assays revealed increased lysis of YAC-1 and B16F10 tumor cells by both NFAT1- and NFAT2-deficient NK cells as compared to wildtype (WT) controls. The inhibitory effect of NFAT transcription factors on NK cell effector function could also be confirmed in vivo by employing WT and NFAT KO animals in syngeneic B16F10 melanoma and RMA-S flank tumor models, which revealed a significantly reduced tumor burden in NFAT1 and NFAT2 KO mice. Comparative analyses with single NFAT as well as NFAT1+NFAT2 double KO and WT animals further confirmed the inhibitory effect of NFAT1 and NFAT2 and pointed to additive effects of NFAT1 and NFAT2 in NK cell tumor immunosurveillance.

Taken together, our results provide the first evidence for a direct functional involvement of NFAT1 and NFAT2 in NK cell antitumor reactivity and identify NFAT as a negative regulator of NK cell function.

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TOO SWEET TO BE TRUE: ARTIFICIAL SWEETENERS ABLATE IMMUNOTHERAPY RESPONSE THROUGH MICROBIOME DISRUPTION.

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Immunotherapy has revolutionized patient treatment across cancer types; however, the majority of patients still do not respond, and the reasons for this are unclear. The gut microbiota has been linked to immunotherapy response in melanoma, but the contributing factors to a 'pro' or 'anti' tumor microbiome remain unknown. Dietary factors, most notably, fiber intake, have been shown to support anti-tumor immunity, while diets high in fat or sugars are associated with poor response. Interestingly, artificial sweeteners (sucralose, saccharin), previously considered to be inert, were recently shown to dysregulate the gut microbiome. Therefore, we hypothesized that microbiome shifts due to artificial sweetener consumption may limit anti-tumor immunity and immunotherapy response in cancer.

Using a mouse model of anti-PD1 responsive cancer (MC38), we sought to determine the impact of sucralose consumption on immunotherapy efficacy. We administered sucralose in the drinking water of mice during tumorigenesis and anti-PD1 treatment and assessed tumor burden, survival, and immune infiltration via spectral immunofluorescence. Lymphocytes were isolated from the tumor and surrounding tissues on day 15 for 5' single cell RNAseq and flow cytometry to assess TCR clonality and T cell function. We assessed potential direct impacts of sucralose on T cells and tumor cells through in vitro cell killing and functional assays. In addition, we characterized shifts to the gut microbiome and metabolites through 16S rRNAseq and metabolomics from the stool during tumor progression and anti-PD1 treatment.

Sucralose, but not sucrose, supplementation in the drinking water ablated response to anti-PD1 in the MC38 mouse model [40-60% CR in controls, 10-20% CR in sucralose]. Interestingly, this phenotype could be recapitulated through select antibiotic treatment or fecal microbe transfer (FMT) from sucralose treated animals. Sucralose reduced T cell functionality [cytokine production, proliferation, and cell killing] and increased T cell exhaustion [inhibitory receptor expression, loss of mitochondria] within the tissue. Most notably, we have found that sucralose consumption correlates with lack of response to immunotherapy in melanoma patients treated with anti-PD1.

Here, we demonstrate for the first time that supplementation of artificial sweeteners leads to drastic shifts in the gut microbiome and a significant reduction in anti-PD1 response in both mouse and man. The gut microbiome is both necessary and sufficient for this loss of response, indicating that artificial sweeteners may represent a barrier to effective immunotherapy. Overall, these findings suggest that select dietary intervention or FMT may be required to boost response to current checkpoint inhibitors.

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A COMBINATION OF ONCOLYTIC HSV-1 AND IN VITRO GENERATED CD103+ DENDRITIC CELLS INDUCES ANTI-TUMOR IMMUNE RESPONSES IN A MOUSE MELANOMA MODEL

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Cancer immunotherapy has become a burgeoning treatment modality, with various approaches showing great promise. One of these approaches is treatment with so-called oncolytic viruses (OVs). The realization that oncolysis represents only the first step in a cascade of immune activation has revolutionized this therapy, resulting in investigation of all kinds of combination treatments aimed at increasing anti-tumor immunity. In this study we aimed to study whether the OV T-VEC alone or in combination with in vitro generated CD103+ DCs could contribute to overall anti-tumor immunity resulting in reduced tumor progression and better survival in a mouse model of melanoma.

CD45.1+ mice were bred and housed at the breeding facility of the Vrije Universiteit Brussel. Both OT-I and C57BL/6 (CD45.2+) mice were obtained from Charles River Laboratories, bred and housed under specific pathogen free conditions at the breeding facility of the VUB and used in experiments between 6 - 10 weeks. The murine melanoma cell line D4M.3A was generated from Tyr::CreER; BrafCA; Ptenlox/lox mice. 3x10⁵ D4M.3A melanoma cells were injected subcutaneously (s.c.) in both flanks. At day 11, tumors were treated with an injection of T-VEC (10⁶ pfu). The treatment was repeated twice with a 3-day interval. Together with the second and third dose, 2-3x10⁶ in vitro generated CD103+ DCs (bone marrow cells differentiated by GM-CSF and Flt-3L) were injected intratumorally in one flank. Incucyte live cell imaging was used to monitor the effect of T-VEC on D4M.3A cells. Flow cytometry was used to assess expression of maturation markers, uptake of tumor cells by DCs, cross-presentation to OT-I cells and calreticulin exposure and cell death (annexin-V/DAPI).

We previously investigated the interaction between T-VEC and human primary DCs and found that both the virus directly and supernatants from human melanoma cells treated with T-VEC induces immune activation on several levels (Tijtgat et al.). Here we wanted to corroborate these findings in a mouse model susceptible to T-VEC. We first showed that D4M3A melanoma cells treated with T-VEC undergo cell death in a dose dependent manner. Significant levels of immunogenic cell death were induced (as measured by calreticulin exposure) suggesting that tumor cells treated with T-VEC are potent stimulators of antigen presenting cells, especially DCs.

When coculturing both the DC2.4 cell line and CD103+ primary DCs, which are the most potent stimulators of anti-tumor immunity, either directly with T-VEC or with supernatant from tumor cells treated with T-VEC, maturation markers CD40, CD80, CD86 and MHC class II were systematically upregulated.

We next investigated the capacity of DCs to take up tumor cells that were treated with T-VEC and found that both DC2.4 and primary CD103+ DCs take up tumor cells, and by using ovalbumin as a model antigen we could also show that this uptake resulted in antigen presentation and T-cell stimulation. Based on these results we wanted to explore the feasibility of using a combination of CD103+ DCs and T-VEC as a novel treatment modality for melanoma. For this purpose, mice were inoculated with D4M.3A tumors at both flanks and the tumor on one flank was injected with sham, T-VEC alone, CD103+ DCs alone or a combination of T-VEC and DCs. Preliminary data show that whereas T-VEC alone results in a small delay in tumor growth, this becomes significant when adding DCs to the treatment.

Here we show the oncolytic virus T-VEC and cross-presenting CD103+ DCs represent a promising combination therapy for the treatment of melanoma, but treatment of other (epithelial) tumors could also be envisaged. We will now investigate in depth the immune compartment within the tumor to establish which mechanisms are responsible for tumor control. We will also combine the DC/T-VEC

treatment with checkpoint inhibitors such as anti-PD-L1 and anti TIGIT, which was shown to improve the effect of T-VEC.

P204

DIFFERENTLY SPECIFIC BTK INHIBITORS IMPAIR ANTI-TUMOR T-CELL IMMUNITY

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The development of T-cell therapies and Bruton Tyrosine Kinase inhibitors (BTKi) have revolutionized the treatment of B-cell malignancies. BTKi differently inhibit off-target (non-BTK) TEC-family kinases: ibrutinib (Ibrut) inhibits IL-2 inducible kinase (ITK) and resting lymphocyte kinase (RLK/TKK) whereas Zanubrutinib (Zanu) mainly inhibits RLK and Acalabrutinib (Acala) has minimal off-target TEC inhibition. BTKi are being combined clinically with T-cell therapies e.g. CAR-T and bispecific antibodies (bsAbs), yet the impact of BTKi on anti-tumor T-cells has had contrasting narratives, with widely differing reports of both immunostimulatory and immunosuppressive effects. Understanding the immunomodulatory effects of different BTKi will be critical to optimal BTKi immunotherapy partner selection. Here we show that inhibition of signaling pathways downstream of ITK and/or RLK in distinct T-cell subsets, has detrimental effects on T-cells, particularly deleterious with Ibrut.

To assess the impact of BTKi, we used spectral flow cytometry to assess T-cell activation and tumor-killing of lymphoma cells in vitro with transgenic T-cells, bsAbs, and CAR-T from murine models, healthy controls and lymphoma patients. To assess in vivo effects, we treated lymphoma-bearing mice with bioluminescent-trackable anti-CD19 CAR-T or anti-GFP (JEDI) T-cells and assessed the impact of BTKi on T-cell trafficking, tumor growth and survival. For gene-editing mechanistic studies, we used Lonza Nucleofection of CRISPR/Cas9 Ribonucleoproteins (RNP) to confirm the independent or redundant roles of ITK and RLK in anti-tumor T-cell efficacy.

In vitro, Ibrut markedly reduced CD8 proliferation, activation and capacity to kill lymphoma cells, whereas Zanu and Acala had minimal effects. Similarly, Ibrut completely abrogated the therapeutic benefit of anti-tumor CD8 cells and markedly impaired anti-tumor efficacy of (mixed CD4/CD8) anti-CD19 CAR-T in vivo, whereas Acala had minimal impact on CD8 T-cells or CAR-T-induced tumor regressions and survival prolongation. To assess BTKi effects on human T-cells, we co-cultured patient or healthy T-cells with lymphoma cells and the CD3xCD19 bsAb blinatumomab. Again, Ibrut diminished both CD8 and CD4 T-cell activation. Amongst CD4 cells, as previously described in patients, Ibrut increased Th1/Th2 ratios but, nonetheless, reduced Th1 CD4 activation. The T-cell impairment observed in these co-culture functional assays might be due to direct inhibition of T-cell TEC-family kinases, or indirect effects, including those on the lymphoma cells altering the tumor:T-cell interaction. To assess direct effects of BTKi on T-cells, we performed spectral phospho-flow, assessing T-cell signaling within minutes of activation. We observed that Ibrut induced relative decrease in activation of TCR downstream signaling nodes e.g. SLP76, Lat, and PLC γ relative to highly-specific BTKi. To assess potential mechanisms of T-cell suppression by non-specific BTKi, we used ITK- and/or RLK-gRNA/cas9 RNP nucleofection to knock out (KO) from anti-GFP CD8 T-cells and observed that ITK was critical for T-cell proliferation and tumor-killing, whereas RLK appeared dispensable. Double KO of ITK and RLK demonstrated greater tumor-killing defects than single ITK KO, suggesting ITK's capacity to compensate for RLK.

Non-specific BTKi such as Ibrut impair CD8 T-cells and CD4/CD8 CAR-T proliferation, activation and tumor-killing in contrast to highly specific BTKi, with similar results seen in murine and human models. Despite numerous datasets showing that Ibrut-treated patients develop improved global T-cell health during therapy, these reports may be confounded by the T-cell suppressive effects of heavy tumor burden, and related immune benefits of tumor debulking. Rigorous comparison of differently specific BTKi suggests that avoiding inhibition of ITK, and possibly RLK, may yield combination BTKi/T-cell therapies with optimal anti-tumor effects in our patients.

P205 VIRTUAL

SINGLE-CELL SEQUENCING REVEALS TUMOR MICROENVIRONMENT (TIME) FEATURES ASSOCIATED WITH MAJOR HISTOLOGICAL RESPONSE (MHR) AFTER NEOADJUVANT CTLA4 AND PD-1 IMMUNE CHECKPOINT BLOCKADE (ICB) IN PATIENTS WITH HPV-POSITIVE HEAD AND NECK SQUAMOUS CELL CARCINOMA

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Concurrent CTLA-4 and PD-1 ICB has demonstrated durable efficacy as neoadjuvant therapy in patients with HNSCC. Therefore, further investigation in the induction or neoadjuvant setting, as well as exploration of the determinants of response, are merited. We investigated features of the TIME associated with MHR following induction CTLA-4 and PD-1 ICB in patients with newly diagnosed HPV-positive head and neck squamous cell carcinoma (HNSCC).

Tumor samples were collected at baseline and on treatment from a cohort of 35 patients enrolled in a phase 2 trial of a 6-week cycle of CTLA-4 and PD-1 ICB induction followed by dose/volume-adapted IMRT (50-66Gy) concurrent with cycle 2. Tumors were then evaluated for histological response and single-cell RNA sequencing and T cell receptor (TCR) sequencing were performed. After stringent clustering and annotation of single cell data, 76,319 CD8+ and 78,622 CD4+ T cells with paired TCR sequence data were identified for comprehensive transcriptomic, TCR repertoire and clonotype analysis.

Twelve (44%) of 27 patients with paired samples (missing data due to COVID) demonstrated a MHR, defined as no more than 10% residual tumor viability on ICB treatment. In the CD4+ T cell compartment, ICB-induced activated regulatory T cells (Tregs) depletion ($p=0.003$, Wilcoxon) was observed in patients with MHR. The depletion was potentially resulted from antibody-dependent cell-mediated cytotoxicity (ADCC) mediated by highly cytotoxic CD16+ NK cells. Furthermore, the CD8+ T cells in patients with MHR were characterized with higher immune response signaling pathways, larger TCR clonality ($p=0.017$, Wilcoxon) prior to ICB treatment. Clonotypes with large clonality predominantly resided in exhausted CD8+ T cell clusters and exhibited a tissue resident memory (TRM) phenotype. Through longitudinally tracing TCR data, over 100 CD8+ T cell clonotypes that expanded significantly ($p < 0.05$, Fisher's exact) post-ICB were identified. Notably, patients with MHR showed significantly higher ICB-induced TCR clonotype expansion compared to non-MHR patients ($p=0.025$, Wilcoxon). Further examination of their functional states revealed higher phenotypic plasticity in patients with MHR, and a variety of T cell modulatory trajectories, including reverse transition from exhausted to less exhausted states and from memory to effector states. A lasso regression model associated T cell expansion, basal 7-gene TRM and 5-gene cytotoxicity scores, and on-treatment proliferation score and cytotoxicity cell proportion with percent reduction in tumor viability ($R^2 = 0.66$).

In HPV-HNSCC, the pre-existing antitumor immune response mediated by CD8+ T cells and NK cells correlates with the response to induction CTLA4 and PD1 ICB. The treatment led to extensive reinvigoration of CD8+ T cell clonotypes with an TRM phenotype and the ones with effector memory phenotype, revealing a remarkable degree of plasticity of distinct T cell clones. Paired RNA and TCR profiling facilitated identification of potentially tumor reactive and ICB-responsive T cells and clonotypes and identified several TIME features associated with tumor cell death.

P206

ANTI-PD-L1 FAB BASED IMMUNOPHOTOTHERMAL LIPID NANO PARTICLE ENABLE TREATMENT OF BREAST CANCER AND ITS METASTASIS

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Over the past decade, various therapeutic trials have made great strides in cancer immunotherapy. Especially, immune checkpoint blockade has been attracted with outstanding efficacy as the regulators of immune cell activity against cancer. Nevertheless, antibody based immune checkpoint blockade have defects including inadequate tumor penetration, and high-cost burden to patients and manufactures. In addition, the diverse chemical action sites of antibodies present difficulties in precise drug attachment. Therefore, in this study, we synthesized the fragment of antigen-binding site (Fab) of the anti-PD-L1 antibody and conjugated it to the drug contained lipid nanoparticle (LNP) in order to utilize both the drug delivery efficiency and the antigen-binding ability of the antibody.

The Indocyanine green (ICG) was incorporated in the LNP for photothermal therapy (PTT) and decorated anti-PD-L1 Fab with the LNPs, which named immunophotothermal LNP (ipLNP). For cancer therapy, 4T1(breast cancer) cells were injected subcutaneously to build the first transplanted tumor in BALB/c. For evaluation of metastatic tumor inhibitory effects of the ipLNP after PTT, these mice received a second transplantation with the same cancer cells, 4T1 breast cancer, by intravenous inoculation.

The accumulation of LNP in 4T1 tumor in the BLAB/c was increased by anti-PD-L1 Fab decoration in the LNPs. In addition, NIR laser irradiation elevated temperature in the tumor, which consequently eliminated 4T1 tumor growth. Furthermore, the mice cured from 1st challenged 4T1 by ipLNP with PTT prevented 2nd challenged lung metastatic 4T1 cancer infiltration, which prevented effect was mediated by T cell-induced anti-cancer immunity.

Thus, these data demonstrated that potential usage of ipLNP as the immuno-photothermal therapeutic material against cancer and its metastasis.

P207

AIRE-EXPRESSING MACROPHAGES IN TUMOR PROGRESSION AND IMMUNOTHERAPY RESISTANCE

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The Autoimmune Regulator (Aire) gene plays an essential role in central immune tolerance through its roles in deletion of self-reactive T cells during thymic selection and regulatory T-cell induction. Outside the thymus, the discovery of extrathymic Aire-expressing cells (eTACs) has defined putative roles for Aire-expressing cells in peripheral tolerance induction, including in autoimmunity, maternal-fetal tolerance, and commensal tolerance. However, any role for these populations in tumor immune evasion remains entirely undefined. Tumor microenvironments (TMEs) are populated by a number of tolerogenic myeloid cells that promote tumor tolerance. However, tumor-resident myeloid populations, particularly macrophages, are highly heterogeneous with some populations promoting tumorigenesis and others inflammation and tumor rejection. Further work is necessary to understand immunosuppressive programs that promote tumor tolerance in myeloid populations and how to therapeutically target these programs. Here we define the biology and function of novel populations of tolerogenic Aire-expressing macrophages in the TME that appear to play critical roles in tumor immune evasion and immune checkpoint resistance across a range of models.

We characterized Aire-expressing cells in a range of mouse tumor models; MC38 (colorectal), B16F10 (melanoma), and AT3 (breast). To detect Aire-expressing cells in the TME, we used an established Aire-GFP reporter mouse; spectral flow cytometry, mass cytometry and single cell RNA sequencing were used to characterize these populations. To specifically target Aire+ cells in the TME, we utilized an established Aire-DTR mouse model. CD8+ T-cell dependence was tested using CD8 blocking antibody in Aire-DTR mice. All experiments were balanced for sex and mice were 6-10 weeks old at tumor injection.

We discovered that a subset of tumor-associated macrophages (TAMs) express Aire in the TME (aTAMs) across multiple mouse tumor models (MC38, B16F10, and AT3). These aTAMs display a unique transcriptional profile characterized by increased immunosuppressive, proliferative, and anti-apoptotic features as well as metabolic reprogramming. Ablation of aTAMs with or without immunotherapy improves tumor control across multiple immunotherapy-resistant mouse tumor models in a CD8 T cell-dependent manner. Ablation of aTAMs additionally promotes inflammatory remodeling of the myeloid compartment and increased activation and granzyme B production from CD8 T cells. Finally, aTAMs can be found and share a similarly immunosuppressive transcriptional profile in publicly available human scRNA-seq tumor datasets across multiple tumor types.

Our data suggest aTAMs represent a phenotypically and functionally distinct Aire-expressing myeloid population in the TME of a variety of tumor models. Elimination of aTAMs improves tumor control, survival, and promotes therapeutically productive immune remodeling of the macrophage and CD8 T cell compartments. Further, specific targeting of peripheral Aire-expressing populations is a novel and promising therapeutic approach for treating a range of cancers. Finally, these insights offer encouraging targets for combination therapy in conjunction with checkpoint inhibitor therapies. Future work will define the specific mechanisms by which aTAMs and Aire contribute to tumor progression and immunosuppression, as well as identifying novel therapeutic targets related to these populations.

P208

CORRELATION BETWEEN THE PIK3CA PATHWAY ALTERATIONS AND CLINICAL OUTCOMES IN PATIENTS WITH HPV-NEGATIVE HEAD AND NECK SQUAMOUS CELL CARCINOMA (HNSCC) TREATED WITH IMMUNE CHECKPOINT INHIBITORS (ICI)

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ICI targeting the PD-1/PD-L1 pathway has demonstrated durable clinical benefits in patients (pts) with recurrent or metastatic (R/M) HNSCC but only in a small subset of pts with a response of rate of < 20%. Identifying mechanisms for ICI resistance may guide optimal pts selection and development of rational combination strategies. Aberrant PI3K/AKT/mTOR pathway activation is an oncogenic driver in many solid tumors including HNSCC. Activating alterations of PIK3CA gene have been suggested to suppress anti-tumor immunity but its impact on ICI response in HNSCC has not been studied.

Retrospective analysis of clinical data of pts with R/M HNSCC with available next-generation sequencing-based molecular test data who received ICI (including immune therapy combination but excluding chemo combination) was conducted to correlate molecular characteristics and clinical outcomes. The association between PIK3CA expression and immune signatures and effector cell infiltrations was analyzed using the HNSCC Cancer Genome Atlas (TCGA) database. HNSCC pt-derived tissues were treated with ICI to explore the correlation between various signaling pathways and immune responses. Lastly, the impact of pharmacologic inhibition of PI3K on the immune gene expression was assessed using various HNSCC cell lines.

A total of 93 pts met the selection criteria for analysis. 33% pts were human papillomavirus (HPV)+ and 60% had ICI as a first-line therapy with 80% receiving anti-PD1 monotherapy. PI3K/AKT/mTOR pathway alteration was seen in 44.1% and was correlated with inferior survival in HPV- pts (HR 1.87, P=0.05). Survival difference was not statistically significant in HPV+ pt group. Additionally, PI3K pathway alteration was associated with shorter progression-free survival (P=0.06) and lower radiographic response rate in HPV- pts. Analysis of HNSCC TCGA data revealed PIK3CA gene expression inversely correlated to CD8+ T-cell infiltration in HPV- HNSCC population. Gene expression and phosphoproteomic analysis of HNSCC pt-derived tissues after ICI treatment showed higher activity of PI3K pathway in the ICI-resistant sample and suppressed INF γ secretion, CD8+ cell infiltration, and CD8 cytotoxic activity. Similar findings were observed in TCGA data demonstrating negative correlation between IFN γ responsive and APM gene with PIK3CA gene expression. Lastly, in vitro pharmacologic inhibition of PI3K pathway with alpelisib and pictilisib induced a significant increase in key immune response gene expressions such as antigen presentation machinery-related, IFN γ responsive, and chemokine genes in PIK3CA mutant but not in wild-type HNSCC cell lines.

We demonstrate that PI3K pathway alteration correlates to poor response to ICI therapy in HPV-HNSCC pts through the remodeling of immune gene expression, thereby promoting immune evasion. While further research is warranted, our finding supports a potential strategy of combining PI3K inhibitors with ICI to improve outcomes in pts with HPV- HNSCC.

P209

HAMPERING IMMUNOSUPPRESSIVE ADENOSINE BY GENOME EDITING FOR THE ADOPTIVE T CELL THERAPY OF COLORECTAL CANCER

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In solid malignancies, tumor infiltrating lymphocytes (TILs) functionality is closely intertwined with metabolic reprogramming within the tumor microenvironment (TME), which supports the increased energy request of cancer cells and suppresses the effector function and cytokines production of the immune infiltrate. Extracellular adenosine, accumulating in the TME for the sequential hydrolysis of ATP by ectonucleotidases CD39 and CD73, has been recently recognized as a new immune checkpoint mediator, which binds preferentially to the A2A receptor (A2AR) to exert its immunosuppressive activity.

To investigate the effects of the adenosine metabolic reprogramming on adoptive T cell therapy (ACT) products for colorectal cancer (CRC), we used CRISPR/Cas9 genome editing technology to disrupt in primary T cells the α chain of the endogenous T cell receptor (TCR) alone or in combination with ENTPD1 (CD39), NT5E (CD73) or ADORA2A (A2AR). To functionally test our engineered T cells, we employed lentiviral transduction to redirect their specificity against the HER-2 antigen, overexpressed in a subgroup of CRC patients, and challenged them with tumor cell lines and patient-derived organoids from primary and metastatic CRC.

We firstly assessed the relevance of adenosine pathway in CRC performing flow cytometry analysis on T cells retrieved from 5 primary CRC neoplastic samples and corresponding patients' peripheral blood. We observed that CD39 and A2AR, but not CD73, were significantly more frequently expressed on TILs compared to peripheral blood lymphocytes and showed a dysfunctional phenotype. Given the importance of the adenosinergic pathway in the implementation of ACT for CRC, by CRISPR/Cas9 technology we generated T cells products in which the α chain of the endogenous TCR has been disrupted alone (TCRed; 88% mean efficiency) or in combination with ENTPD1 (TCRedCD39ko; 92% mean efficiency), NT5E (TCRedCD73ko; 70% mean efficiency) or ADORA2A (TCRedA2ARko; 89% mean efficiency). The manipulation procedure did not perturb edited T cells expansion, CD4/CD8 ratio or memory differentiation. After the HER-2-specific TCR transduction, our engineered T cells efficiently eliminated tumor cells when challenged with tumor cell lines and patient-derived organoids from primary and metastatic CRC. Importantly, TCRedCD39ko T cells displayed superior killing ability compared to TCRedCD73ko, TCRedA2ARko or TCRed T cells. To gain insights into the ATP/Adenosine pathway into T cell functionality, we repeated the killing experiments in the presence of saturating concentration of exogenously added adenosine: in this setting, the functional advantage of TCRedCD39ko T cells was nullified, while TCRedA2ARko T cells were still able to eliminate tumor cells and resistant to adenosine-mediated immune suppression. The phenotypic characterization, proliferation capability and differential cytokines production ability by TCRedCD39ko and TCRedA2ARko T cells after a chronic stimulation with HER-2 antigen are currently under evaluation.

We showed the promising role of the editing of adenosine-signaling genes in ACT for CRC, that we will further exploit in additional in vitro and in vivo environments mimicking the immunosuppressive TME and in combinatorial therapeutic approaches.

What's next in the pipeline of melanoma treatment

P210

NUCLEAR LOCALIZATION OF THE SUPPRESSIVE PROTEIN, GLYCOPROTEIN A REPETITIONS PREDOMINANT (GARP), IS LINKED TO POOR PATIENT PROGNOSIS IN MELANOMA AND GLIOBLASTOMA

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Biomarkers are the backbone of individualized medicine, providing invaluable information on disease progression and the likelihood of therapy response, among other things. With highly aggressive and treatment resistant malignancies, like melanoma and glioblastoma (GB), it is the utmost priority to discover novel biomarkers in order to optimize treatments regimens and outcomes of cancer patients.

Glycoprotein A repetitions predominant (GARP), a surface activation marker of regulatory T cells (Treg) has desirable properties of a biomarker. GARP is overexpressed in the suppressive tumor microenvironment (TME) by tumor cells and cells that contribute to immunosuppression alike, including activated Treg and platelets. Secretion of GARP (sGARP) into the TME aids in the inhibition of anti-tumor immune responses (e.g. induction of peripheral Treg and M2 macrophages, inhibition of effector T cells) mediated by the protein's function as an activator of the immunosuppressive cytokine, transforming growth factor beta. Notably, GARP was recently discovered in melanoma and GB to be even more enriched in the nucleus. The upregulation of GARP in the TME coupled with its multi-faceted role in suppression in the TME, make GARP a promising biomarker candidate.

Previous work has begun to analyze GARP in its different forms (e.g. sGARP, GARP+ Treg, GARP+ tumor tissue) as a biomarker for various cancers types (e.g. prostate, lung) and shown initial links between GARP, tumor aggressiveness, and overall survival (OS). However, studies have yet to explore nuclear GARP (GARPNU+) and GARP mRNA as possible candidates for novel cancer biomarkers. This study aimed to evaluate GARPNU+ and GARP mRNA in tumor tissues for the first time as prognostic biomarkers for melanoma and GB.

Retroactive analyses of GARP transcript levels in human tumor tissue correlated to OS were performed using OncoLnc (n=152 GB, n=458 skin cutaneous melanoma, upper and lower percentiles: 50/50). GARPNU+ in human tumor tissue was analyzed via immunohistochemistry (n=35 GB, WHO stage IV - 2016 GB, primary; n=111 melanoma, stages I-IV, primary and metastatic, cutoffs: low (50%, n=19) and high (>90%, n=16) GARPNU+ cells) and correlated to OS. Patient samples were obtained with informed consent.

Although all melanoma and GB patients expressed GARP mRNA, no correlation between GARP transcript levels and OS was observed. GARPNU+ was expressed in both melanoma and GB tissues, regardless of disease staging. Every patient in the GB cohort expressed GARPNU+ but varied in their frequency of GARPNU+ cells. Division of the cohort into "low" or "high" frequency of GARPNU+ revealed a significant correlation between high frequency of GARPNU+ and reduced OS in GB (Medians low: 12 months, high: 4 months; p=0.0026). Analysis of the melanoma cohort is ongoing. Future studies are planned to increase patient cohort size, to expand this work to other cancer types, and to evaluate GARPNU+ as a predictive biomarker for therapy response.

In summary, our results indicate for the first time that GARPNU+ – not GARP transcript levels – is associated with survival in cancer patients and suggest the potential application of GARPNU+ as a prognostic biomarker for melanoma and GB.

P211

TARGETING NKG2DL WITH BISPECIFIC ACD16/ACD3 FUSION PROTEINS ON TRIPLE NEGATIVE BREAST CANCER

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Triple negative breast cancer (TNBC) is a highly aggressive subtype of breast cancer with poor response to conventional systemic treatment. In recent years, treatment has undergone many changes including the implementation of immune checkpoint inhibitor-based immunotherapy in routine treatment. Despite these efforts, relapse rates in TNBC remain high. Accordingly, there is a medical need for novel immunotherapeutic approaches. NKG2D ligands (NKG2DL) are expressed on cancer cells but typically not on healthy tissue. They potently stimulate reactivity of natural killer (NK) cells and CD8+ T cells that express the activating NKG2D receptor. Targeting NKG2DL is currently under investigation in clinical trials utilizing chimeric antigen receptor (CAR) T or NK cell therapy formats. While most of these studies are focused on leukemia patients, some also include patients with solid tumors, e.g. TNBC.

We developed bispecific fusion proteins (BFPs) that consist of the extracellular NKG2D receptor domain fused with either anti-CD3 (NKG2D-CD3) or anti-CD16 (NKG2D-CD16) Fab fragments, which enables simultaneous targeting of multiple NKG2D ligands (NKG2DLs). We functionally characterized the BFPs in various in vitro assays with TNBC cell lines (n=8) and PBMC of TNBC patients (n=19).

First, we characterized expression of the NKG2DL MICA, MICB, ULBP1-4 on various TNBC cell lines and found MICA and ULBP2 to display the highest surface expression for all investigated cell lines, whereas ULBP4 was overall absent. Targeting TNBC cells with NKG2D-CD3 or NKG2D-CD16 was found to efficiently activate T cells and NK cells, respectively, of healthy donors in vitro, resulting in their degranulation and release of IFN γ , Granzyme and Perforin. In short term lysis assays, pronounced killing of TNBC cells was achieved by NKG2D-CD16, whereas NKG2D-CD3 required a longer time to demonstrate effective anti-tumor activity. Long-term killing assay revealed that both NKG2D-CD16 and NKG2D-CD3 were successful in inducing lysis of TNBC cells, with the killing ability of T cells stimulated by NKG2D-CD3 being more pronounced than that of NK cells stimulated by NKG2D-CD16. Notably, PBMCs from TNBC patients undergoing chemotherapy were likewise found to exhibit significant NK cell and T cell activation and tumor cell lysis upon stimulation with NKG2D-CD16 or NKG2D-CD3.

These findings provide evidence that NKG2D-based NK cell and T cell engagers may alone and in combination with chemotherapy constitute a beneficial treatment option for TNBC patients.

P212

COMBINED ALECSAT AND ANTI-PDL1 IMMUNOTHERAPY INHIBITS TRIPLE-NEGATIVE BREAST CANCER GROWTH AND METASTASIS

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Adoptive cell transfer immunotherapy is gaining momentum as an appealing approach for advanced-stage cancer treatment. However, generating sufficient numbers of immune cells that possess the ability to selectively identify and eliminate cancer cells, while avoiding the emergence of resistant clones, remain a major challenge. To address this challenge, we have recently developed a novel procedure called ALECSAT (Autologous Lymphoid Effector Cells Specific Against Tumor Cells). This technique enables the selection, expansion, and maturation of polyclonal lymphocytes derived from peripheral blood, endowed with the capability to identify, and eradicate malignant cells. Previous preclinical and clinical studies have emphasized the importance of long-term persistence and evasion of immunosuppression as critical properties for achieving therapeutic efficacy.

To assess the therapeutic potential of ALECSAT cells in the context of triple-negative breast cancer, we employed immunodeficient mice challenged with either cell lines or three distinct patient-derived xenograft (PDX) models of triple-negative breast cancer. These mice were subsequently treated with ALECSAT cells, both with and without concurrent anti-PDL1 therapy. The cell line models received partly HLA-matched allogeneic ALECSAT cells, whereas the PDX models were treated with autologous ALECSAT cells. The impact of these treatments on primary tumor growth, metastasis formation, and overall survival was evaluated.

While ALECSAT monotherapy effectively impeded the formation of experimental and spontaneous metastases in the cell line model, it exhibited limited efficacy in inhibiting primary tumor growth and failed to improve overall survival in the PDX models. In contrast, the combination of ALECSAT cells and anti-PDL1 therapy exerted a potent inhibitory effect on primary tumor growth, almost completely abrogated metastasis, and significantly prolonged the survival of mice bearing cell line- and PDX-tumors.

The combination therapy of ALECSAT and anti-PDL1 demonstrates encouraging anti-cancer responses in both the cell line-derived xenograft model and the autologous PDX models of advanced triple-negative breast cancer. These promising findings warrant further investigation, not only in the context of triple-negative breast cancer, but also in other malignancies. The synergistic activity observed with the combination therapy holds immense potential for future therapeutic strategies and warrants continued exploration in the field of cancer treatment.

P213

THE CLINICAL DEVELOPMENT OF MRNA-GALSOMES AS A THERAPEUTIC MRNA LIPID NANOPARTICLE VACCINE AGAINST CANCER.

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During the Covid-19 crisis, mRNA vaccines have gained global recognition for being generally safe and highly effective although questions remain on the mode of action and inflammatory nature of this novel vaccine platform (Verbeke R. et al. JCR 2021). Following intramuscular injection of two doses, these mRNA vaccines were characterized by high titers of antibodies protecting the host from COVID-19. However, for therapeutic cancer vaccine development, the activation and presence of cytotoxic CD8 T lymphocytes (CTLs) underpins vaccine efficacy. Therefore it is debatable whether the m1 Ψ -modified mRNA-LNP vaccine technology is sufficiently capable of inducing effective immunity in this context. Within our research group, we developed the mRNA Galsome platform where N1-methyl-pseudouridine (m1 Ψ) modified mRNA is co-formulated with the potent broad-spectrum adjuvant alpha-Galactosylceramide (α GC) in lipid nanoparticles (LNPs). Within the context of therapeutic cancer vaccination, this platform technology has important advantages such as the empowerment of conventional T cell responses, broadening of the immune response by activating NKT- and NK cells while simultaneously reverting the immune depressed tumor microenvironment by altering its myeloid cell composition (Verbeke R. et al. ACS Nano 2019). Based on promising preclinical data, showing effective control of tumor growth in a B16 mouse melanoma model, we are currently setting-up a phase I clinical trial in lung cancer patients in close collaboration with Ghent University Hospital. Apart from the establishment of a GMP-compliant production system, we also investigated the impact of several vaccine parameters like LNP on the immunogenicity of m1 Ψ -modified mRNA vaccines. This clinical translation process comprised the fine-tuning of the LNP formulation, mRNA construct and administration route where we investigated the impact of these parameters on the reactogenicity and immunogenicity with an emphasis on the induction of robust T cell responses in mice.

mRNA-vaccines and mRNA-Galsomes were formulated with m1 Ψ - mRNA OVA encoding for either fLuc or ovalbumin. The mRNA vaccines were prepared with differently composed LNPs and administered in mice through different routes of administration to evaluate mRNA delivery, tolerability and immunogenicity. Within these experiments, we also investigated whether mRNA vaccines could benefit from the inclusion of the α GC adjuvant. In addition, mRNA vaccines were evaluated on an in vitro human PBMC model to confirm preclinical reactogenicity.

The highest antigen-specific T cell responses were obtained after IM injections of mRNA-LNPs containing α GC with a clear dose-sparing effect exerted by the adjuvant. We also observed clear differences in reactogenicity using different LNP formulations which was also confirmed in an ex vivo human PBMC model.

Taken together, we believe that the mRNA-Galsome platform of α GC-adjuvanted mRNA-LNPs has great potential for cancer vaccination.

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INDUCTION OF NK CELL REACTIVITY AGAINST ACUTE MYELOID LEUKEMIA BY A FC-OPTIMIZED B7-H3 ANTIBODY

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Acute myeloid leukemia (AML) is the most frequent acute leukemia in adults. Although treatment options of AML have improved during the last decade, so far, no anti-tumor antibodies are clinically approved for AML therapy. The crucially important factor for the therapeutic success of monoclonal antibodies (mAbs) in cancer treatment is their ability to mediate antibody-dependent cellular cytotoxicity (ADCC) through natural killer (NK) cells. Recently, the coreceptor of immune-checkpoint B7 family B7-H3 (CD276) has emerged as a promising target for AML immunotherapy, due to its profound expression on leukemic blasts of AML patients.

Here we provide preclinical characterization of our previously developed B7-H3 mAb 8H8 in AML. By introduction of amino acid substitutions (S239D/I332E) in the Fc part, 8H8-SDIE provides enhanced affinity to the activating Fc receptor CD16 on NK cells. We characterized B7-H3 expression and binding of 8H8 on AML cell lines (n=12) and patient samples (n=64) and employed 8H8-SDIE in various in vitro assays

Flow cytometric analyses showed that 8H8-SDIE bound specifically to the target antigen at saturating doses of approximately 1 µg/mL on primary AML patients' samples. Co-cultures of allogeneic peripheral blood mononuclear cells (PBMC) with B7-H3-positive primary AML cells, revealed that B7-H3-SDIE induced significant NK cell activation measured by CD69 and CD25 expression. In line, determination of CD107a upregulation confirmed significant induction of NK cell degranulation by our construct. Additional analysis of the supernatants by LegendPlex showed 8H8-SDIE treatment-induced secretion of immunomodulatory IFN γ and TNF as well as enhanced levels of Granzyme B, Granulysin, and Perforin mediating NK cell effector functions. No effects were observed in the presence of the corresponding iso-SDIE control. Target cell-restricted lysis of AML cell lines and primary AML patients' samples by 8H8-SDIE was potently induced in Europium and FACS-based cytotoxicity assays, while iso-SDIE treatment did not induce lysis of leukemic cells.

Taken together, we here introduce a novel attractive immunotherapeutic compound potently inducing NK cell anti-leukemic reactivity as a beneficial treatment option for AML.

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IMMUNE CHECKPOINT BLOCKADE MITIGATES SYSTEMIC INFLAMMATION AND AFFECTS CELLULAR FLIP-EXPRESSING MYELOID-DERIVED SUPPRESSOR CELLS IN NON-SMALL CELL LUNG CANCER PATIENTS

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Cancer cells favor the generation of myeloid cells with immunosuppressive and inflammatory features, such as myeloid-derived suppressor cells (MDSCs), which can support their growth and progression. The anti-apoptotic molecule, cellular FLICE (FADD-like interleukin-1 β -converting enzyme)-inhibitory protein (c-FLIP), which acts as an important modulator of caspase-8, is required for the development and function of monocytic (M)-MDSCs. This study aimed at assessing the effect of immune checkpoint inhibitor (ICI) therapy on circulating immunological landscape, including FLIP-expressing MDSCs, in non-small cell lung cancer (NSCLC) patients.

Circulating immune cells and plasma concentrations of inflammatory cytokines in NSCLC patients receiving ICI were prospectively collected before the initiation of immunotherapy (T0) and during the first clinical evaluation (T1). The immunosuppressive functions of M-MDSCs were tested in vitro. Changes in the frequencies of immune cells, M-MDSC-associated properties, and cytokine levels were correlated with patients' outcome.

Thirty-four NSCLC patients were enrolled and classified as progressors (P; 18 patients) or non-progressors (NP; 16 patients), according to the Response Evaluation Criteria in Solid Tumors and clinical evaluation. Using t-distributed stochastic neighbor embedding (t-SNE) and cluster analysis, we demonstrated that NP patients at T0 showed an increased amount of monocytes and NK cells, as well as inflammation-associated soluble mediators such as TNF- α , GM-CSF, and IL-2. A reduction in the pro-inflammatory IL-6, IL-8, and IL-1 β was detected in NP patients after ICI treatment. We also identified a reduction of c-FLIP expression in M-MDSCs in NP patients at T1, even though NP and P patients showed the same level of expression at T0. In agreement with the c-FLIP expression, monocytes isolated from both P and NP patients displayed similar immunosuppressive functions at T0; however, this pro-tumor activity was negatively influenced at T1 in the NP patient cohort.

ICIs can mitigate systemic inflammation and impair tumor-dependent immunosuppression in NP NSCLC patients by affecting c-FLIP expression in M-MDSCs.

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MODI2, A VACCINE TARGETING HOMOCITRULLINATED SELF-EPITOPES, STIMULATES POTENT CD4-MEDIATED ANTI-TUMOUR RESPONSES AS A THERAPY FOR SOLID CANCERS.

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The tumour microenvironment (TME) is subject to stressful conditions such as nutrient deprivation, genotoxic stress, and hypoxia. These conditions force cancer cells to undergo autophagy where cellular proteins are targeted for degradation as a mechanism to generate energy and promote cell survival. Stresses within the tumour microenvironment can also mediate post-translational modifications (PTM) of self-proteins. Homocitrullination is the conversion of lysine residues to homocitrulline which can generate neoepitopes and bypass self-tolerance.

We use mouse models to characterise immune responses by cytokine ELISpot assay to vaccination with four homocitrullinated self-peptides and assess responses for tumour therapy in murine models of melanoma, breast cancer and colorectal cancer. We also assess expression of the self-antigens in human tumour microarrays by immunohistochemistry and the available CD4 repertoire to the homocitrullinated self-peptides by culture and cytokine ELISpot assay in PBMCs of healthy humans.

We demonstrate that the presentation of homocitrullinated self-peptides on MHCII to CD4 T cells can stimulate strong Th1 responses which can be generated by vaccination and subsequently can mediate tumour therapy in mouse models of melanoma, breast cancer and colorectal cancer. Healthy humans also show a repertoire of CD4 T cells able to respond to homocitrullinated self-peptides suggesting repertoires are not deleted and human lung, colorectal, breast and pancreatic tumours are shown to express the self-antigens that are targets of the Modi2 vaccine. Despite this, peptides that stimulate strong T cell responses are often hydrophobic and have complications with large scale manufacture and solubility. Here we demonstrate the use of a self-assembling nanoparticle technology ("SNAPvax") to co-deliver four homocitrullinated peptides and adjuvant in nanoparticles of a precise size and composition that is optimized for manufacturing ease and T cell induction. This formulation ("Modi2") provided improved peptide solubility and sterile filtration in aqueous buffer, simplifying key process steps, and was shown to stimulate strong Th1 responses and tumour clearance in mouse models.

We propose the Modi2 SNAPvax vaccine formulation has potential for translation into clinic in several cancer indications.

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IDENTIFICATION OF FACTORS INVOLVED IN DOUBLE STRANDED RNA-INDUCED CELL DEATH THROUGH GENOME-WIDE CRISPR SCREENS

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Double-stranded RNAs (dsRNAs) are potent immunostimulatory nucleic acids of viral origin but also physiologically produced by mammalian cells. Increased levels of dsRNAs, due to viral infection or failure of endogenous repression, trigger an immune response that leads to cytokines production or cell death. Recent studies demonstrated that several cancer types already contain sufficient quantities of dsRNA to activate immune responses. Moreover, some clinical trials proved the efficacy of Polyinosinic-polycytidylic acid (pl:C), a synthetic analog of dsRNA, as adjuvant treatment in breast cancer patients, in terms of overall survival and longer relapse-free survival. The 2',5'-oligoadenylate synthetase (OAS)-RNASEL system is widely thought to be the main responsible for dsRNA-induced cell death, through mechanisms not fully understood.

To elucidate these mechanisms, we employed candidate-based and genome-wide genetic approaches. As death-inducing stimulus we used the combination of pl:C, and Interferon- α (IFN- α), which upregulates OAS enzymes thus maximizing the efficiency of dsRNA sensing in IFN-saturating condition. As model systems we used both the A549 (highly responsive to pl:C) and HT29 (low-responsive) cell lines.

RNASEL-KO cells were, as expected, refractory to IFN- α +pl:C induced cell death; interestingly however, interfering with protein synthesis or RNA transcription with Cycloheximide (CHX) or Actinomycin D (ActD), caused RNASEL-KO cells to undergo apoptosis, although CHX/ActD alone had no cytotoxic effect. This finding suggests that the activation of RNaseL-independent systems is different from those activated by canonical dsRNA receptors. Transient genetic ablation through siRNA showed that this novel death-inducing pathway is independent of other canonical dsRNA sensors such as TLR3, RIG-I, MDA5 and PKR. We then performed a Genome-wide clustered regularly interspaced short palindromic repeats (CRISPR)-based screening and integrative analysis to prioritize gene targets that favour/inhibit dsRNA-dependent cell death. The screen revealed that RNA surveillance, RNA Polymerase II Transcription Initiation, Mitochondrial translation and DNA Repair genes have a protective role against dsRNA increase. Given the involvement of these hits in replication interference recovery and RNA:DNA hybrids removal, we hypothesize the possibility of dsRNA-dependent RNA:DNA hybrids formation, causative of replication stress, DNA damage and cell death. Moreover, among multiple candidates with synergistic/inhibitory role with dsRNA, we validated BRCA1, commonly mutated in hereditary forms of breast, ovarian and several other cancers, as a potential translationally relevant hit. In addition, the immunofluorescent staining of gamma-H2AX, used for the quantification of DNA double-strand breaks, revealed that IFN- α +pl:C treatment induces a significant increase of DNA double-strand breaks compared to control conditions.

In conclusion, we reveal the existence of a RNASEL-independent mechanism of cell death, which at least partially relies on the activity of BRCA1. These findings may pave the way for the use of dsRNA-inducing therapy for synthetic lethality in the context of BRCA1-mutated tumors.

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TARGETING INTERLEUKIN-1B TO IMPROVE CHEMOIMMUNOTHERAPY IN LUNG CANCER

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Anti-PD-1/PD-L1 immunotherapy to reactivate CD8 T-cell function has revolutionised the treatment of metastatic non-small cell lung cancer. Despite this efficacy, most patients have intrinsic resistance to these checkpoint inhibitors, notably due to a low level of immune infiltration. In order to find new avenues for combating resistance to immunotherapy, we investigated the involvement of IL-1 β in the response to chemoimmunotherapy. IL-1 β , whose production can be induced by the inflammasome pathway and caspase-1 activation, is a cytokine that can be either beneficial or deleterious, depending on the cancer models and cytotoxic agents used.

In the context of lung cancer, IL-1 β appears to be pro-tumour in the absence of treatment. Several pre-clinical studies have shown that IL-1 β promotes the proliferation of cancer cells, angiogenesis and the recruitment of type 2 macrophages to the tumour. These observations were reinforced by the CANTOS (Canakinumab Antiinflammatory Thrombosis Outcome Study) study (Novartis Pharmaceuticals, 2020). In this study including 10,061 patients with cardiovascular disease, treatment with canakinumab, an antibody targeting IL-1 β , reduced the incidence of lung cancer as well as its mortality. Data from these studies showed that blocking IL-1 β could be a preventive strategy for high-risk individuals. However, no studies have investigated the role of IL-1 β in the efficacy of standard treatments, namely a combination of chemotherapy and immunotherapy.

The aim of this project is to study the role of IL-1 β in the response to chemoimmunotherapy, with a view to target this interleukin and improving the response to treatment in lung cancer.

We used the LLC1 (Lewis Lung carcinoma cell line) model with an activating mutation of KRAS and overactivation of the MAP kinase pathway. The treatment used for this project was a doublet of cisplatin/pemetrexed chemotherapy in combination with a MEK inhibitor, trametinib. An anti-PD1 was also added to the treatment for the in vivo experiments. The expression of CXCL10 (a chemokine that enables the recruitment of CD8 T cells) in vitro as well as the monitoring of tumor growth and gene expression in vivo were studied using various Crispr-Cas9-modified cells (NLRP3, AIM2, Caspase-1, IL-1 β , IL1R1 and CXCL10). In addition, in vitro and in vivo experiments were carried out on modified cancer cells overexpressing IL-1 β or by adding recombinant IL-1 β .

Our in vitro and in vivo results show a beneficial effect of IL-1 β in the response to cisplatin/pemetrexed/anti-PD-1 treatment in combination with a MEK inhibitor, trametinib. Indeed, the combination of treatments induces IL-1 β production by cancer cells following the formation of an inflammasome and caspase-1 activation. In this model, IL-1 β acts in an autocrine manner on cancer cells and induces the production of CXCL10, a chemokine that enables the recruitment of CD8 T cells at the tumour site thanks to the CXCR3 receptor present on the surface of these immune cells.

In conclusion, our results show that IL-1 β is able to sensitize a tumor resistant to chemoimmunotherapy and consequently to improve its efficacy.

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MTFF2-MSA (MTNX-1700) SUPPRESSES TUMOR GROWTH AND INCREASES SURVIVAL IN ANTI-PD-1 TREATED CT26.WT SUBCUTANEOUS AND CT26-LUCIFERASE ORTHOTOPIC SYNGENEIC COLORECTAL CANCER MODELS BY TARGETING MDSCS IN BALB/C MICE

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Myeloid-derived suppressor cells (MDSCs) in the tumor microenvironment are potential therapeutic targets in immune checkpoint cancer therapy, particularly for cancers that are unresponsive to anti-PD-1 therapy. It has previously been demonstrated that trefoil factor family 2 (TFF2), a secreted anti-inflammatory peptide, can partially suppress MDSC expansion and partially activate tumor immunity through agonism of the CXCR4 receptor. We investigated whether a novel fusion protein, murine TFF2-murine serum albumin (mTFF2-MSA), has single-agent activity and can improve the therapeutic effects of anti-PD-1 in CT26.wt subcutaneous and CT26-Luciferase (CT26-Luc) orthotopic syngeneic mouse models of advanced colorectal cancer (CRC).

Two syngeneic colon carcinoma mouse models were developed using the CT26.wt and CT26-Luc CRC cell lines grafted subcutaneously and orthotopically, respectively, into BALB/C mice. We generated a recombinant fusion protein, designated mTFF2-MSA, which contains murine TFF2 fused to murine serum albumin (MSA), for the purpose of increasing half-life and reducing the frequency of dosing. Mice subsequently received mTFF2-MSA, anti-PD-1 antibody (clone 29F.1A12 for subcutaneous study; clone RMP-1-14 for orthotopic study) or a combination of mTFF2-MSA and anti-PD-1. Tumor volume and survival were measured. At the endpoint, flow cytometry was performed on the blood, bone marrow, tumor, and lymph nodes, to examine treatment-induced effects on cellular immune profiles.

In the CT26.wt model, tumor growth was suppressed by mTFF2-MSA, anti-PD-1 and by the combination of mTFF2-MSA and anti-PD-1 by 16%, 40% and 60%, respectively. Survival in the CT26.wt model on Day 30 treated with vehicle, mTFF2-MSA, anti-PD1 and the combination of mTFF2-MSA and anti-PD-1 was 0%, 40%, 60% and 60%, respectively. In the CT26-Luc model, mTFF2-MSA, anti-PD-1, and the combination of mTFF2-MSA and anti-PD-1 suppressed tumor growth by 42%, 94%, and 94%, respectively. In the CT26-Luc model, neutrophils were significantly reduced in the blood in all treatment groups by flow cytometry. In the bone marrow, a significant reduction in total macrophages, M2 macrophages, and neutrophils was also observed but only in the group treated with anti-PD-1 and mTFF2-MSA. In the axillary lymph node, there was a significant reduction in TOX+ cells in both CD4+ and CD8+ T-cells in all treatment groups. In the tumor, there was a significant reduction in total macrophages and M2 macrophages in all treatment groups, while NK cells were also increased, but only in the combination anti-PD-1/mTFF2-MSA treated group.

mTFF2-MSA has single agent activity and is additive to anti-PD-1 antibody checkpoint inhibition in treating two syngeneic (subcutaneous and orthotopic) mouse models of advanced CRC.

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PRELIMINARY REPORT OF CARPETS PHASE 1 CLINICAL TRIAL OF GD2-CAR-T CELL THERAPY IN PATIENTS WITH GD2-POSITIVE MALIGNANCIES

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GD2 is a validated cancer immunotherapy target. GD2 is a disialoganglioside and mainly expressed by tumors of neuroectodermal origin: neuroblastoma, small cell lung cancer, and melanoma. A growing number of reports indicate that GD2 expression also in sarcomas, triple negative breast cancer, non-small cell lung cancer, adult glioblastoma, and paediatric gliomas.

GD2 is highly restricted in its normal tissue expression but is expressed at low levels on CNS neurons, peripheral nerves, skin melanocytes, and mesenchymal stromal cells.

CARPETS is a phase 1 study of the safety and immune effects of an escalating dose autologous GD2 chimeric antigen receptor-expressing peripheral blood T cells in patients with GD2-positive metastatic melanoma and refractory solid tumours. The co-primary objectives were CAR-T cell manufacturing feasibility and safety. The secondary objectives were CAR T cell persistence, documented tumour responses, and assessment of bystander immunity.

The GD2-CAR-T cells expressed a third generation CAR comprising the 14g2a GD2-binding ectodomain fused to the CD28 transmembrane domain and containing CD28, OX40, and CD3zeta intracellular signaling domains. The GMP-manufactured replication-incompetent murine gamma-retroviral vector included upstream of the CAR, an inducible caspase-9 suicide gene.

In the first 6 patients, the original manufacturing protocol led to poor in vivo persistence and activation-induced T-cell death (AICD).

Hence, we modified the protocol to include Miltenyi's Transact Activation Reagent and TexMACS Media along with expansion in IL-7 and IL-15 and the G-rex chamber. We obtained more central memory CAR-T cells and reduced AICD.

The CARPETS trial was closed to recruitment after enrolment of 12 patients with GD2-positive malignancies. Co-primary objectives of CAR-T cell manufacturing feasibility and safety were met. Fifteen CAR-T cell products were manufactured, 14 products administered, with one product failing batch release criteria because of bacterial contamination with a skin commensal. Fourteen infusions were administered safely with grade 1 adverse events attributable to infusions e.g., bad taste of D5W and fever.

Of the secondary objectives, data will be shown for all subjects concerning CAR-T cell persistence by DNA quantitative PCR and flow cytometry of the anti-idiotype-detected CAR molecule together with serum cytokine measurements. Although tumor responses were not apparent by RECISTv1.1, some correlative science data of interest will be shown. Bystander immunity has not yet been investigated.

In addition, clinical and laboratory data will be presented for the GD2 immunohistochemistry that was used for patient selection, and such patient-specific characteristics of administered CAR-T cell products as CAR surface expression, fold-expansion of CAR-T cells, CD3 purity at the end of cellular expansion, CD4: CD8 ratios, and T-cell memory and 'exhaustion' phenotypes.

We recruited seven patients with melanoma, four with colorectal cancer, and one with sarcoma. The clinical data showed GD2-CAR-T cell manufacturing for several solid tumour indications was feasible

and CAR-T cell administration was safe. Although there was no evidence of tumour shrinkage attributable to the CAR-T cell therapy. Unpublished laboratory data indicate evidence of target engagement, and CAR-T cell expansion and persistence. Our preliminary conclusions include:

- 1) That lymphodepleting chemotherapy seemed to be required for CAR-T expansion in vivo.
- 2) The altered ex vivo manufacturing process half-way through the trial seemed to result in higher levels of circulating CAR-T cells.
- 3) Concurrent BRAF/MEK inhibitor therapy in melanoma patients were not sufficient to observe positive effects on in vivo CAR-T cell characteristics such as expansion, persistence, and immunophenotype that had been previously hypothesised to result after oncogenic protein blockade-mediated the tumor ingress of T cells.

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THE ADAPTABLE AFFINITY DRUG CONJUGATE (ADACTM) TARGETING CD40 FACILITATE MODULAR NEOANTIGEN PEPTIDE DELIVERY THROUGH AFFINITY-BASED DRUG CARGO DELIVERY

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Agonistic anti-CD40 therapy has shown promising results in pre-clinical models but the narrow therapeutic window upon intravenous (i.v) administration limits its use. Additionally, monoclonal agonistic CD40 therapies depend on antigen presentation, in addition to its inherent immune stimulation capacity, for efficient tumor-specific T cell activation. We have developed an Adaptable Drug Affinity Conjugate (ADAC) that simultaneously facilitates co-delivery of CD40 stimulation and antigen delivery to enable subcutaneous drug administration and local immune activation. ADAC is based on a novel agonistic CD40 antibody equipped with scFvs, which facilitates non-covalent binding through high-affinity to a peptide tag (pTag). Synthetic long peptide (SLP) cargos including the pTag allow for rapid cargo loading without the need for complex conjugation chemistry or purification methods.

The phage-display selected novel agonistic CD40 antibody was evaluated for agonistic activity in vitro using cultures of human primary B cells and monocyte-derived dendritic cells (moDCs). After conversion into the bispecific format, we assessed the ability to induce both CD8+ and CD4+ T cell proliferation in vivo by using the model SLPs, gp10020-39 (CD8), OVA252-264A6K (CD8) and OVA323-339 (CD4). Lastly, the anti-tumor efficacy was evaluated in the TC-1 model using the clinically evaluated selicrelumab as a benchmark.

Our novel STRIKE-1001 CD40 antibody had high agonistic activity in the IgG2 format with an affinity of 2nM compared to selicrelumab 0.4nM and did not block the CD40L interaction to Fc-CD40. Converting the monoclonal STRIKE-1001 into the ADAC format (STRIKE-2001) did not impact its agonistic activity. Further, STRIKE-2001 induced antigen-specific proliferation of CD8+ (10-15 fold) and CD4+ T cells (2-7 fold) in draining lymph nodes compared to non-linked cargo. Additionally, dual delivery of CD8 and CD4 epitopes significantly enhanced the CD8 T cell activation and proliferation in vivo. While a significant tumor growth reduction, compared to control, was confirmed with peptide-cargo loaded STRIKE-2001 by subcutaneous (s.c) delivery at the non-tumor site in the TC-1 model. Additionally, the benchmark selicrelumab delivered i.v. with a 3x higher dose of did not control tumor growth. Lower toxicity was noted with s.c. administration of STRIKE-2001 compared to systemic delivery of selicrelumab. This favorable toxicity profile may be linked to the relative slow systemic release upon s.c administration. Pharmacokinetic data of STRIKE2001 demonstrate a bioavailability of approx. 60% and a Cmax of 12.7ug/ml peaking at 24-48 h after s.c. administration compared to i.v administration reaching Cmax of 136ug/ml after 30 min.

Thus, that local administration together with co-delivery of the antigen with the agonistic effect of the CD40 antibody in the ADAC format can reduce toxicity and rely on local lymph node engagement to prime tumor-specific T cells. The developed ADAC drug candidate, STRIKE-2001, is in pre-clinical development and is envisioned to allow for tailored neoantigen delivery for individualized cancer vaccine strategies in the clinic.

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INTRADERMAL VACCINATION WITH A LEUKEMIC CELL BASED CANCER VACCINE INDUCES FUNCTIONAL CD8 T-CELL RESPONSES TO COMMON TUMOR ANTIGENS IN PATIENTS WITH BLOOD-BORNE AND SOLID TUMORS

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Priming the immune system to respond to tumor associated antigens (TAA) is challenging, due to the fact that these are self-antigens and poorly immunogenic. However, due to the overexpression of these antigens in a wide variety of cancer types, they are highly attractive targets for cancer vaccine trials. Many of these vaccine trials have either used peptides or proteins, in combination with adjuvants, viral vectors or have been administered as DNA vaccines, and they have shown the possibility to induce T cell responses toward these TAA's.

Vididencel is a leukemic cell derived cancer vaccine, which expresses a variety of tumor associated antigens (TAA) such as WT1, PRAME, RHAME and Mucin, which all are frequently upregulated in blood-borne and solid tumors. Vididencel is currently investigated in clinical trials in AML patients (NCT03697707), which are in complete remission, but have measurable residual disease, as well as in Ovarian cancer patients (NCT04739527), with high grade serous ovarian cancer, after debulking surgery and chemotherapy, without residual disease by CT-scan.

Patients in these two studies were all given 4 biweekly priming doses of vididencel, followed by 2 booster doses at week 14 and 18. Peripheral blood mononuclear cells (PBMC) for T-cell response analysis were taken before start of vaccination and at several timepoints thereafter (AML study week 6, 11, 18, 20 and 32 and ovarian study at week 4, 10, 14, 18 and 22), ficoll isolated and stored in LN2 until use. Data was available for analysis from 20 AML patients and 7 ovarian cancer patients. Assessment of the functional T cell responses was done using the golden standard, human IFN γ ELISPOT using 15-mer overlapping peptide pools to WT1 and PRAME for all patients, additionally RHAMM was assessed in AML patients and NY-ESO1 and MAGEA3/A4 in ovarian cancer patients. PBMC were stimulated for several days with the respective peptide pools or controls and restimulated for the IFN γ ELISPOT. Vaccine induced response were defined as at least a 2-fold increase in spot number over baseline responses.

Vaccine-induced responses (VIR) were observed in 17/20 AML patients, against WT1, PRAME or RHAMM, and in 6/7 evaluable ovarian cancer patients, against WT1, PRAME, NY-ESO or MAGEA3/A4. PRAME responses were observed early after the first 4 doses, with already VIRs measurable at week 4, increasing to week 6. WT1 responses observed, were lower, and increased after the 4th dose. Additional IFN γ ELISPOT performed in AML patients also showed VIR to RHAMM, especially during and after the booster doses. Although NY-ESO1 is not expressed by vididencel, during and after booster doses, NY-ESO1 responses were detected in ovarian cancer patients.

In the AML patients the number of observed VIRs correlate with clinical responses, with the highest number of VIRs in patients who had a reduction or clearance of their residual disease (data presented atEHA2023, ASH2022). Data in the ovarian cancer study is still too preliminary to correlate it with clinical outcome.

Induction of functional T cell response to TAAs is shown in patients with AML and Ovarian cancer, indicative for the possibility to prime and boost these T cells against TAA's expressed by vididencel in patients. Although the number of IFN γ secreting T-cells to PRAME and WT1 was lower in the AML patients, the kinetic of the T-cell responses was comparable in both patient cohorts. Additionally, despite NY-ESO1 not being expressed by vididencel, an effective T cell responses was measured. This could indicate that due to tumor cell lysis, additional highly expressed antigens might be better

presented to the immune system, inducing a T cell response.

In conclusion: vididencel is a cell based vaccine able to induce a plethora of tumor associated antigen responses in both leukemic and solid tumor patients.

P224

THE OBESITY PARADOX IN MELANOMA IMMUNOTHERAPY: UNDERSTANDING MOLECULAR MECHANISMS IN THE MODULATION OF THE TUMORAL MICROENVIRONMENT BY FATTY ACIDS

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Obesity is a complex medical condition and is usually considered a factor for increased risk and worse outcome of several diseases, including cancer. However, recent studies of immune checkpoint inhibitor (ICI) immunotherapy have shown that obese melanoma patients were surprisingly associated with better overall survival. This paradox could have several explanations, but one possible hypothesis is that obesity-related changes in the tumor microenvironment (TME) directly affect the efficacy of ICI therapy.

To elucidate the mechanisms of the obesity paradox, dietary changes and different ICI therapies (anti-PD1, anti-CTLA4 and the combination of anti-PD1/anti-CTLA4) should be further investigated, with a focus on T cell exhaustion mechanisms, which are key in determining the outcome of antitumor responses and sensitivity to ICIs. Furthermore, the effect of obesity on other cells involved in the TME, such as antigen presenting cells (APC), is still poorly understood. These cells modulate T cell responses, acting as co-stimulators or co-inhibitors. Many studies have investigated the role of myeloid cells in systemic obesity-associated inflammation, but not specifically in the context of immunotherapy against melanoma.

Our preliminary results suggest that tumor growth in obese mice was increased and partially slowed by ICI therapy. At the same time, MC38 cells showed distinct IFN γ -dependent expression profiles, mainly involving metabolic changes, which need to be further investigated in the context of the obese phenotype.

Our studies aim to dissect the molecular mechanisms regulating the role of obesity in the context of ICI therapy to potentially identify novel biomarkers and pharmacologically-tunable pathways against melanoma.

P225

PHARMACOLOGICAL TARGETING OF IL-1 SIGNALING DISRUPTS CANCER-INDUCED SYSTEMIC IMMUNOSUPPRESSION AND IMPROVES THERAPEUTIC VACCINE RESPONSE IN HPV16-INDUCED CANCER

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We are studying a genetically-engineered mouse model (GEMM) of HPV16-induced cervical cancer replicating key features of advanced cervical cancer patients, including an abundance of “myeloid-derived suppressor cells” (MDSC) in the periphery and resistance to immunotherapies. We previously reported that such myeloid cells, particularly neutrophils, orchestrate systemic immunosuppression (SIS) resulting in impaired cytotoxic T cell/dendritic cell functions and associated failure of therapeutic vaccination (Galliverti et al., Cancer Immunol Res. 2018, Galliverti et al., Cancer Immunol Res. 2020). Our objective is to dissect the molecular and cellular mechanisms leading to the differentiation, expansion, and accumulation of immunosuppressive neutrophil populations both in the periphery and the tumor microenvironment (TME). Our strategic goal is to identify novel SIS-disrupting therapeutic approaches that enable efficacious anti-tumor immunity in the context of a nanoparticle-based E7 long peptide (NP-E7LP) vaccine that otherwise has no efficacy.

We hypothesized that soluble factors produced within the TME are transmitted systemically and act at a distance on myeloid cell reservoirs, thereby inducing SIS. Therefore, we performed a proteomic analysis of the blood of tumor-bearing mice and employed single-cell RNA sequencing of key organs involved in SIS to investigate the underlying signaling mechanisms. We identified six different subsets of neutrophils, ranging from progenitor cells to 'immunosuppressive' subtypes with 'MDSC-like' features. Interestingly, neutrophils from the periphery differed from those found in the TME, where we identified a tumor-specific N5 subset recently described as pro-tumoral in lung cancer. By integrating the proteomic and transcriptomic data, we identified soluble factors produced in the TME, with matched receptors expressed on neutrophils in the periphery.

In parallel, we derived cancer cell lines from spontaneous squamous cell carcinoma arising in the HPV16 GEMM that exhibited distinctive characteristics once injected subcutaneously in control mice. One cell line, H16sc-IS1, fully recapitulated the phenotype observed in our GEMM, whereas the others (e.g., 'H16sc-ImP') did not. We reasoned that key soluble factors would be shared by de novo and H16sc-IS1 tumors but not others lacking associated SIS, allowing us to refine our list of candidates. Notably, three cytokines from the IL-1 cytokine superfamily (IL-1 α , IL-33, and IL-36 β) linked to poor prognosis in patients caught our attention.

We therefore systemically administered these cytokines to control mice, using G-CSF as a positive control. Co-injections of IL-1 α , IL-33, and IL-36 β without G-CSF induced an expansion of suppressive myeloid cells, whereas G-CSF alone did not. When mice bearing H16sc-ImP tumors that do not induce SIS were similarly treated, suppressive neutrophils were expanded in bone marrow and spleen as well as in the TME, which is otherwise devoid of neutrophils. Notably, treatment with these cytokines dampened the NP-E7LP vaccine response in H16sc-ImP-tumor-bearing mice.

We next performed pharmacological blockade of IL-1 α , IL-33, and IL-36 β using an anti-IL1RAP monoclonal antibody, targeting their shared co-receptor. IL1RAP was primarily expressed by neutrophils, both in the periphery and TME. Anti-IL1RAP monotherapy resulted in reduced tumor

growth in H16sc-IS1-tumor-bearing mice, with decreased abundance of immunosuppressive neutrophils in the periphery and evident reprogramming of those in the TME. Importantly, anti-IL1RAP treatment improved NP-E7LP vaccine response in H16sc-IS1-tumor-bearing mice, concomitant with the induction of tumor-specific CD8 T cell responses.

In summary, we have implicated the IL-1 signaling pathway in cancer-induced SIS, which can be targeted pharmacologically to improve response to therapeutic vaccination. These results may be of relevance to advanced cervical cancer patients with demonstrable SIS.

P226

PERSONALIZED ADJUVANT DENDRITIC CELLS VACCINATION IN MELANOMA PATIENTS: INSIGHTS INTO IMMUNOLOGICAL LANDSCAPE

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In the last 20 years we have treated more than 80 advanced melanoma patients (pts) with a tumor lysate-loaded autologous DC vaccine, observing a clinical benefit of 54.1% and an ORR of 63.6% to subsequent chemotherapy. Additionally, the analysis of post-vaccine biopsies highlighted the vaccine ability to induce intratumoral CD8 up-regulation ($p=0.0195$). These data suggest a potential improvement of the activity of sequential chemotherapy and a greater efficacy in preventing relapse in pts with minimal residual disease status.

We designed a randomized phase II trial (EudraCT no. 2014-005123-27) to provide a complementary autologous DC vaccination to pts with resected stage III/IV melanoma, with the aim to: a) evaluate whether specific immunization against tumour cells improves relapse free survival (RFS) compared to the standard follow-up; b) define biomarkers of DC vaccine clinical activity. Overall, from 2015 to 2019 a total of 18 eligible pts were included in this study, 10 of whom received 6 monthly DC vaccination cycles combined with IL2 administration (arm A), and 8 pts were included in the observational cohort (arm B).

Patients receiving DC vaccination have shown a better RFS compared to the observational cohort (median 6.6 months (arm A) vs 5.2 months (arm B), ns). Intriguingly, favorable trends in arm A, even if not significant, were observed for female pts (median 15.5 months (female) vs 3.3 (male), pts with less than 60 years (median 22.5 months (age < 60) vs 4.7 months (age ≥ 60), and with wild-type BRAF status (median 22.5 months (BRAF WT) vs 3.8 months (BRAF mut).

Patient's HLA class I and II (loci A-B-C-DR and DQA/DQB) were characterized by means of sequence-specific oligonucleotide probe reverse hybridization method. We found that not relapsed (NR) arm A pts ($n=3$) presented a maximal heterozygosity at HLA class I loci (HLA-A, HLA-B, or HLA-C), carried an HLA-A*02 profile and two of them had the HLA-B*35 supertype, already described to be correlated with favorable clinical outcomes.

The immunological response against selected tumour-associated antigens (Melan-A, PMEL, Tyrosinase, MAGE-A3, NY-ESO1 and survivin) was evaluated on arm A pts at baseline, after the fourth vaccine (VAX4) and at the end of treatment (EOT) applying IFN- γ ELISPOT assay. An increased number of specific anti-survivin and anti-NY-ESO1 spot forming cells (SFCs) was observed along treatment. In particular, the average number of SFCs/5x10⁵ PBMCs was 68, 68 and 103 for survivin and 0, 3 and 12 for NY-ESO1 for the baseline, VAX4 and EOT timepoint, respectively. A cytokine profiling has been performed on patient's plasma samples by means of multiplex ELISA. We found a significant increment of cytokine levels in EOT compared to baseline samples. In particular, in NR pts we observed an increase of INF α ($p=0.038$) and IL-8 ($p=0.030$), while in relapsed (R) pts we found a significant increment of IL-4 ($p=0.009$) and IL-9 ($p=0.032$). Conversely, no significant modulation of circulating cytokines was observed in arm B during the follow-up.

At the periphery we found a prominent increment of the percentage of total lymphocytes after DC vaccination (R $p=0.008$, NR $p=ns$), a consistent increase of the Lymphocyte to Monocyte Ratio (R $p=0.034$, NR $p=ns$), and a decrease of the Neutrophil to Lymphocyte Ratio (R $p=0.040$, NR $p=ns$). At the tumor site we found an increased number of intratumoral CD8+ T cells in the tissue biopsies

collected before vaccination in NR (3958 ± 2003 positive cells/mm²) compared to R ($1925 \pm 867,7$ positive cells/mm²) pts.

Overall our data show the safety profile of DC vaccination as adjuvant treatment in Melanoma pts and highlight peculiar traits of immunomodulation, especially in R pts, that will be further explored with multiparametric flow cytometry to better understand the contribution of individual immune subpopulations, and to guide the design of new immunotherapy combinations as upfront therapeutic strategies.

P227

STRIKE2001-KRAS, A NOVEL CD40-TARGETED AGONISTIC ANTIBODY CARRYING KRAS PEPTIDE CARGO VIA A MODULAR DRUG CARGO LOADING PRINCIPLE (ADAC™) LEADING TO DUAL AGONISM AND ANTIGEN-PRESENTATION IN VIVO.

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Agonistic CD40 targeting antibodies have demonstrated promising pre-clinical efficacy and success in various cancer models. However, clinical studies have revealed moderate responses for anti-CD40 monotherapy, likely due to the narrow therapeutic window as a result of the administration route e.g. infusion. To improve efficacy and reduce toxicity, the bivalent anti-CD40 agonist named STRIKE2001 was developed. STRIKE2001 combines immune stimulation and antigen delivery, resulting in efficient T cell activation, a crucial component lacking in current clinical trials.

STRIKE2001 is a humanized IgG2 antibody comprising two peptide-binding single chain variable fragments (scFv) linked to the CH3 domain and expressed as a recombinant protein. These scFv bind to a short non-immunogenic peptide tag (pTag). Mutated KRAS peptides are then synthesized by solid-phase peptide synthesis along with the pTag, creating a flexible tumor antigen delivery system based on the Adaptable Drug Affinity Conjugate (ADAC) platform. The final product, STRIKE2001-KRAS, aims to treat patients with KRAS mutated cancer such as non-small cell lung cancer (NSCLC), colorectal cancer and pancreatic cancer.

The development of STRIKE2001-KRAS has been successful, initial process development shows that STRIKE2001 is stable, processable and yields high expression (5.8 g/L). Species justification demonstrate that STRIKE2001 binds both human and cynomolgus CD40. Furthermore, selectivity screening of >6000 human proteins indicates no unspecific interaction or off-target binding by STRIKE2001. In addition to its favorable developability, STRIKE2001-KRAS extends the in vivo half-life of the KRAS peptides from minutes to 4.5 hours, as shown by PK study performed in hCD40 transgenic mice, improving peptide cargo exposure and reducing variability in immune responses postulated to be linked to peptide stability and lymph node exposure. Additionally, the selected pTag-KRAS peptides are able to induce KRAS-specific T cell responses in humanized HLA transgenic mice, confirming that the addition of the pTag to the KRAS peptides does not affect the processing and presentation of the KRAS peptides, and this was further confirmed for other human model antigens in vitro. Moreover, the pTag itself did not induce any immune responses in vivo or in long-term PBMC cultures.

These results confirm that STRIKE2001-KRAS is a highly effective antigen delivery system.

P228

EFFICACY AND PHARMACODYNAMIC BIOMARKERS OF MITAZALIMAB IN COMBINATION WITH CHEMOTHERAPY IN PRECLINICAL MOUSE MODELS

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Mitazalimab is a CD40 targeting agonistic monoclonal IgG1 antibody. Targeting CD40 with mitazalimab in combination with chemotherapy induces anti-tumor effects both by licensing dendritic cells to activate tumor-specific T cells, and by triggering macrophage polarization. Subsequently, macrophages degrade the extracellular matrix in the tumor stroma, increasing the sensitivity to chemotherapeutic agents, and favoring T-cell influx. Currently, mitazalimab is being evaluated in a Phase 1b/2 trial (NCT04888312) in combination with mFOLFIRINOX. The objective of the present study was to assess the anti-tumor efficacy of mitazalimab and FOLFIRINOX in a mouse pre-clinical model, and to investigate pharmacodynamic biomarkers in peripheral blood, induced at early time points after treatment.

The ability of mitazalimab to augment the response to chemotherapy was demonstrated in human CD40 transgenic (hCD40tg) mice bearing MB49 syngeneic tumors. hCD40tg mice were subjected to a dosage regimen of three weekly cycles, containing either vehicle (dextrose), mitazalimab, FOLFIRINOX (oxaliplatin, irinotecan, 5-fluorouracil, and folinic acid), or mitazalimab and FOLFIRINOX in combination. Repeated administration of mitazalimab together with FOLFIRINOX induced long-term survival, and tumor volume control.

Peripheral blood was collected from the mice at the end of the first cycle and subjected to RNA-sequencing to identify pharmacodynamic biomarkers. Exploratory data analysis confirmed that samples clustered according to treatment regimen, where the groups containing mitazalimab displayed the highest transcriptomic similarity. Differential gene expression analysis revealed 3356 differentially expressed genes between the groups, and 710 differentially expressed genes between the two mitazalimab containing groups. In addition, pathway enrichment and gene set variation analyses highlighted examples of transcriptional activity induced by immune- and chemo-therapeutic agents. Mitazalimab, induced high expression of genes involved in response to type-1 IFN as well as extracellular matrix organization. On the other hand, FOLFIRINOX boosted pyrimidine metabolism; a process that is associated with 5- fluorouracil.

In conclusion, mitazalimab synergizes effectively with FOLFIRINOX, inducing long-term survival in a preclinical tumor model. The pharmacodynamic biomarkers of mitazalimab identified here are in agreement with the data from the phase 1 study of mitazalimab in patients with advanced stage tumors (NCT02829099). Together, these data support the ongoing clinical phase 1b/2 study (NCT04888312) of mitazalimab in combination with mFOLFIRINOX.

P229

PROGRESSION TO MYELOMA REFLECTS ACCELERATING IMMUNOSENESCENCE, LOSS OF TUMOUR MHC CLASS I EXPRESSION, AND TUMOUR GROWTH-ASSOCIATED INFLAMMATION

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Multiple Myeloma (MM) is a haematological malignancy characterized by the accumulation of malignant plasma cells in the bone marrow (BM). MM is preceded by two pre-cancerous precursor conditions with accelerating risk of progression: Monoclonal Gammopathy of Undetermined Significance (MGUS, ~7% progress in 5 years) and Smouldering MM (SMM, ~50%). However, current risk models are unable to identifying patients at imminent risk of progression. Tumour genetic features are similar between SMM and MM, suggesting progression is driven by tumour-extrinsic factors in the BM immune microenvironment.

MM has one of the highest mutational burdens among haematological malignancies and several reports describe in vitro T cell anti-tumour activity. However, the BM in MM does not accumulate exhausted T cells¹, suggesting unknown patterns of T cell differentiation within the tumour-reactive compartment. We set out to characterise evolving immune processes during the progression of MM.

1. L. Zheng et al., Science 374, eabe6474 (2021).

We generated a dataset of published and newly-generated BM single-cell RNA sequencing (scRNAseq) composed of >914,000 cells from patients with MM (n=46), MGUS (n=19) and SMM (n=28) and non-cancer controls (n=58), some with single-cell TCR sequencing. scvi was used for integration. Regression and principal component analysis were used for differential abundance. Tumour cells were identified via their unique combination of immunoglobulin genes. Local tumour infiltration was assayed using flow cytometry (BM aspirate % CD138+ cells).

We characterise two immune profiles (immunotypes), composed of immune cells co-associated in abundance.

The first immunotype reflected aging-like differentiation, including a loss of naive T and B and haematopoietic progenitors, and an enrichment of differentiated T and NK cells. This immunotype correlated with age in controls (compositional PCA, $R=0.68$, $P<0.001$), reflecting healthy aging. This immunotype was enriched in MM patients independent of age ($P=0.004$), rising with advancing severity (MGUS to SMM to MM), such that patients had increasingly prematurely-aged immune composition. This immunotype correlated with tumour MHC class I (MHC-I) expression ($R=0.43$, $P=0.007$), which fell between SMM to MM (HLA-C, $P=0.029$), thus patients whose tumours expressed the lowest MHC-I were the most prematurely-aged. In patients, clonal terminal memory CD8+ T cells expressing a cytolytic gene set enriched in non-virally-annotated clones correlated with tumour MHC-I ($R=0.33$, $P=0.002$). Together this suggests an accumulation of immune memory, reminiscent of healthy aging, occurs alongside tumour immune editing.

The second immunotype was composed of inflammatory cells, including Interferon Stimulated Gene (ISG)-expressing T and B cells and inflammatory CD4+ regulatory T cells. This immunotype was enriched in osteoarthritic non-cancer controls alongside MM (not MGUS or SMM) patients. This immunotype was pronounced in marrows with the highest local tumour infiltration and the most proliferating tumour cells (% MKI67+ tumour), suggesting tumour growth-associated inflammatory

niche remodelling. ISG+ effector T cells shared T cell receptors with aging-like immunotype cytolytic CD8+ memory cells, so may represent the acute source of memory T cells accumulating in MM.

Age-independent, aging-like (immunosenescent) differentiation in parallel to tumour down-regulation of MHC-I suggests an accumulation of historical anti-tumour responses leading up to MM progression. Inflammatory cells reflecting local tumour burden and growth may represent anti-tumour responses occurring in situ. Together, these processes may represent the acute and memory phase of tumour-reactive T cell responses in the BM of MM. Understanding the development of anti-tumour immunity during MM progression may improve predictive risk models of progression and identify patients who will benefit the most from early immune intervention strategies.

P230

VACCINATION STIMULATING POST-TRANSLATIONAL MODIFICATION SPECIFIC TH1 RESPONSES REPOLARISES THE TUMOUR ENVIRONMENT TO REDUCE SUPPRESSIVE LAP EXPRESSING T CELLS

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Post translational modification (PTM) of proteins has the potential to alter the ability of T cells to recognize major histocompatibility complex (MHC) class -I and class -II restricted antigens and T cells recognising PTMs often escape central tolerance. PTM of proteins such as citrullination and homocitrullination can occur in the tumour microenvironment and CD4 T cells targeting these modifications can be harnessed for tumour therapy.

Human CD4 T cell repertoire in cancer patients and healthy donors is analysed by flow cytometry to homocitrullinated and citrullinated self-peptides for proliferation by CFSE dilution, cytokine release and cell surface marker expression. Lung cancer microarrays were analysed for presence of citrullinated vimentin and LAP staining by immunohistochemistry. Vaccination with citrullinated self-peptides in murine models was assessed for vaccine specific immune responses by cytokine ELISpot assay and LAP expressing CD4 T cells in murine tumours were analysed by flow cytometry. The effect of vaccination with citrullinated self-peptides on tumour therapy and the LAP expressing CD4 T cell population in tumours was assessed in a mouse tumour model.

In this study we demonstrate a repertoire of CD4 T cells in healthy donors and lung and ovarian cancer patients to citrullinated and homocitrullinated self-peptides that have escaped central tolerance. In cancer patients some of these responses appear attenuated showing an inverse correlation between proliferation and IFN γ secretion and a suppressive LAP expressing phenotype. We demonstrate in lung cancer microarrays that the presence of PTM, in particular citrullinated vimentin, affords a good survival prognosis but suggest that LAP expressing CD4 T cells may polarise responses and promote tumour growth. In mice models we show the presence of higher levels of LAP expressing CD4 T cells in tumours compared to spleens and demonstrate that vaccination stimulating Th1 responses to tumour expressed citrullinated antigens reduces LAP expressing CD4 T cells in tumours and promotes tumour therapy.

This data suggests that vaccination stimulating PTM specific Th1 responses can overcome an immunosuppressive tumour environment.

P231

TUMOUR ASSOCIATED cDC DYSFUNCTION IS CO-TRANSFERRED TO LYMPH NODE RESIDENT cDC WITH ANTIGEN

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T cell responses against infections and cancer are directed by conventional dendritic cells (cDC) in lymph nodes distant from the site of challenge. It has been shown that T cells primed against tumours are only partially activated compared to that seen during infection but the reason for this is unknown. Migratory cDC which travel from the tissue to the lymph node have been shown to assume a regulatory phenotype in the tumour microenvironment and these drive initial T cell activation but also transfer antigen to lymph node resident cDC. These resident cells also have essential roles defining the character of the resulting T cell response, however, it is unknown how they can appropriately process and present diverse sets of antigens to suitably direct responses given their spatial separation. Given this lack of understanding of how resident cDC interpret the character of received antigen it is unclear whether the tumour can affect the phenotype of distal antigen bearing resident cDC.

Here using a novel strain of influenza A and an i.v. tumour model utilising a modified melanoma model we use advanced light microscopy and flow cytometry to track antigen from the site of challenge to the lymph node and characterise the phenotype of antigen bearing cDC subsets. Utilising trackable poly(I:C) we also show the effect of co-transferred adjuvants on resident cDC. Finally using mixed bone marrow chimeras it is possible to identify cell-intrinsic signals allowing harmonisation of lymph node resident cDC with peripheral cDC activation.

Here we show that acquisition of antigen from influenza and tumours drive distinct phenotypes of cDC activation in the lung. Tissue and lymph node cDC activation is harmonized and this is independent of previously proposed mechanisms including type I interferon or CD4 T cell help. Instead resident cDC mirror peripheral cDC activation as a consequence of co-transfer of contextual cues. As such, in the tumour, incomplete cDC activation in the tumour microenvironment (TME) is mirrored by lymph node resident cDC, whilst during influenza infection pathogen associated molecular patterns co-transferred with antigen drive TLR signalling in the resident cDC and their subsequent robust activation.

This co-transfer mechanism explains how individual antigens can be handled distinctly by resident cDC and how signals driving poor tumoral cDC activation further impact the lymph node. This helps explain previous suggestions of lymph node cDC dysfunction in the absence of overt immunosuppression. Importantly this also demonstrates that addition of adjuvant at the tumour site has the potential to also specifically influence antigen bearing lymph node resident cDC to improve anti-tumour T cell responses. Our findings clarify how tissue context dictates antigenic and, consequently, T cell fate in the lymph node and how this is disrupted in the setting of cancer.

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ANTI-SIALYL-DI-LEWIS A CAR T CELLS FOR EFFECTIVE ANTI-TUMOUR THERAPY

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Targeting cancer-associated glycans has been shown to be a viable option for cancer immunotherapy. Tumour cells show altered glycan expression that can be exploited to differentiate between cancer and self, but this requires the use of highly specific anti-glycan antibodies. We have previously described a number of anti-glycan antibodies that can recognise cancer-associated glycans on tumour cells. These include an antibody that specifically targets sialyl-di-Lewis a which is overexpressed on many cancer types including pancreatic, colorectal, gastric, ovarian, and lung. Chimeric antigen receptor (CAR) T cell therapy has the potential to target tumours with all the advantage of an antigen-specific T cell response, but without the dependence on MHC-presentation. Here we have engineered third generation anti-sialyl-di-lewis a CAR T cells to target tumour cells.

The constructs were designed with the binding sequences in either the light heavy (LH) or heavy light (HL) orientations to determine the optimal configuration for the CAR T cells. Glycan binding of the scFv was assessed by ELISA and SPR and cell binding by flow cytometry. In vitro T cell activation was measured by flow cytometry, cytokine release by ELISA and target tumour cell killing by LDH release assay. CAR T cells were assessed in vivo in NSG mouse model implanted with COLO205 tumour cells.

ELISA assays showed that the HL had a better affinity and lower EC50 than the LH orientation (EC50 1×10^{-6} and 1×10^{-5} , respectively). The kinetics of the antibody-glycan binding were further assessed using SPR which demonstrated that the HL orientation had a higher on/off rate than the LH orientation. Flow cytometry-based assays confirmed binding of the scFV to cancer cell lines. The third generation CAR T cells were then assessed in vitro and in vivo for target specific activation and killing. In vitro the anti-glycan CAR T cells showed CD69 activation, IFN γ release and tumour cell killing over a range of E:T ratio's. CAR T activation and tumour killing was observed for the sialyl-di-lewis a positive human COLO205 cancer cell line, but not for the sialyl-di-lewis a negative AGS cells. These results confirm that sialyl-di-lewis a CAR T activation and tumour killing occurs in a glycan-dependent manner. Despite the lower binding affinity, the LH orientation CAR T cells show comparable activation and killing to the HL orientation CAR T cells with some evidence that the LH CAR T cells were effective at lower E:T ratios particularly at earlier timepoints. The sialyl-di-lewis a -targeting CAR T cells were subsequently assessed in vivo in NSG mouse models. NSG mice implanted with COLO205 cells were treated CAR T cells and showed tumour regression compared to treatment with non-transduced T cells.

Together this data showed that anti-sialyl-di-lewis a CAR T cells can be engineered to induce effective tumour control. Our results suggest that targeting cancer-associated glycans using CAR T cell technology has potential and may lead to new avenues for cancer immunotherapy.

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UNRAVELING CHALLENGES TO ACHIEVE OPTIMAL EFFICACY OF CAR T THERAPY IN OSTEOSARCOMA

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Osteosarcoma (OS) is a malignant bone tumor that predominantly affects adolescents. Despite significant progress in treatment, pediatric patients with high-grade tumors continue to face dismal survival outcomes, with rates falling below 30%. While chimeric antigen receptor T (CAR-T) cell therapy has shown remarkable success in hematological malignancies, its efficacy in treating solid tumors, including OS, remains a challenge yet to be fully addressed. The primary objective of this study is to investigate the key obstacles hindering the effectiveness of NKG2D-CAR T cell treatments in OS. Additionally, we aim to explore the potential of combination therapies, particularly those incorporating armed oncolytic adenovirus ICOVIR15K (OAd), in overcoming these limitations and enhancing therapeutic outcomes.

In this study, we developed an optimized 3D hypoxic in vitro model to investigate the challenges of NKG2D-CAR T cell therapy in OS. OS cells were cultured in ultra-low adherent plates under controlled hypoxic conditions to simulate the tumor microenvironment. Co-culturing OS spheroids with NKG2D-CAR T cells allowed us to examine cytokine expression, cytotoxicity, and T cell activation patterns. To address immunosuppression challenges, we infected OS cell lines with an armed oncolytic adenovirus (OAd) engineered to express additional transgenes such as IL15, CXCL10, and an NKG2D ligand. In vitro studies evaluated transgene expression in OS and NKG2D-CAR T efficacy, while an in vivo model followed the behavior of NKG2D-CAR T cells after infection. This comprehensive approach enhances our understanding of NKG2D-CAR T cell therapy in OS and provides potential strategies to overcome current limitations.

Our study demonstrated comparable antitumoral efficacy of NKG2D-CAR T cells in both hypoxic spheroids and normoxic conditions, also supported by their expression of cytokines such as INF γ and TNF α . Additionally, we observed maintained expression of immune checkpoint inhibitory pathways and NKG2D ligands under hypoxic culture conditions. However, when we combine NKG2D-CAR T cells with OAd did not result in enhanced oncolysis of OS tumor cell lines. Nevertheless, we observed increased CAR T cell activation and cytotoxicity when utilizing other solid tumor cell lines. Interestingly, our combination approach utilizing OAd facilitated enhanced in vivo infiltration of CAR-T cells into OS tumors. These results provide valuable insights into the challenges of improving CAR-T therapy efficacy for OS and offer promising directions for the development of effective therapies targeting OS and other similar malignancies.

The NKG2D-CAR T therapy faces numerous challenges. Interestingly, neither three-dimensional structure nor hypoxia appears to be determining factors for CAR T activation in OS. Exploring other limiting factors, in an effort to overcome the immunosuppressive status of OS, we investigated a combination therapy of OAd with CAR T. However, in vitro studies did not demonstrate any synergy, unlike in other solid tumors, although in vivo experiments indicated that OAd increased CAR T homing to OS. Further segmented and detailed studies are needed to investigate all factors that may affect the efficacy of CAR T in solid tumors resistant to immunotherapies, such as OS.

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LOCAL DELIVERY OF INTERLEUKIN-12 ARMED WITH PD-1/PD-L1 BLOCKING NANOBODIES USING A SELF-AMPLIFYING RNA VECTOR ENHANCES ANTITUMOR RESPONSES

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Immunostimulatory cytokines are promising therapeutics for cancer therapy since they can remodel the tumor microenvironment; however, their use is often hampered by a limited efficacy and an unfavorable toxicity, especially when they are administered systemically. Different strategies can be used to optimize their therapeutic potential, such as local administration to concentrate their action in the tumor site, or protein engineering to increase tumor targeting or to provide additional effector functions.

We developed cytokines “armed” with antibodies, i.e. immunocytokines (ICKs), by fusing mouse interleukin-12 (IL-12) with single domain antibodies (nanobodies) that block the interaction between mouse and human PD-1 and PD-L1, previously described by us. These ICKs were designed from either naturally occurring double-chain IL-12 (dcIL12) or from a synthetic single-chain IL-12 version (scIL12), and were fused to one or two nanobody copies by flexible linkers. The different constructs were cloned into a self-amplifying RNA vector based on Semliki Forest Virus (SFV) to evaluate their correct expression in vitro and their antitumor potential.

First, correct expression of the different ICKs was confirmed in vitro by infecting cells with SFV vectors encoding these chimeric proteins. Similar levels of secreted ICKs and control IL-12 were achieved in all cases. The preservation of the biological functions of both components of the ICKs was confirmed by PD-1/PD-L1 binding assays, and by activation of mouse primary splenocytes in vitro. To evaluate their antitumor effect, ICKs were delivered locally into tumors using SFV viral vectors in a mouse model of colon adenocarcinoma. The SFV vector provides a high and transient level of expression of the therapeutic transgene and induces potent adjuvant effects due to the RNA self-amplification, which leads to the induction of type I interferon responses and apoptosis in infected cells. In this model, SFV vectors encoding ICKs fused to the anti-PD-1 nanobody (Nb11) showed a more potent antitumor effect than the cytokine alone or the ICKs carrying the anti-PD-L1 nanobody. In terms of safety, local administration of these vectors did not induce toxicity in mice at the doses tested. One ICK based on scIL12 (scIL12-Nb11) was selected for further evaluation. SFV vectors encoding scIL-12 or scIL12-Nb11 led to an increase in the infiltration of CD8+ T cells in tumors. Mechanistically, the increased antitumor effect of scIL12-Nb11 compared to scIL12 could be partially attributed to the targeting of the ICK to PD-1+ cells. Within the tumor context, these cells predominantly consist of antigen-experienced CD8+ T cells, including a subset that is tumor-specific. The antitumor effect of scIL12-Nb11 was also confirmed by a non-viral delivery method of SFV self-amplifying RNA, since this approach could be more easily translated to the clinic. For this purpose, a plasmid carrying the SFV replicon encoding the ICK was delivered locally by intratumoral electroporation.

We believe that these new “armed” cytokines and the self-amplifying RNA vectors used for their delivery could have a strong potential for cancer immunotherapy, helping to overcome the immunosuppressive tumor microenvironment. In addition, the ICKs described in this work could be readily humanized by substituting the murine cytokine by the human counterpart, making them clinically relevant agents.

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ANTI-TUMOR ACTIVITY AND SAFETY OF ACTENGINE IMA203 TCR-T IN PATIENTS WITH PRAME-POSITIVE SOLID TUMORS

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T cell receptor (TCR)-based adoptive cell therapy holds great promise to unlock the peptide-HLA target space for the treatment of patients with metastatic solid tumors, for which a high unmet medical need still exists. IMA203 is an autologous cell therapy utilizing a human TCR engineered to target the PRAME (preferentially expressed antigen in melanoma) peptide-HLA complex with high affinity and specificity. PRAME is a multi-cancer target with a high prevalence across many different solid tumor types, such as cutaneous and uveal melanoma, sarcoma subtypes, uterine, ovarian, lung, triple-negative breast and head and neck cancer, among others.

The ongoing trial (NCT03686124) evaluates the safety and anti-tumor activity of IMA203 TCR-T in patients with recurrent/refractory solid tumors. HLA-A*02:01+ patients are screened for PRAME expression by RT-qPCR analysis of archived or fresh tumor tissue. After lymphodepletion with Cy/Flu (500 mg/m² and 30 mg/m² IV x 4d), IMA203 is infused followed by low-dose IL-2 (1 mil IU SC daily x 5d, then twice daily x 5d).

As of April 4, 2023, 27 patients were treated in the phase 1a dose escalation phase at dose level (DL) 1-4 and 11 patients were treated in the phase 1b dose expansion cohort A at DL4/DL5, the provisional RPD2, applying an improved manufacturing process. All 38 patients were heavily pretreated with a median of 4 prior systemic therapies. At baseline, tumor burden was 113 mm (median sum of longest diameters of target lesions) and 63% of patients had elevated serum LDH levels. Infused cell doses ranged from 0.08x10⁹ to 2.09x10⁹ in phase 1a, and from 1.3x10⁹ to 8.84x10⁹ IMA203 T cells in phase 1b. TEAEs were manageable with most common events being cytopenias (100%), CRS (92% G1-2, 3% G3) and ICANS (13% G1-2). Importantly, so far no on-target, off-tumor toxicities were observed. IMA203 achieved confirmed objective responses in different solid tumor types, including PD1-refractory cut. melanoma, uveal melanoma, platinum-resistant ovarian cancer, synovial sarcoma and head and neck cancer. Objective response rate (ORR) across 18 patients in phase 1a and phase 1b treated at DL4/5 above 1x10⁹ IMA203 T cells was 61% (11/18) according to RECIST1.1 at week 6 post infusion. Most patients treated until cut-off were PD1-refractory melanoma patients (cutaneous, uveal and melanoma of unknown origin), for whom an ORR of 70% (7/10) at week 6 and a confirmed ORR of 56% (5/9) at month 3 was observed at DL4/5 above 1x10⁹ IMA203 TCR-T cells in phase 1a and phase 1b. Manufacturing enhancements including serum-free transduction and monocyte depletion implemented in phase 1b resulted in improved features of the cell product and increased IMA203 T cell levels in patients. Eleven patients were treated with these improved IMA203 T cells and achieved an initial ORR of 64% (7/11) at week 6 and a confirmed ORR of 67% (6/9) at month 3. 5 of 7 responders had an ongoing response at 9 (cutaneous & uveal melanoma), 6 (cutaneous melanoma), 3 (ovarian cancer), and 1.5 months (synovial sarcoma) post infusion. At a median follow-up (FU) of 8.5 months, median duration of response (DOR) was not reached (min 1.3+, max 8.8+ months). Translational data was consistent with clinical outcome: PRAME-specific T cells showed favorable engraftment and persistence in patients and were detectable in all evaluable post treatment tumor tissues with the

degree of infiltration being associated with objective responses. Objective responses were observed at all PRAME expression levels in the tumor, ranging from levels just at the mRNA threshold to >35-fold above the threshold.

IMA203 TCR-T showed a manageable tolerability and promising clinical activity in heavily pre-treated cancer patients. The data highlight the broad clinical potential of IMA203 in different PRAME+ cancers, including PD1 refractory melanoma.

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CIRCULATING EARLY MYELOID DERIVED SUPPRESSOR CELLS AS BIOMARKERS FOR EARLY DETECTION OF LUNG CANCER

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Lung cancer (LC) patients, despite innovative cancer treatments such as target and immunotherapies, still have the poorest five-year survival rate. In this context the possibility of an early detection can anticipate therapeutic intervention, avoiding aggressive therapies thus prolonging disease-free and overall survival. Therefore, the identification of new circulating early biomarkers still represents a relevant clinical need.

Lung cancer (LC) patients (n=63) and heavy smokers (HS) cancer free-subjects (n=52) were evaluated for the enrichment of specific subtypes of myeloid cells. Circulating early myeloid derived suppressor cells (eMDSCs) and low-density neutrophils (LDNs) were assessed by flow cytometry on peripheral blood mononuclear cells (PBMCs). Early MDSCs were defined as CD11b+CD33+CD15-CD14- cells among Lineage negative (Lin-) HLA-DR- PBMCs, whereas LDNs were defined as CD66b+CD15+ cells within the gate of CD11b+ PBMCs. Mature and immature subtypes were discriminated according to the expression of CD10.

Performing a sex- and smoking -matched case-control analysis in 39 LC and 39 HS subjects we were able to compare the capacity of specific myeloid population to discriminate lung cancer patients from heavy smokers. The analysis showed significantly higher values of eMDSC (OR=1.12; 95% Confidence Interval (CI) 1.03-1.21), total LDN (CD66b+CD15+; OR=1.23; 95%CI 1.04-1.45) and of mature CD10+ LDN (OR=1.30; 95%CI 1.05-1.61). Moreover, within the overall cohort of 63 LC patients, we analyzed the distributions of the different myeloid populations according to stage (I-II vs III-IV). In this comparison, total LDN (CD66b+CD15+; Kruskal-Wallis (KW) Test p-value=0.014), and mature CD10+ LDN (KW test p-value=0.006) showed significant different distributions according to tumor stage, observing higher values in patients with advanced stage. On the contrary, no difference among stages was observed for the eMDSC population.

These findings point to eMDSCs as an early marker for lung cancer detection whereas LDN could be as well associated with disease progression.

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THE MICROBIAL METABOLITE DESAMINOTYROSINE ENHANCES T-CELL PRIMING AND CANCER IMMUNOTHERAPY WITH IMMUNE CHECKPOINT INHIBITORS

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Inter-individual differences in response to immune checkpoint inhibitors (ICI) remain a major challenge in cancer treatment. The composition of the gut microbiome has been associated with differential ICI outcome, but the underlying molecular mechanisms remain unclear, and therapeutic modulation challenging.

We established an in vivo model to treat mice with the type I interferon (IFN-I)-modulating, bacterial-derived metabolite desaminotyrosine (DAT) in order to improve ICI therapy. Broad spectrum antibiotics were used to mimic gut microbial dysbiosis and associated ICI resistance. We utilized genetic mouse models to address the role of host IFN-I in DAT-modulated antitumor immunity. Changes in gut microbiota were assessed 16S-rRNA sequencing analyses.

We found that oral supplementation of mice with the microbial metabolite DAT delays tumor growth, and synergistically promotes ICI immunotherapy. DAT-enhanced antitumor immunity was associated with more activated T cells and natural killer cells in the tumor microenvironment, and was dependent on host IFN-I signaling. Consistent with this, DAT potently enhanced expansion of antigen-specific T cells following vaccination with an IFN-I-inducing adjuvant. DAT supplementation in mice compensated for the negative effects of broad-spectrum antibiotic-induced dysbiosis on ICI-mediated antitumor immunity. Oral administration of DAT altered the gut microbial composition in mice with increased abundance of bacterial taxa that are associated with beneficial response to ICI immunotherapy.

We introduce the novel therapeutic use of an IFN-I-modulating bacterial-derived metabolite to overcome resistance to ICI cancer immunotherapy. This approach is a promising strategy particularly for patients with a history of broad-spectrum antibiotic use and associated loss of gut microbial diversity.

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IMPAIRED ACTIVATION OF PLASMACYTOID DENDRITIC CELLS VIA TOLL-LIKE RECEPTOR 7/9 AND STING IS MEDIATED BY MELANOMA-DERIVED IMMUNOSUPPRESSIVE CYTOKINES AND METABOLIC DRIFT.

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Plasmacytoid dendritic cells (pDCs) infiltrate a large set of human cancers. IFN- α produced by pDCs drives growth arrest and apoptosis in tumor cells and modulates innate and adaptive immune cells involved in anti-cancer immunity. However, the activation state and clinical relevance of pDCs infiltration in cancer is still largely controversial. In Primary Cutaneous Melanoma (PCM), pDCs density decreases over disease progression and collapses in metastatic melanoma (MM). Moreover, the residual circulating pDC compartment is defective in IFN- α production, as result of lactic acid exposure.

Peripheral blood pDCs, isolated from buffy coats of healthy donors, were exposed to melanoma cell lines supernatants (SN-mel) and stimulated with R848, CpG-A or ADU-S100. The IFN- α and CXCL-10 production was tested by intracellular flow cytometry and ELISA. We also performed a bulk RNA-sequencing on SN-mel-exposed pDCs resting or stimulated with R848. On human melanoma biopsies, we assessed the expression of human myxovirus resistant protein 1 (MxA), as surrogate of IFN- α production. Moreover, to test endogenous dsDNA-cGAS activation, we analyzed pDC-enriched PCM cases by using proximity ligation assay (PLA). To assess the presence of TGF- β in the melanoma microenvironment, we performed RNAscope assay on PCM tissue sections and measured the amounts of TGF- β 1 and TGF- β 2 on SN-mel by ELISA.

Our findings from microscopic, functional and in silico analyses indicate that the melanoma milieu directly impairs IFN- α and CXCL-10 production by pDCs via both Toll-like Receptor -7 and -9 and cyclic GMP-AMP synthase (cGAS)-stimulator of interferon genes (STING) signaling pathway. Based on transcriptomic data, we proposed that TGF- β production by melanoma cells and metabolic drift represent relevant mechanisms enforcing pDC-mediated melanoma escape.

The introduction of immunotherapies, such as immune checkpoint blockades (ICBs), has significantly improved the outcome of MM patients. Unfortunately, half of the surgically resected patients relapses and do not show a durable response to ICBs, suggesting the need for novel therapeutic strategies. Our findings might propose a new window of intervention for novel immunotherapy approaches empowering the innate compartment of melanoma.

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BICYCLE TICA™ - BICYCLIC PEPTIDES FOR TUMOR-TARGETED IMMUNE CELL AGONISM

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Bicycles are novel synthetic bicyclic peptides which can potently and selectively bind cell surface proteins. Their small size, tissue penetration, and rapid clearance make them an ideal modality for precision-guided delivery of cytotoxins or radionuclides to tumors, as well as anti-tumor immune agonism.

They have a modular format that allows for the generation of bispecific compounds that can target and engage distinct cellular targets.

Bicycle Tumor-Targeted Immune Cell Agonists (Bicycle TICAs) have demonstrated potent, target specific activity in vitro, as well as anti-tumor activity in preclinical tumor models. BT7480, a Nectin-4/CD137 (4-1BB) Bicycle TICA™, and BT7455, an EphA2/CD137 Bicycle TICA™, induce cytokine production and increased immune cell infiltration resulting in tumor regression and complete responses in Nectin-4 and EphA2 positive syngeneic mouse models. This platform has been extended to the development of NKp46-targeted NK-TICA™ molecules which demonstrate the induction of cytokine secretion and tumor killing by primary human NK cells, in vitro, in a target dependent manner.

In this poster we will illustrate these two applications of the Bicycle® technology to tumor immunotherapy.

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EFFLUX OPTIMIZED TOLL-LIKE RECEPTOR 7/8 AGONIST GLYCOCONJUGATES FOR TREATMENT OF MULTI-DRUG RESISTANT BRAF WILD-TYPE MELANOMA VIA BYSTANDER ASSISTED IMMUNOTHERAPY

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Recurrent Multi-Drug Resistant (MDR) melanoma evades the action of chemotherapeutics by drug efflux, a process that uses Multidrug Resistance Protein 1 (MRP1) and related ABC transport proteins to actively transport drugs from within cancer cells to the extra cellular space. That said, the tumor microenvironment remains rich with tumor infiltrating lymphocytes and tumor associated macrophages which have proven viable targets for multiple types of systemic and locally administered immunotherapy. Based on these two observations, we envisioned that an immunotherapeutic prodrug which is activated by MRP1 could prove uniquely valuable in treating MDR solid tumors. Here we disclose BAIT719, a novel imidazoquinoline glycoconjugate (BAIT719), which is optimized for efflux via MRP1 yet retains potency as an immunotherapeutic through agonism of Toll-Like Receptor 7/8.

BAIT719 was synthesized as a glycoconjugate using methods previously reported by our group. Male 10 week old C57BL/6 mice were used according to protocol# 1077_2025 approved by the Miami University IACUC. The B16-F10-LUC2 cell line was obtained from ATCC and the MDR phenotype was developed by culturing with escalating doses of doxorubicin until stable culture at 1µM was obtained.

In-vitro, BAIT719 was metabolized by B16 melanoma cells, and MRP1-mediated efflux of the TLR7/8 agonist metabolite activated a model TLR reported cell line (RAW-Blue) as well as primary murine Bone Marrow Derived Dendritic Cells (BMDCs). Stimulation (measured by inflammatory cytokine secretion) was promoted by MRP1 expression and enhanced for MDR relative to non-MDR melanoma. In-vivo, BAIT719 was well tolerated when administered IP daily for 10 days. Dose escalation studies revealed lower systemic inflammation (as measured by body weight and serum cytokines) relative to control TLR7/8 agonist metabolite with a >2-fold higher maximum tolerable dose. BAIT719 was subsequently evaluated in an SQ MDR-B16 solid tumor model, for its potential to cause tumor growth delay via Bystander Assisted Immunotherapy. Here we examined depletion of myeloid derived suppressor cells in addition to other changes in the immunological remodeling of the tumor microenvironment. Synergistic efficacy with anti-PD-L1 checkpoint inhibitor was also observed.

In summary, we have developed a novel immunotherapeutic with potential to link TLR7/8 agonism to mechanisms of Multi-Drug Resistance. We have demonstrated this mechanism of action in MDR-melanoma, but because drug efflux is common to most MDR cancers, we envision this strategy of Bystander Assisted Immunotherapy could be broadly applicable to other chemoresistant solid tumors as well.

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BNT162B2 INDUCED MONOCYTE STATES LINK INFLAMMATORY MEDIATORS AND LIPID METABOLISM UNDER IMMUNE CHECKPOINT THERAPY

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In patients undergoing BNT162b2 vaccination under immune checkpoint therapy, we previously described the frequent coordinated induction of a set of serum cytokines (IL-6, CXCL8, IL-2, CCL2). These cytokines were frequently associated with cytokine-release syndrome, a clinically relevant and sometimes lethal adverse event. However, none of our patients developed clinically relevant CRS symptoms. It remained unclear why these patients were clinically resistant to CRS symptoms, where cytokines originated from and which cell populations they affected.

To understand the cellular immune response in these patients, we performed single cell RNA sequencing of peripheral blood mononuclear cells (PBMC) on 18 matched pre and post vaccination samples from 8 patients with CRS-associated cytokine release from a prospectively planned cohort study (DRKS00022890). Using the Milo algorithm, we retrieved cell states correlated with CRS related cytokines and analyzed their gene expression patterns using differential expression and Spectra factor analysis.

We identified a specific state of classical monocytes which positively correlated with CRS associated cytokines and increased in abundance after vaccination (vaccine-induced "VI" monocytes). By contrast naïve B cell states were negatively correlated, and tumor-reactive CD8 T cells uncorrelated with CRS-related cytokines and vaccination status. Our VI-monocyte state also expressed multiple inflammatory cytokines including CXCL8, CCL2 suggesting that they might contribute to vaccination-induced CRS cytokine release. The cognate receptors for these cytokines were expressed on diverse immune cell types indicating far reaching cell-cell interaction networks within PBMC. To pinpoint gene programs characterizing VI-induced monocytes we used the factorization method Spectra. Spectra identified gene programs of lipid metabolism to be overexpressed in VI-monocytes suggesting a link between these processes and CRS-related cytokine release and pinpointed TNF and IL17 signaling as possible inducers of this monocyte program.

Our results suggest that BNT162b2 vaccination induces a specific monocyte state with gene programs of lipid metabolism which expresses multiple vaccine induced CRS related cytokines offering a possible target for modulation of vaccine-induced inflammatory responses under cancer immunotherapy. In line with cohort studies suggesting safety of BNT162b2 vaccination during cancer immunotherapy, tumor-reactive T cells were unaffected by the vaccination.

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CLEAR CELL MORPHOLOGY COULD BE THE MOST SENSITIVE FOR IMMUNOTHERAPY IN OVARIAN CANCER

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Programmed death-ligand 1 (PD-L1) was expressed in various gynecology tumors. High-grade ovarian cancers could be a potential target for immune anti-PD-L1 modulate therapy. Antibodies targeting PD-L1 molecules are emerging cancer therapeutics. This study was designed to evaluate the expression of the PD-L1 marker in high-grade ovarian cancer types and evaluate its prognostic potential

The study included 18 patients with ovarian high-grade serous cancer (HGSC) and 11 patients with clear cell cancer (CCC) histology type, both in the International Federation of Gynecology and Obstetrics (FIGO) stage I. The expression of the PD-L1 marker was measured by tissue microarray-based immunohistochemistry. Expression levels of PD-L1 were correlated with the presence of tumor-infiltrating lymphocyte (TIL) and other histopathology parameters.

HGSC ovarian cancers predominantly had low PD-L1 expression, while CCC ovarian cancers had high PD-L1 expression ($p < 0.001$). PD-L1 expression did not show significant differences considering analyzed parameters other than histology type (localization, size, FIGO stage, lymphovascular invasion, tumor necrosis, and presence of TIL) among all ovarian cancers. There was no statistically significant difference in any of the tumor characteristics within histological types of ovarian cancers

PD-L1 expression was significantly higher in clear cell histology type than in high-grade serous ovarian cancers in FIGO I stage.

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CD40 AGONIST SYSTEMICALLY DRIVES THE DIFFERENTIATION OF TUMOR-SPECIFIC AND BYSTANDER CD8 T CELLS INTO A HIGHLY ACTIVATED PD1+ KLRG1+ CYTOTOXIC CD8 T CELL SUBSET

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Although preclinical studies of agonist anti-CD40 antibody (α CD40) have demonstrated its promising potential as a cancer immunotherapy, such results have not yet translated reproducibly to patients, highlighting the need for a better understanding of the mechanism of action and biomarkers of therapeutic response. α CD40 is known to activate dendritic cells, resulting in increased expression of MHCII, co-stimulatory receptors, and IL-12, and ultimately driving the expansion of CD8 T cells. However, it remains unknown what CD8 T cell subsets expand in response to α CD40, and specifically if α CD40 expands subsets of exhausted CD8 T cells similarly to anti-PD1/PDL1 checkpoint blockade. In addition, despite the importance of peripheral immune engagement in response to cancer immunotherapy, how α CD40 differentially affects the phenotype of CD8 T cells in the tumor versus the periphery has not been explored.

Using mass cytometry and flow cytometry, we investigated the impact of α CD40 on CD8 T cell phenotype and function in the tumor microenvironment and peripheral immune system of AT3 breast and MC38 colorectal cancer models seven days post-treatment. This study was collaboratively supported by the imCORE network.

Single dose administration of α CD40 significantly slowed AT3 and MC38 tumor growth over seven days. We identified a PD1+ KLRG1+ CD8 T cell subset that is dramatically expanded in α CD40-treated mice. This PD1+ KLRG1+ CD8 T cell subset was unique to α CD40 treatment, as anti-PDL1 did not elicit a similar population. PD1+ KLRG1+ CD8 T cells expressed intermediate levels of inhibitory receptors such as CD39, CD38, TIM3, and LAG3 compared to short-lived effector cells observed in acute *Listeria monocytogenes* infection and terminally exhausted CD8 T cells in the tumor (Tex-term, PD1+ CD39hi). They also expressed a similar amount of Granzyme B and Perforin compared to Tex-term, suggesting similar cytotoxicity. Since there can be non-tumor-specific bystander CD8 T cells in tumors, we used a tetramer to determine if PD1+ KLRG1+ CD8 T cells were tumor-specific. A significant fraction of tumor-specific CD8 T cells adopt this PD1+ KLRG1+ phenotype after α CD40 treatment, while there was no difference in the frequency of tetramer+ Tex-term. This increased frequency was observed systemically across the blood, tumor-draining lymph node, spleen, and tumor with the greatest change in magnitude occurring in the blood and tumor. Paradoxically, α CD40 treatment of healthy mice also elicited expansion of a similar magnitude of PD1+ KLRG1+ CD8 T cells in the blood, suggesting that α CD40 may be acting non-specifically on CD8 T cells regardless of antigen-specificity and that these cells may be highly activated as opposed to exhausted.

We propose a model where in tumor-bearing mice, α CD40 drives the differentiation of both tumor-specific and non-specific CD8 T cells into a highly activated cytotoxic PD1+ KLRG1+ state. Thus, even though α CD40 may consistently elicit dramatic expansion of CD8 T cells, many of these cells may not be tumor-specific and may have little anti-tumor function. Further delineation of phenotypic differences between tumor-specific and non-specific PD1+ KLRG1+ CD8 T cells could identify biomarkers for effective CD8 T cell responses after treatment with α CD40. In addition, therapeutic strategies that target the effect of α CD40 to tumor-specific CD8 T cells rather than bulk CD8 T cells may be more effective than α CD40 alone.

P244

ONCOFETAL TENASCIN C-REDIRECTED CAR T CELL THERAPY FOR PEDIATRIC SOLID AND BRAIN TUMORS

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The lack of targetable antigens is a considerable roadblock for pediatric solid and brain tumor chimeric antigen receptor (CAR) T cell therapy. To expand the array of targets, we developed a pipeline that identifies upregulated exons in the pediatric brain and solid tumors compared to normal tissues. From our screen of 1,532 tumor samples, we identified oncofetal Tenascin C (TNC), an extracellular matrix protein with known contributions to cancer malignancy, as a potential CAR target. We evaluated the expression of the C domain of TNC (C.TNC) at RNA and protein levels in an array of pediatric solid and brain tumor PDXs and cell lines. The C.TNC isoform was expressed in all samples, with high-grade glioma (HGG) and osteosarcoma (OS) having the highest expression.

Next, we developed a CAR against the C domain (C.TNC-CAR) with a CD28 costimulatory domain and achieved ~60% retroviral transduction efficiency in primary T cells.

C.TNC-CAR T cells effectively kill solid tumor cell lines in vitro and produce cytokines upon target interaction. We confirmed antigen specificity using a C.TNC negative cell line (CCRF) and CAR-specific killing using a C.TNC-CAR with mutated ITAMs to abrogate CAR CD3z signaling. However, the in vivo efficacy of C.TNC-CAR T cells against LM7 (OS) or DIPG007 was transient.

To improve the in vivo activity of C.TNC-CAR T cells, we designed a constitutively active IL-18 cytokine receptor with extracellular leucine zippers and intracellular IL-18 chains (zip18R). We validated that the expression of zip18R can activate its downstream adapter molecule, MyD88, using a reporter cell assay. Co-expression of zip18R and C.TNC-CAR improved the ability of T cells to kill LM7 recursively in repeat stimulation assay. In vivo, zip18R expression in C.TNC-CAR T cells sustained their antitumor activity against LM7, resulting in a significant survival advantage.

In conclusion, we have shown that we can target tumors that secrete oncofetal protein TNC with C.TNC-CAR T cells and that their antitumor activity can be significantly improved by activating IL-18 signaling via zip18R. Thus, our results warrant further exploration of C.TNC-directed T-cell immunotherapy for pediatric solid and brain tumors.

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MODELING EDB SPLICE VARIANT OF FIBRONECTIN (FN1)-REDIRECTED CAR T CELLS IN SYNGENEIC OSTEOSARCOMA MODELS

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The EDB splice variant of fibronectin (FN1) is expressed in a broad range of adult and pediatric solid tumors. We had previously shown (Wagner et al. Cancer Immunol Res. 2021) that EDB, which is 100% homologous between human and mouse, can be targeted with CAR T cells in xenograft models without overt toxicity. The goal of this project was now to evaluate EDB-CAR T cells in immune competent osteosarcoma (OS) models and study the effects of EDB-CAR T cells on the tumor microenvironment (TME).

We generated a murine EDB-CAR with a CD28z signaling domain and demonstrated that EDB-CAR T cells recognize and kill a panel of EDB+ murine osteosarcoma (OS) cell lines (F420, F331, Myc29) in an antigen dependent manner. To evaluate their antitumor activity in vivo, we adoptively transferred EDB-CAR T cells into Myc29- or F331-bearing mice without lymphodepleting chemotherapy.

EDB-CAR T cells induced significant (Myc29 model) and transient (F331 model) anti-tumor activity, and in both models, tumors ultimately progressed. Initial mechanistic studies in the Myc29 model revealed that intra-tumoral EDB-CAR T cell expansion peaked 5-6 days post CAR T cell infusion. Based on this finding we administered 2 doses of CAR T cells or IL-2 to enhance EDB-CAR T cell expansion and/or persistence. However, neither intervention prevented tumor recurrence. Analysis of infiltrating immune cells of the TME revealed that at peak expansion of EDB-CAR T cells (5-6 days post infusion) there was an influx of neutrophils in the TME, which was followed by an influx of B cells 12-13 days post infusion.

In summary, we demonstrate here that murine EDB-CAR T cells have antitumor activity in immune competent OS models without systemic toxicities. EDB-CAR T cells induced a sequential influx of neutrophils and B cells into tumors, and studies are under way to determine if these cells limit the effector function of EDB-CAR T cells within the TME.

P247**ASSESSING THE EFFICACY OF IMMUNOTHERAPY COMBINED WITH CHEMOTHERAPY VERSUS CHEMOTHERAPY ALONE IN NON-SMALL CELL LUNG CANCER: A SYSTEMATIC REVIEW AND META-ANALYSIS OF RANDOMIZED CLINICAL TRIALS**

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Non-small cell lung cancer (NSCLC) remains a primary source of cancer-related mortality worldwide. The emergence of immunotherapy has challenged the efficacy of chemotherapy as a mainstay treatment, leading to a keen interest in combining these two modalities. The survival benefits of combining these two treatment modalities across various demographic and clinical parameters are yet to be fully understood. This study aimed to compare the survival outcomes of NSCLC patients treated with immunotherapy combined with chemotherapy versus chemotherapy alone. This comparison was performed across diverse demographic and clinical parameters to provide a robust evidence base for clinical decision-making.

A comprehensive search of databases, including MEDLINE, EMBASE, the Cochrane Library, and ClinicalTrials.gov, was conducted for randomized controlled clinical trials (RCTs) published until March 2023. Only RCTs that compared survival outcomes of the two treatment strategies across various parameters in NSCLC patients were included. Statistical analysis was conducted using R software (version 4.0.3) with metafor and meta packages. Hazard ratios (HR) for progression-free survival (PFS) and overall survival (OS), as well as relative risk (RR) for response rate and major adverse events, were calculated using random-effects models to account for inter-study heterogeneity.

A total of 13 eligible RCTs were included, involving 6,677 patients (3,476 in the combined treatment group and 3,201 in the chemotherapy-only group). Combining immunotherapy and chemotherapy demonstrated superior PFS and OS across all subgroups compared to chemotherapy alone. In non-squamous histology, significant improvements were observed in PFS (HR=0.58, 95% CI 0.54-0.63, $P<0.01$, $I^2=33\%$) and OS (HR=0.72, 95% CI 0.64-0.81, $P<0.01$, $I^2=50\%$). Squamous histology also revealed enhanced PFS (HR=0.50, 95% CI 0.43-0.59, $P<0.01$, $I^2=58\%$) and OS (HR=0.66, 95% CI 0.56-0.78, $P<0.01$, $I^2=42\%$). In gender-specific analysis, both male (PFS: HR=0.52, 95% CI 0.48-0.57, $P<0.01$, $I^2=34\%$; OS: HR=0.69, 95% CI 0.63-0.77, $P<0.01$, $I^2=32\%$) and female patients (PFS: HR=0.61, 95% CI 0.51-0.72, $P<0.01$, $I^2=33\%$; OS: HR=0.69, 95% CI 0.52-0.90, $P<0.01$, $I^2=66\%$) demonstrated significant improvements in PFS and OS. The combination treatment significantly improved PFS and OS for both younger (below 65 years, PFS: HR=0.50, 95% CI 0.45-0.55, $P<0.01$, $I^2=16\%$; OS: HR=0.63, 95% CI 0.55-0.73, $P<0.01$, $I^2=47\%$) and older patients (65 years or more, PFS: HR=0.59, 95% CI 0.54-0.66, $P<0.01$, $I^2=0\%$; OS: HR=0.79, 95% CI 0.70-0.89, $P<0.01$, $I^2=0\%$). Patients with no smoking history (PFS: HR=0.59, 95% CI 0.46-0.77, $P<0.01$, $I^2=48\%$; OS: HR=0.74, 95% CI 0.59-0.93, $P<0.01$, $I^2=41\%$) and those who are former or current smokers (PFS: HR=0.51, 95% CI 0.47-0.56, $P<0.01$, $I^2=33\%$; OS: HR=0.69, 95% CI 0.63-0.77, $P<0.01$, $I^2=39\%$) showed significant improvement in PFS and OS. Patients with central nervous system (CNS) metastasis (PFS: HR=0.44, 95% CI 0.36-0.55, $P<0.01$, $I^2=0\%$; OS: HR=0.46, 95% CI 0.33-0.61, $P<0.01$, $I^2=0\%$), as well as patients without CNS metastasis (PFS: HR=0.58, 95% CI 0.48-0.70, $P<0.01$, $I^2=66\%$; OS: HR=0.71, 95% CI 0.63-0.81, $P<0.01$, $I^2=0\%$), exhibited significantly improved PFS and OS. Similar results were found in patients with and without liver metastasis. The response rate was significantly higher with combination treatment (RR=1.54, 95% CI 1.39-1.70, $P<0.01$, $I^2=62\%$), but so was the rate of major adverse events (RR=1.31, 95% CI 1.12-1.52, $P<0.01$, $I^2=58\%$).

The combination of immunotherapy and chemotherapy demonstrated improved survival outcomes across a range of demographic and clinical parameters in NSCLC patients compared to chemotherapy alone. However, an increase in major adverse events was observed, necessitating

careful patient selection and monitoring. Further large-scale RCTs are needed to confirm these findings and to inform personalized treatment strategies in NSCLC.

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DIETARY FATTY ACIDS SHAPE THE IMMUNE MICROENVIRONMENT OF METASTATIC OVARIAN CANCER

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Standard treatments such as surgery and chemotherapy have limited effectiveness in treating ovarian cancer. Immunotherapy has emerged as a promising approach to complement these standard treatments. However, the hostile microenvironment of ovarian cancer inhibits the generation of effective T cell responses, posing a challenge for the success of immunotherapies. It remains unclear whether dietary habits contribute to immune cell dysfunction and immunosuppression in ovarian cancer patients.

To address this gap, we investigated the impact of dietary fatty acids on the progression of ovarian cancer using a pre-clinical mouse model that resembles the advanced stages of the disease in humans.

Our findings reveal that a diet rich in n-3 (omega-3) fatty acids significantly prolonged the survival of ovarian cancer-bearing mice compared to diets enriched in saturated fatty acids (lard) or n-6 fatty acids (corn oil). This improved survival was accompanied by a reduction in tumor burden in the omentum, a major site for ovarian cancer metastasis. Prostaglandin E2 (PGE2), a bioactive lipid derived from arachidonic acid (AA) present in cell membranes, facilitates malignant progression by suppressing anti-tumor immunity. We found that dietary n-3 fatty acids that compete with AA for incorporation into cell membranes, effectively reduced PGE2 levels at tumor sites. Furthermore, a diet rich in n-3 fatty acids increased the proportion of TNF α -expressing CD4+ and CD8+ T cells, as well as natural killer (NK) cells at diverse tumor locations. We also observed accumulation of B cells in the peritoneal cavity of ovarian cancer hosts fed a diet rich in n-3 fatty acids and this increase inversely correlated with the proportion of malignant cells at the same site. A diet rich in n-3 fatty acids consistently improved overall survival by 20% in immunocompetent mice. However, in mice lacking T cells and B cells (RAG2-deficient mice), the increase in survival was only of 3%. These immune changes were accompanied by significant alterations in the composition of the gut microbiota among the different diets, suggesting a link between dietary n-3 fatty acids, gut microbiota, and endogenous immune responses that contribute to the delay of ovarian cancer progression.

In summary, our study demonstrates that dietary n-3 fatty acids can modulate the gut microbiota and support anti-tumor immune responses, resulting in delayed ovarian cancer progression. These findings provide valuable insights into the potential benefits of specific dietary interventions in combination with immunotherapy for more effective ovarian cancer treatment in the clinic.

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INSIGHT INTO THE ANTIGENIC TARGETING OF LEUKEMIA BY TCR-REDIRECTED iNKT CELLS

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AML (acute myeloid leukemia) is currently treated with chemotherapy and allogeneic HSCT (hematopoietic stem cell transplantation). However, disease relapse remains high urging the need for novel immunotherapeutic approaches. We have shown that AML express the non-polymorphic CD1c and are efficiently targeted by T cell engineered to express a TCR (DN4.99) specific the tumor-associated lipid mLPA restricted by CD1c, suggesting a new approach for donor-unrestricted adoptive cell therapy of this disease. iNKT (natural killing T) cells, a subset of T lymphocyte, express a conserved semi-invariant TCR that recognizes lipid antigens presented on the monomorphic CD1d molecules. They play a crucial role in tumor immunosurveillance either through direct antigen recognition or indirectly by modulating suppressive myeloid cells in the tumor microenvironment. Importantly, they can control GVHD, promote GVL (graft versus leukemia), and are able to migrate to the bone marrow.

Due to these appealing features for adoptive cell therapy, we are investigating the ability of mLPA-redirected iNKT cells to control the leukemia in vitro and in a CD1c-humanized mouse model.

We found that mouse iNKT cells engineered with the human DN4.99 efficiently kill in vitro two mouse leukemia cell lines RMA and C1498 transduced with CD1c. This recognition was further enhanced by pulsing the tumor cells with synthetic mLPA, suggesting a direct correlation between the lipid antigen concentration in the leukemia cells and the efficiency of T cell recognition. mLPA is an ether-bond lipid, implying a peroxisomal origin through a biosynthesis pathway involving the AGPS (alkylglycerone phosphate synthase) enzyme. We hence transduced the RMA-CD1c and C1498-CD1c cells with AGPS to assess whether its overexpression could enhance mLPA synthesis and, in turn, their recognition by DN4.99 TCR-iNKT cells. We found that iNKT cells recognized and killed substantially better either leukemia cells transduced with AGPS compared to the WT.

We are currently gaining further insights into the ability of DN4.99 TCR-redirected iNKT cells to control CD1c-expressing leukemia progression in vivo and the cell-endogenous mLPA antigen synthetic pathway that could be harnessed for improved leukemia targeting.

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INHIBITION OF MUTANT IDH1 ENABLES TUMOR-REACTIVE T-CELL RESPONSES AND SYNERGIZES WITH IMMUNE CHECKPOINT INHIBITION IN A HUMANIZED GLIOMA MOUSE MODEL

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An immunosuppressive environment orchestrated by the oncometabolite 2-Hydroxyglutarate (2-HG) is a hallmark of IDH1 mutant gliomas. Tumor secreted 2-HG is imported by infiltrating T cells, suppressing T-cell activation and proliferation. Inhibition of mutant IDH1 (mIDH1) using small molecule inhibitors (IDH1i) has shown therapeutic response in pre-clinical studies. Moreover, the recently reported Phase III trial with Vorasidenib, showed a significant improvement in patient progression-free survival with low grade IDH1/2 mutant gliomas, further exemplifying the therapeutic potential of IDH1i. However, the inhibition of the mIDH1 enzyme activity has primarily been evaluated in its ability to block gliomagenesis. Its contribution towards remodelling the tumor immune microenvironment and the consequent impact of reduced levels of 2-HG on rescuing T-cell function warrants thorough investigation.

To address this, we developed a novel syngeneic mIDH1 glioma model in MHC-humanized A2.DR1 mice and investigated the tumor immune microenvironment of mice treated with either immune checkpoint blockade or IDH1i – BAY1436032, using single-cell RNA and T-cell receptor sequencing

IDH1i treatment potentiated the accumulation of infiltrating T-cells with an increased abundance of T-cell subsets with a tumor-reactive phenotype and reduced regulatory T-cells. Concurrent with our previous findings that mIDH1 induces a CD4⁺ T-cell response, we observed an enrichment of CD40L⁺ CD4⁺ and PD1⁺CD4⁺ T-cells. Receptor-ligand analyses revealed a restoration of intercellular communication and upregulation of T-cell activation pathways. Combined with the observation that the top T-cell clones were more abundant within the PD1⁺ CD4⁺ T-cells, these results indicated a tumor-driven clonal expansion of CD4⁺ T-cells. We then combined IDH1i treatment with immune checkpoint blockade and observed a synergistic therapeutic benefit and reduced tumor growth compared to IDH1i monotherapy.

Cumulatively these findings suggest an immunotherapeutic role of BAY1436032 in addition to blocking IDH1 mutation-induced gliomagenesis. The reduction of 2-HG levels by IDH1i facilitates an immune-permissive tumor microenvironment, enabling a functional anti-tumor immune response further exploitable by immune checkpoint blockade. In turn, these findings support clinical trials testing the efficacy of IDH1 inhibitors in combination with adjuvant immunotherapies such as vaccines or immune checkpoint inhibitors in patients with IDH1 mutant gliomas.

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IN VIVO MACROPHAGE ENGINEERING REPROGRAMS THE TUMOR MICROENVIRONMENT LEADING TO ERADICATION OF LIVER METASTASES

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The liver hosts an immunosuppressive microenvironment, which supports the seeding of cancer cells originating liver metastases. Current pharmacological treatments fail in the presence of liver metastases, in part due to immunosuppressive signals that, in the liver, limit immune activation. Therefore, it is of pivotal importance identifying new interventional tools that activate the immune system in the liver unleashing adaptive immunity against liver metastases.

We developed a new lentiviral vector (LV) platform, termed IFN α LV, for in vivo genetic engineering of tumor-associated and liver resident macrophages, enabling locally sourced delivery of IFN α to liver metastases. Selective transgene product expression is driven by a macrophage specific promoter and fine-tuned by microRNA target sequences.

Genetic engineering of liver macrophages by in vivo IFN α LV delivery in distinct syngeneic mouse models of colorectal cancer (CRC) and pancreatic ductal adenocarcinoma (PDAC) liver metastases delayed tumor growth up to complete tumor eradication in some mice. Dose-response studies showed that IFN α expression was well-tolerated at all tested doses, with no major alteration of blood parameters, absence of autoreactive antibodies or no histopathological abnormalities in all tested organs. Molecular characterization of the tumor microenvironment in IFN α LV-treated mice by employing spatial transcriptomics, showed upregulation of interferon-stimulated genes preferentially in metastatic and peri-metastatic liver areas. Moreover, in responder mice, by employing single cell transcriptomics, we found that IFN α LV induced tumor-associated macrophage (TAM) immune reprogramming, enhanced MHC-I/MHC-II restricted antigen presentation and CD8 T cell activation. CD8 T cell depletion in mice hosting liver metastases attenuated the therapeutic effects exerted by IFN α LV, indicating that CD8 T cells may play a key effector function. On the other hand, depletion of IFN α receptor in both CD4 and CD8 T cells did not prevent IFN α LV therapeutic activity, suggesting that IFN α may promote CD8 T cell function indirectly, for example by enhancing antigen presentation.

On the other hand, mice not responding to IFN α LV displayed a high number of immunosuppressive T regulatory type 1 (TR1)-like cells, downregulated MHC-II related genes in antigen presenting cells, increased IL10 signaling in the tumor microenvironment and upregulated expression of exhaustion markers in CD8 T cells. Blocking IL10 prevented accumulation of TR1-like cells in liver metastases, but also diminished the therapeutic effect of IFN α LV, probably due to a key role of IL10 in T cell rejuvenation. Simultaneous combination of CTLA4 blockade and IFN α LV delivery resulted in strong synergistic effect, leading to hyper expansion of bonafide tumor antigen-reactive T cell clones and tumor regression up to complete response in most treated mice.

By performing RNA sequencing analysis of liver metastases from CRC patients, we found a positive association between expression of interferon-stimulated genes, CTLA4 and TR1-like cell signature supporting a link between IFN α activation and expression of CTLA4 as well as expansion of TR1-like cells in liver metastases. This observation supports the rationale for combining CTLA4 blockade with IFN α LV in liver metastatic CRC patients.

In summary, we developed a gene-based platform that upon a single well-tolerated intravenous LV infusion rapidly promotes a therapeutic response against liver metastases by enabling immune activation. Furthermore, we found that CTLA4 expression and TR1-like cells may promote immune evasion in presence of IFN α . These results provide the basis for a new off-the-shelf therapeutic strategy with rapid translation potential for cancer patients with severe unmet medical need.

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SINGLE VERSUS MULTIPLE INJECTIONS OF ONCOLYTIC ADENOVIRUSES IN A CLINICAL TRIAL SUGGESTS STRATEGIES TO IMPROVE CANCER VIROTHERAPY THROUGH IMMUNE-EVASION STRATEGIES

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Oncolytic virotherapy shows great promise for treating high-grade gliomas by leveraging virus-mediated oncolysis to stimulate anti-tumor immune responses. However, there has been a knowledge gap regarding the presence or consequence of neutralizing antibodies in brains during virotherapy of gliomas. This study investigates the impact of neutralizing antibodies developed in patients during a phase 1 clinical trial of our oncolytic adenovirus Delta-24-RGD for the treatment of recurrent malignant gliomas. It also explores a novel approach of hexon swapping to overcome immune responses and optimize oncolytic potential.

Sera were collected from patients with recurrent malignant gliomas who were treated with Delta-24-RGD. A chimeric virus Delta-24-RGD-H43m was engineered by swapping the hypervariable regions of the viral capsid protein hexon. Immunized mice bearing murine glioma GSC005 were treated with Delta-24-RGD or Delta-24-RGD-H43m.

In our phase 1 clinical trial, a single intratumoral injection of Delta-24-RGD in glioma patients induced neutralizing antibodies in less than half of the cases. Remarkably, 20% of these patients achieved survival beyond three years. Conversely, multiple oncolytic adenovirus injections led to neutralizing antibodies in 80% of patients but resulted in no long-term survivors. This inverse relationship prompted further investigation into the role of neutralizing antibodies in glioma virotherapy. Immunofluorescence analysis of Delta-24-RGD-treated murine brain tumors revealed colocalization of antibodies and viral proteins, suggesting neutralization of the virus. We hypothesized that antibody-mediated neutralization limits the efficacy of Delta-24-RGD and engineered a chimeric virus called Delta-24-RGD-H43m to counteract neutralization and enhance oncolysis. As hypervariable regions of the hexon protein are the major binding targets of neutralizing antibodies, Delta-24-RGD-H43m incorporated hypervariable regions from the rare serotype 43 into the prevalent serotype 5-based Delta-24-RGD. Delta-24-RGD-H43m demonstrated comparable infectivity, replication, and oncolytic capabilities to the parental virus, as confirmed by electron microscopy images, western blot, and cell viability assays. A homology-based folding prediction algorithm revealed distinct surface structures of Delta-24-RGD-H43m, its parental virus, suggesting that antibodies against serotype 5 will not bind to the chimeric virus. Indeed, clinical trial patient sera evaluation showed that Delta-24-RGD-H43m displayed significant resilience against the inhibitory effects of neutralizing antibodies compared to the parental virus ($P=0.01$). In murine models with pre-existing immunity to adenovirus, Delta-24-RGD-H43m treatment led to a significantly extended median survival and a higher percentage of long-term survivors than the parental virus ($P=0.05$).

Our data underscore the efficacy of hexon swapping to overcome dominant immune responses against oncolytic viruses and provide a strategy to increase the effectiveness of virotherapy for gliomas. These findings revealed valuable insights into the interactions between oncolytic viruses and the host immune system and paved the way for translating immune-evading virotherapies to the clinical setting.

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EXPLORING NONO AS A POTENTIAL INNATE IMMUNE SENSOR FOR ONCOLYTIC ADENOVIRUS IN GLIOMA TREATMENT

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Despite aggressive therapies, glioblastoma (GBM) patients face a median survival of just seven months after tumor progression. To address this challenge, oncolytic virotherapy has emerged as a promising approach, offering potential for reprogramming the immunosuppressed microenvironment of GBMs and enhancing anti-tumor immunity. Among the oncolytic adenoviruses, Delta-24-RGD has demonstrated favorable results in phase I/II clinical trials (NCT00805376, NCT03178032, and NCT02798406). However, the immune response of patients has functionally cleared the virus, limiting its effectiveness to approximately 20% of patients. In the context of gliomas, Non-POU Domain Containing Octamer Binding (NONO) has been identified as an overexpressed gene associated with poor patient survival.

We identified upstream regulators in response to adenovirus infection using bulk RNA sequencing. Immunoprecipitation was used to identify possible protein interactions, later confirmed by liquid chromatography mass spectrometry. Innate immune activation was tested using qPCR and confirmed using western blot. Virus replication was assayed using qPCR and virus titration.

Through bulk RNA sequencing, our study revealed a remarkable seven-fold upregulation of the NONO pathway during adenoviral infection. Western blot analyses further confirmed a significant increase in NONO expression following Delta-24-RGD infection. Utilizing mass spectrometry analyses, we identified cellular and viral members of the NONO interactome. Immunoprecipitation assays validated these findings and demonstrated the binding of NONO to adenoviral proteins during infection, as well as its association with the foreign DNA sensor cyclic GMP AMP synthase (cGAS). We further investigated the functional role of NONO using shRNA knockdown and found that NONO was necessary for inducing innate immune activation in response to adenovirus infection. Notably, we observed a striking increase in type I interferon after virus infection, which was reversed in NONO-null cells. Of interest, adenovirus replication was enhanced in NONO knockdown cells. Consequently, we identified NONO as a previously unrecognized innate immune sensor of adenoviruses. Moreover, the interaction between NONO and adenoviral proteins suggests a novel double-sensor mechanism involving the detection of both foreign DNA and viral proteins.

Our findings highlight the importance of considering the interactions between NONO and adenoviral proteins when designing the next generation of oncolytic adenoviruses for glioma treatment. By harnessing the insights gained from this study, we aim to enhance the efficacy of oncolytic virotherapy and pave the way for improved outcomes in glioblastoma patients.

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THE TUMOR MICROENVIRONMENT ARCHITECTURE CORRELATES WITH EARLY RECURRENCE OF HEAD AND NECK SQUAMOUS CELL CARCINOMA

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Emerging evidence suggest that not only the frequency and composition of tumor-infiltrating leukocytes but also their spatial organization might be a major determinant of tumor progression and response to therapy. However, most studies focused only on how the fine tumor architecture can discriminate tumor subtypes rather than evaluate its prognostic potential.

Here we performed an explorative, prospective clinical study to assess whether structures within the tumor microenvironment can predict recurrence after salvage surgery in Head and Neck Squamous Cell Carcinoma (HNSCC).

We employed co-detection by indexing (CODEX) multiparametric imaging and flow cytometry to measure the major leukocyte subsets and an ad-hoc computational framework to identify and analyze discrete cell types and cellular neighborhoods (CN) and correlate the presence of CT and or CN with clinical parameters and recurrence.

As suspected, we found that flow cytometry underestimated the number of PMN-MDSC and neutrophils in the tumor, suggesting caution in interpreting flow cytometry and scRNAseq from tissues undergoing significant processing.

Unsupervised clustering identified the expected leukocyte subsets but also additional populations such, for example, “capsule cells” that, when present, contour the neoplastic nests.

Cell neighborhood and cell-to-cell spatial analysis reveal the presence of 11 distinct cellular neighborhoods. Five CNs were characterized by homotypic cell-to-cell interactions (cold tumor, neutrophil rich, MDSC rich, NK CN, M1 rich), while in six CNs cells interacted with cells of a different type (Tertiary lymphoid structure (TLS) Type 1, TLS type 2, Hot tumor CN, vasculature CN, peritumoral CN, and Stroma CN).

Finally, we employed tensor deconvolution and uni- and multivariate analysis to evaluate whether the tumor composition in terms of cell type and cellular neighborhood discriminates the tumors of patients that recur from those of patients that remain tumor-free. We found that the frequency of type 1 tertiary lymphoid structure composed of CD31^{high}CD38^{high}plasma cells is associated with the lack of recurrence after surgery in HNSCC.

Our data support the notion that the structural architecture of the tumor microenvironment plays an essential role in tumor progression and indicate that type 1 tertiary lymphoid structures and long-lived CD31^{high}CD38^{high}plasma cells are associated with a good prognosis in HNSCC.

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INCREASING BREAST CANCER IMMUNOGENICITY VIA BIFUNCTIONAL RNA THERAPEUTICS TARGETING THE SPLICEOSOME MACHINERY

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Even with the FDA approval of Atezolizumab in combination with nab-Paclitaxel for PDL1-positive triple-negative breast cancer, metastatic breast cancer is still deadly. The modest impact of checkpoint inhibitor therapy in this disease can be partially explained by the relatively low tumor mutation burden, the low expression of neoantigens, and the overall low immunogenicity of metastatic breast cancer compared to other malignancies in which checkpoint inhibitors are highly effective. RNA sequencing and MHC-peptidome analyses of tumors from patients undergoing checkpoint inhibition therapy revealed the critical contribution of intron-derived epitopes in the neoantigen pools. In breast cancer, these intron-derived neoantigens are poorly expressed because of the constitutive upregulation of the spliceosome machinery.

We hypothesize that a targeted silencing snRPE, a key component of the spliceosome machinery, can increase breast cancer immunogenicity and synergize with anti-PD1 in eradicating metastatic breast cancer. To test this hypothesis, we generated a smart bifunctional molecule (PS03) composed of an RNA aptamer that recognizes both mouse and human metastatic breast cancer cells and siRNA against the spliceosome component snRPE. We tested our smart RNA therapeutic in vitro and in vivo for its specificity, silencing efficacy, and antitumor effect as monotherapy or in combination with anti-PD1 antibody.

In vitro, the simple addition of PS3 to the 4T1 cell lines silenced snRPE by more than 90%. Similarly, given systemically in 4T1-bearing mice, PS3 significantly inhibited snRPE expression on neoplastic cells but not in normal tissues, as demonstrated by digital image cytometry. Chronic PS3 administration was safe and restrained 4T1 progression in immunocompetent mice, whereas it did not affect the tumor of immunodeficient NSG mice indicating an immune-mediated antitumor mechanism. Additionally, PS3 synergized with anti-PD1 therapy and inhibited the formation of lung metastases. Spatial multiplex immunofluorescence and RNA sequencing studies are being performed to determine how PS3 treatment modulates the metastatic microenvironment and to identify the treatment-induced intron-derived antigens.

In summary, our innovative bifunctional RNA therapeutic is well-tolerated and can increase tumor immunogenicity and the efficacy of checkpoint inhibition therapy. Since our aptamer recognize both mouse and human breast and prostate metastatic cancers, is invisible to the immune system, and can be chemically manufactured with high purity, PS03 represents a new class of safe immune therapeutics that can be easily translated into the clinic and have a significant impact on metastatic tumors, the primary cause of cancer mortality.

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THE TRACKER BIOBANK

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Immunotherapy has been a game-changer in the treatment of advanced non-small cell lung cancer (NSCLC). Unfortunately, this benefit is seen in only a subset of patients and mechanisms of resistance are still being studied. In lung cancer research, acquisition of adequate tissue specimens that support multiple lines of scientific enquiry has been a longstanding challenge. We have established a consumer co-designed biobank, Tissue Repository of Airway Cancers for Knowledge Expansion of Resistance (TRACKER), to collect clinically-annotated longitudinal biospecimens to enable research towards discovery of biomarkers, characterisation of immunotherapy resistance mechanisms and development of novel therapeutic strategies.

Primary tumour, malignant and non-malignant lymph node(s), bronchoalveolar lavage fluid, and peripheral blood is taken from treatment naïve patients referred for endobronchial ultrasound for diagnosis and staging of suspected advanced NSCLC. Patients commencing immunotherapy have blood samples taken after the first cycle (week 3), third cycle (week 9), and then at the time of re-staging scans (week 12, week 24 etc.). Patients who develop disease progression are referred for repeat EBUS. Coded samples are processed and cryopreserved for molecular analysis including CiTE-seq, flow cytometry, ctDNA analysis.

TRACKER was initiated in 2021 and has two established recruitment sites in Victoria (Austin Health, RMH) and three labs (WEHI, ONJCRI, PMCC). Despite COVID-19 related delays, we have recruited 45 participants with stage III-IV lung cancer (mean age 68.5, males 67%) with 48 EBUS tissue samples taken at baseline and 87 liquid biopsies obtained over course of treatment (range 1-6/patient) to July 2023. Multi-omics analysis of biospecimens has commenced. Plans are underway to expand the partnership to include 7 sites and 5 labs in NSW, QLD, VIC and WA over the next 3 years. Samples and data can be made available for collaborative research project upon application.

Collectively, this program will boost translational research and increase collaboration while avoiding wastage of valuable tissue samples and reducing research silo mentality.

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IDENTIFICATION OF IMMUNE-RELATED TUMOR-SPECIFIC GENE EXPRESSION SIGNATURES IN CD4- AND CD25-ENRICHED CELL POPULATIONS FROM TUMOR AND BLOOD SAMPLES FROM BREAST CANCER PATIENTS

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A more detailed understanding of breast cancer biology in terms of transcriptional profiles and immunological gene signatures might lead to identification of novel biomarkers and targets for immunotherapy. A previous study identified distinct transcriptional features of regulatory T cells from tumor tissue compared to peripheral blood and normal breast tissue providing a rationale for targeting of specific tumor-infiltrating immune cells through cell- and tissue-specific gene expression.

In the current study, we analyzed differential gene expression in tumor-infiltrating and peripheral blood CD4+ T cells and CD25+ regulatory T cells from breast cancer patients and healthy female blood donors. RNA sequencing was performed on enriched CD4+ and CD25+ immune cell populations that were obtained from tumor tissue and from isolated peripheral blood mononuclear cells using magnetic labelling and column-based isolation.

The gene expression profiles of peripheral blood CD4+ and CD25+ cells from breast cancer patients resembled the profile of cells from healthy controls more than their tumor-infiltrating counterparts. There was a significant downregulation of genes involved in anti-inflammatory immune responses and T cell activation in tumor-infiltrating CD25+ cells compared with peripheral blood CD25+ cells, and a significant upregulation of the immune regulatory CD24 marker in both tumor-infiltrating CD4+ and CD25+ cells. Expression of CD24 has been associated with poor prognosis in breast cancer and its function is suggested to resemble that of PD-1.

The results indicate the involvement of mechanisms of immune tolerance in the tumor microenvironment by significant downregulation of genes involved in anti-inflammatory immune responses in regulatory T cells and upregulation of the immunosuppressive CD24. The presented results confirm the concept of how differential gene expression signatures can lead to the identification of tumor-specific markers with a potential as therapeutic targets that may improve immunotherapy, which is targeted directly at the tumor microenvironment. Further investigations are planned to elaborate and confirm the presented results.

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CLINICAL AND BIOLOGICAL ACTIVITY OF FLX475, AN ORAL CCR4 ANTAGONIST, IN COMBINATION WITH PEMBROLIZUMAB IN ADVANCED CANCER

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FLX475 is a potent and selective CCR4 antagonist, designed to block the recruitment of immunosuppressive regulatory T cells (Treg) into tumors without affecting healthy tissues. Blocking migration of Treg into the tumor microenvironment (TME) has the potential to restore antitumor immunity and provide an additive effect with a variety of conventional and immunotherapy-based approaches to overcome immune resistance and broaden clinical efficacy. In a recent interim clinical update from the ongoing FLX475-02 Phase 1/2 trial (NCT03674567), evidence of monotherapy and combination activity were reported. FLX475 monotherapy induced complete responses in two of the six evaluable subjects enrolled with EBV+ NK/T cell lymphoma. In checkpoint inhibitor naïve non-small-cell lung cancer (NSCLC), 4/13 subjects (31%) had confirmed partial responses (PRs) following treatment with the combination of FLX475 and pembrolizumab. In this analysis we present biomarker data from patients with a broad range of tumor types treated with the combination of FLX475 and pembrolizumab.

Circulating Treg (CD25+CD127-/low CD4+) were analyzed by flow cytometry. CD8 and FOXP3 positive cells in tumor biopsies were quantified by immunohistochemistry (IHC). RNAseq data derived from tumor biopsies prior to, and after approximately 6 weeks of treatment with FLX475/pembrolizumab were compared to published biopsy data from anti-PD-1 treated patients. Gene set variance analysis, immune deconvolution and machine learning approaches were used to interrogate these datasets to identify differences conferred by FLX475.

FLX475/pembrolizumab treatment results in a small but significant increase in proportion of circulating Treg by day 21 of treatment. Transcriptomic analysis of paired biopsies from both anti-PD-1 (publicly available datasets) and FLX475/pembrolizumab regimens significantly increased T cell infiltration immune signatures (CD8A, GZMB, IFNG, PRF1) and the published “expanded immune gene signature” (associated with response to pembrolizumab) indicating potentially turning cold tumors hotter. However, FLX475/pembrolizumab prevented coordinated increase of Foxp3+ Treg in the TME. This is consistent with the finding that significant increase in expression of CCR4 and its ligands CCL17 and CCL22 were only observed in biopsies of patients receiving anti-PD-1 treatment compared to FLX475/pembrolizumab. Supervised classification between responders and non-responders identified that baseline gene expression profiles of FLX475/pembrolizumab patients are distinct and better predictors of response than profiles associated with anti-PD-1 response. Positive predictive features of FLX475/pembrolizumab response were statistically enriched in Treg related gene sets in contrast to anti-PD-1 predictive features consistent with our therapeutic hypothesis.

FLX475/pembrolizumab therapy results in beneficial changes in the TME consistent with our proposed mechanism of action. Baseline markers associated with favorable response are different for the combination treatment compared to anti-PD-1 monotherapies suggesting that new populations of patients might benefit from the FLX475/pembrolizumab combination.

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A20 ORCHESTRATES CD8 T CELL EXHAUSTION IN THE TUMOR MICROENVIRONMENT

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CD8 T cell exhaustion poses a critical barrier to effective cancer immunotherapy. Within the tumor microenvironment (TME), activated CD8 T cells undergo repetitive stimulation, transitioning from a stem-like progenitor exhausted stage (Tpex) before entering a terminally exhausted state (Tex), defined by progressive and irreversible loss of cytotoxicity. Importantly, Tpex and Tex universally express the checkpoint molecule PD-1; although PD-1 blockade enhances Tpex proliferation and cytotoxicity, it ultimately fails to rescue these cells from the Tex fate. We therefore remain in dire need of immunotherapeutic targets that can durably invigorate Tpex or prevent the Tex fate altogether, which requires a better understanding of Tex differentiation and PD-1 signaling in general.

Intriguingly, previous multi-omic analyses have shown a correlation between the Tpex-to-TeX transition and decreased NF-kappaB activity, alongside increased expression of A20, a multi-functional ubiquitin-modifying protein and key inhibitor of the NF-kappaB pathway. Additionally, our investigation discovered a strong positive correlation between expression of A20 and Tex markers in two human cancer transcriptomic databases (TCGA and UCSF Immunoprofiler). This data hints at a central role for A20 in Tex differentiation, though this hypothesis has yet to be directly interrogated.

To address this gap, we leveraged our unique panel of A20 transgenic mice to investigate the relationship between A20 and Tex in the TME. Using knock-in mice with A20 domain-specific inactivating mutations, we observed significantly delayed tumor growth in multiple models (B16F10, CT26 and MC38) when A20's zinc finger 7 (ZF7) linear ubiquitin-binding domain was deficient. Conversely, inactivation of A20's enzymatic zinc finger 4 (ZF4) ubiquitin ligase or OTU deubiquitinase domains had no impact on tumor growth. Strikingly, nearly half of mice homozygous for the ZF7 inactivating mutation rejected MC38 tumors outright, while no rejection was observed in their wild-type (WT) littermates. Tumor rejection in ZF7 mice relied on CD8 T cells and specifically on CD8 T cell-intrinsic ZF7 inactivation. Interestingly, our full spectrum flow cytometry analysis revealed no quantitative difference in total or PD-1+ CD8 T cells between WT and ZF7 tumors. Instead, while most CD8 T cells in WT tumors displayed a Tex phenotype (PD-1+ Ly108- TIM-3+), a substantially higher proportion of ZF7 CD8 T cells remained in the Tpex state (PD-1+ Ly108+ TIM3-) with evidence of effector-memory CD8 T cell differentiation (KLRG1+ or CD27+). Finally, and surprisingly, PD-1 blockade failed to synergize with ZF7 inactivation; in WT but not ZF7 mice, PD-1 blockade enhanced tumor suppression and increased the Tpex fraction, indicating a mechanistic overlap between PD-1 blockade and ZF7 inactivation.

Collectively, these findings underscore A20 as an orchestrator of Tex differentiation, while highlighting its potential involvement in the enigmatic PD-1 signaling axis. A20 therefore constitutes a crucial intracellular checkpoint, and its ZF7 domain specifically emerges as an attractive target for cancer immunotherapy.

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ANTIGEN-SPECIFIC CYTOTOXIC T LYMPHOCYTE GENERATION FOR CANCER IMMUNOTHERAPY BY ARTIFICIAL IMMUNOGENIC CELL DEATH LIPID NANOPARTICLES

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For effective cancer therapy, induction of immunogenic cell death (ICD) of cancer cells are being studied to treatment of cancer and prevention of recurrence and metastasis of cancer. However, these strategies showed limited effect in the prevention of recurrence and metastasis.

Artificial ICD lipid nanoparticles (AiLNPs) were synthesized by LNPs with surface insertion of cancer cell surface proteins and decorated with immunogenic cell death molecules, HMGB1 and Calr. These LNPs were formulated into the smallest liposomes by a sonication and followed by an extruder. The LNPs were then decorated with DAMPs by Ni-NTA and his-tag interaction. Using a membrane protein isolation kit, the cancer cell membrane proteins were harvested, and proteins were inserted into the lipid bilayer. For cancer therapy, CT-26-iRFP (carcinoma) cells were subcutaneously inoculated to build the tumor in BALB/c mice. Moreover, to determine the anticancer effect of AiLNPs against LLC1(metastatic lung cancer), LLC1-iRFP cells were intravenously inoculated to build the tumor in C57BL/6 mice. On day 6 tumor injection, mice were treated with AiLNPs every 3 days.

Therefore, in this study, to overcome this limitation, we developed AiLNPs, which contained cancer cell membrane protein and decorated with immunogenic cell death molecules, HMGB1 and Calr. Based on decorated HMGB1 and Calr, the AiLNPs efficiently targeted to dendritic cells (DCs) in the spleen and lymph node and promoted activation of those cells. In addition, AiLNP-induced activation DC promoted tumor cell membrane-specific helper and cytotoxic T lymphocyte (CTL) activation, which showed specific killing of membrane protein contained tumor cells in vitro. Consequently, AiLNPs administration inhibited CT-26 and LLC1 tumor growth in BLAB/c and C57BL/6 mice, respectively. Furthermore, AiLNPs synthesized with human breast cancer membrane proteins promoted human peripheral blood activation DCs and CTLs, which activated CTLs selectively attacked human breast cancer cells.

Therefore, these data suggested that AiLNPs are expected to be used as a patient-specific cancer therapeutic vaccine to prevent cancer recurrence and metastasis.

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ENHANCEMENT OF GENETIC VACCINE EFFECTIVENESS BY SPATIOTEMPORAL COORDINATED DELIVERY OF GENETIC ADJUVANTS

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In vivo transfer of genetic information encoding an antigen is the base of genetic vaccination. Scientists had worked on genetic vaccines against infectious diseases and cancer for decades before SARS-CoV-2 spillover, but COVID-19 pandemic accelerated scientific discoveries and resulted in an unprecedented genetic vaccination campaign scale showing a successful story.

However, we are assisting the dawn of genetic vaccination where the interaction between genetic vaccine itself and the complexity of immune system has been only in part unveiled. Immune system is a very complex network of biological signals with changing properties along the time and space axes. For this reason, such notions of classical vaccinology (based on protein subunits, inactivated or attenuated viruses) cannot be easily transferred to genetic vaccination. The potency of classical vaccines is highly dependent on adjuvants that recruit innate arm or immune system in antigen injection site to create a milieu that potentiates adaptive immune response.

Classical adjuvants (e.g., alum and Adjuvant Systems AS series) have been tested in combination with genetic vaccines without substantial benefits. We reasoned that while for classical vaccines, the antigen of interest is administered as drug substance itself, genetic vaccines are administered as a sort of pro-drug where transcription (for viral vectors) and translation (for mRNA) need to occur before antigen is displayed to host's immune system. In this context, asynchronous activation of innate arm of immune system can impede the production of the antigen as transcription and translation shutoff have evolved as first defense when innate immune response is triggered. Genetic vaccines themselves actually trigger innate immunity where years of optimization allowed to identify a good compromise between activation of innate immune arm and antigen translation (e.g., modified nucleosides into IVT mRNAs, such viral vectors).

With the aim to improve genetic vaccine efficiency, for hard to hit infectious diseases agents and cancer, we reason to co-delivery the antigen of interest with an adjuvant molecule administered by the same delivery platform as encoded nucleic acid. The co-delivery of the antigen and adjuvant gene with the same platform technology ensure their spatiotemporal coordinated expression and display to immune system. We generated a proof of concept of this approach with adenovirus encoded antigens (i.e., cancer and infectious disease antigens) co-delivered with a second adenovirus encoding an immunomodulatory molecule able to significantly improve the quantity and quality of immune response. While the choice of encoded immunoadjuvant used for proof of principle was data-driven, here we implemented an in silico prioritization algorithm that allowed us to identify 100 potentially immunostimulatory genes to encode as genetic vaccine adjuvants. We implemented a high-throughput technology to rapidly encode these genes as both adenovirus vectorized and as encoded into In Vitro Transcribed (IVT) RNA formulated into LNPs. Enhancement in immunogenicity of these encoded adjuvants is in vivo tested by co-delivery with Spike model antigen delivered in the same matching delivery platform (i.e., Adenovirus and LNP-RNA).

This screening allowed us to identify several genetic adjuvants able to enhance both T and humoral response. We are also characterizing the mechanism of action for those immunomodulators with significant biological activity.

In conclusion, while genetic vaccines are yet revolutionizing the vaccinology and immunotherapy, there is still room to explore to make these platforms more effective. Genetic adjuvants can improve the quality and quantity of immune response in both cancer and infectious diseases. The easily

manipulation of nucleic acid delivery platforms (i.e., adenovirus and mRNA) make genetic adjuvants feasible to be rapidly translated into the clinic.

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N-GLYCOSYLATION INHIBITION HINDERS IMMUNOSUPPRESSIVE TUMOR MICROENVIRONMENT CELLS IMPROVING CAR T CELL EFFICACY

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Adoptive transfer of CAR T cells demonstrated impressive results against B-cell malignancies, but still limited efficacy against solid tumors. In this context, multiple challenges need to be overcome, including poor tumor recognition and strong immunosuppression within the tumor microenvironment (TME). Our Unit has recently reported that pharmacological inhibition of N-glycan synthesis in cancer cells increases CAR T cell efficacy by improving tumor recognition and preventing T cell exhaustion. In this project, we investigated the role of N-glycosylation blockade on TME cells in the context of colorectal cancer (CRC) and pancreatic adenocarcinoma (PDAC)-derived liver metastases and CEA-specific CAR T cell therapy.

We characterized the phenotypic and transcriptional profile of de-glycosylated M2 macrophages (M2-M) and Hepatic stellate cells (HepSCs) and we performed in vitro functional assays (tripartite co-cultures, suppressive assays and released-cytokines analysis) including these TME key players. To evaluate the effect of N-glycosylation blockade on TME cells in vivo, we exploited immunodeficient mice reconstituted with a human immune system (huSGM3), engrafted intra-hepatically with tumor cells and treated with CEA CAR T cells.

In vitro studies revealed that N-glycosylation inhibition abolishes the ability of both TME cells to restrain T cell proliferation and increases the elimination of cancer cell lines (BxPC3 and LoVo) and patient-derived tumor organoids (PDOs from CRC-liver metastases). Interestingly, these effects were associated with profound phenotypic and transcriptional changes in M2-M and HepSCs. In particular, the treatment was able to inhibit M2-polarization in terms of surface markers expression, IL-10 secretion and gene expression profile, and was shown to hinder the activation of HepSCs and inhibit the PD-1/PDL-1 axis. Importantly, in the humanized mouse model, we observed that N-glycosylation inhibition increases CEA CAR T cell antitumor activity, in terms of survival, and this is associated with the down-regulation of immunosuppressive genes in tumor-infiltrating human immune cells.

Overall, these data suggest that blocking N-glycosylation can help overcome multiple barriers that currently limit CAR T cell efficacy in solid tumors, acting not only on tumor cells, but also on immunosuppressive tumor microenvironment cells.

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STABILITY OF THE GUT MICROBIOTA DURING ANTI-PD1 IMMUNOTHERAPY DEFINES COMPLETE RESPONSE IN MELANOMA PATIENTS.

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The advent of immune checkpoint inhibition (ICI) therapy markedly improved the outcome for melanoma. However, response remains heterogeneous, with about half of the patients being refractory or developing relapse. Although a causal link between the gut microbiota and modulation of antitumor response has been established, current knowledge is limited to findings from cross-sectional analyses. Here, we follow the gut microbiota of melanoma patients over the course of anti-PD1 therapy, to delineate gut changes related to host response and identify gut and host factors involved.

To study response-related gut microbiota changes during ICI, patients with unresectable melanoma from two Italian Hospitals (n=23) were followed at baseline and over the course of anti-PD1 immunotherapy (over 13 months) to collect fecal and blood samples. Patients were annotated following RECIST 1.1 classification and PFS. Additionally, baseline fecal samples from tumor-free subjects were used as reference to compare gut diversity trends between response groups at therapy. Finally, cross-study validation was carried on metagenomes (n=281) from Europe (n=4), UK (n=2) and USA (n=3) baseline cohorts.

Our results demonstrate that the gut microbiota is fairly variable during ICI therapy. However, changes are less pronounced among complete responders (CR), especially at later cycles. We identify and validate a core of longitudinally stable gut microbiota taxa in CR, which comprise mostly Clostridia taxa. These core CR taxa associate consistently with positive systemic markers, supporting their immune modulatory potential. At the functional level, our data demonstrate a key role for specific bacterial cell components in driving a productive immune response.

Overall, we propose microbiota stability during ICI therapy as a consequential feature of an immune-beneficial Clostridia-rich gut among complete responders.

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SMALL PEPTIDES AS INHIBITORS TARGETING IMMUNE CHECKPOINT PD-1/PD-L1

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Programmed death-1 (PD-1) and Programmed death-ligand 1 (PD-L1) are one of immune checkpoints responsible for the inhibition of the immune system. Activated T-lymphocytes express PD-1 on their surfaces and tumor cells frequently escape from the immune response by expressing PD-L1 [1]. Inhibiting the immune checkpoints may elicit anticancer effects [1]. Several therapies using monoclonal antibodies against PD-1 or PD-L1 are available. Despite encouraging patient results, this type of treatment has many side effects and is very expensive. Therefore, there is a constant need for developing immunotherapies with small-molecule inhibitors of the PD-1/PD-L1 interaction (e.g. peptides), which may overcome the common problems related to antibodies [1,2,3]. Peptides can be an excellent complement to antibody therapy.

Based on the crystal structure of the PD-1/PD-L1 complex and MM/GBSA calculations a series of peptides, potential inhibitors, were designed and synthesized [3]. To study the protein-peptide interactions the immunoenzymatic assay (ELISA) and surface plasmon resonance (SPR) were used. Next, the effect of peptides on cell viability (cell lines - CHO-K1, Jurkat E6.1 and TCS Ctrl - modified BW5417) was tested. The ability of the selected peptides to compete with the PD-1 or PD-L1 protein for binding to PD-L1 or PD-1, respectively, present on the cell surface was investigated. Also, their ability to inhibit the formation of the PD-1/PD-L1 complex in a test by restoring NFAT-mediated luciferase expression or by measuring the expression of eGFP under the transcription factor NF-κB was determined. For the peptides with the best inhibitory properties, the spatial structure was determined using the nuclear magnetic resonance (NMR) technique and they were then docked to their receptor by using molecular modeling methods.

Using the MM/GBSA computational technique, the amino acid residues in both proteins, responsible for stabilizing the structure of the PD-1/PD-L1 complex, were determined. Approximately forty linear or cyclic peptides were then designed and synthesized. On the basis of tests to examine the binding of the peptides to PD-1 or PD-L1 proteins, a dozen peptides were selected for further cellular testing, during which the inhibitory properties of the peptides against PD-1/PD-L1 complex formation were examined. The results of these studies indicated that two peptides had the strongest inhibitory properties and disrupted PD-1/PD-L1 protein binding in cellular assays, restoring the PD-1 signaling pathway. Peptides with inhibitory properties are characterized by their cyclic structure.

Synthesized and tested in our project peptides may provide the groundwork for the future design of more potent checkpoint inhibitors. Especially in light of the fact that clinical trials focusing on the development of immuno-oncology therapies using peptides and peptidomimetics are becoming more common.

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P-TYPE PILUS PAPG PROTEIN ELICITS TLR2-MEIDATED IMMUNE ACTIVATION FOR CANCER IMMUNOTHERAPY

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Adhesion protein of pilus in the Escherichia coli (E. coli) has been shown to contribute induction of inflammation. E. coli contained two type of pilus, which are type I and P type pilus. Effect of the type I pilus adhesion protein, FimH, has well-defined in the induction of immune activation in the mouse and human. However, no studies have been conducted on the function of PapG, E. coli P type adhesion protein in the immune stimulatory effect. In this study, we examined that purified recombinant PapG in the induction of immune activation.

The immune activation effect of PapG in bone marrow derived dendritic cells (BMDCs), splenic DC, and human peripheral blood DCs (PBDCs) was analyzed by flow cytometry and ELISA. In silico studies and TLR2-knock out (KO) mice were used to confirm that DC activity of PapG is dependent on toll-like receptor 2 (TLR2). To confirm adjuvant effect of PapG, B16-OVA tumor-bearing CD45.1 congenic mice transplanted with OT-I and OT-II cells were used. Moreover, the antitumor efficacy of PapG was evaluated in C56BL/6 with B16-OVA tumor.

The PapG treatment promoted dramatic changes in dendritic morphology in BMDCs. Moreover, PapG induced upregulation of co-stimulating molecules, expression of major histocompatibility complexes (MHC) I and II, and production of pro-inflammatory cytokines in BMDCs and splenic DCs. Based on in silico study, the PapG effectively bound to mouse and human TLR2. The induction of DC activation was failed in the TLR2-knock out mice by PapG treatment, which indicated the PapG induced TLR2-dependent activation of DCs. Furthermore, we evaluated adjuvant effect of PapG and found that combination treatment of PapG and ovalbumin (OVA) promoted OVA-specific T cell proliferation, cytokine production, and cytotoxicity, consequently it elicited anti-cancer immune response against OVA-expressing B16 melanoma. In addition, PapG induced activation of human PBDCs and their subsets in the TLR2 dependent manner. The activation conventional DC2 by PapG promoted syngeneic T cell proliferation and activation.

These data demonstrated that PapG could be an effective immunostimulant for cancer immunotherapy.

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THE RNA BINDING PROTEIN FMRP IS INVOLVED IN SYSTEMIC IMMUNOSUPPRESSION OF HPV16-INDUCED CANCER.

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Human papillomavirus (HPV) infections can lead to squamous cell carcinomas, in particular cervical cancer, the fourth most frequent cancer in women. Although a 'therapeutic' HPV16-E7 peptide vaccine exists, it has minimal efficacy for cervical carcinomas, and as such, developing new immunotherapeutic approaches is a priority.

We are studying a genetically engineered mouse model (GEMM) of cervical cancer (K14-HPV16/H2b) that phenocopies the multistage progression implicated in human cervical carcinogenesis. Systemic immunosuppression (SIS) appears at the early stages of HPV16-induced carcinogenesis, associated with an increased abundance of immunosuppressive myeloid-derived suppressor cells (MDSCs) in bone marrow and spleen.

We have previously investigated a 'next-generation' nanoparticle-conjugated E7long-peptide vaccine (NP-E7LP) that has demonstrable efficacy in conventional tumor transplant models driven by the HPV16 oncogenes. However, this vaccine is not effective in the GEMM model due to this SIS. The current goals of our project are to understand how HPV16-expressing tumors induce SIS and then to identify means to disrupt SIS and increase the efficacy of therapeutic cancer vaccines.

The fragile X mental retardation protein (FMRP) has previously been shown to mediate tumor evasion and is highly expressed in human cervical cancer compared to many other cancers. Disruption of FMRP expression in cancer cells reprograms the otherwise immunosuppressive tumor microenvironment to become immunostimulatory, inducing CD8 T cell recruitment and activation, leading to tumor control.

We hypothesized that FMRP might be involved in the underlying molecular mechanisms of SIS within our cervical cancer models.

Single-cell RNA sequencing (scRNA-seq) data were analyzed to evaluate FMRP's cancer network signature - which is diagnostic of its TME-programming activity - in our cervical cancers model and in human cervical cancer datasets.

In comparing FMRP-high vs FMRP-low cancer cells, we observed a correlation between FMRP-high cancer cells and increased expression of immunosuppressive cytokines. We then used a syngeneic cancer cell line derived from our GEMM (LT2), which recapitulates the GEMM's SIS phenotype. LT2 cells had a FMRP activity signature, and knock-down (KD) of FMRP downregulated candidate immunosuppressive chemokines and cytokines. Congruently, the secretome of FMRP KD cells was also found to rescue CD8 proliferation and decrease the expression of immunosuppressive genes in CD11b⁺ cells in cell culture bioassays, opposite to the wild-type secretome. Next, an in-vivo experiment using an inducible shRNA in LT2 cells demonstrated that an FMRP-KD reduced the myeloid expansion indicative of SIS in bone marrow and spleen, slowed down tumor growth, and promoted a CD8 T cell influx into the tumor.

Collectively, our results suggest FMRP regulates the induction of SIS, in addition to orchestrating an intensely immuno-suppressive tumor microenvironment. We are now working to further illuminate its role in SIS, as well as to determine whether the down-regulation of FMRP is sufficient to both relieve

SIS and reprogram the immunosuppressive TME, thereby improving the otherwise ineffectual response to therapeutic HPV17 E7 long-peptide vaccines.

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INVESTIGATE TUMOR NEOANTIGEN RECOGNITION BY CIRCULATING AND TUMOR-INFILTRATING T LYMPHOCYTES IN ESOPHAGEAL ADENOCARCINOMA

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Locally advanced esophageal adenocarcinoma (EAC) is a very aggressive tumor that is currently treated with neoadjuvant chemotherapy (nCT), alone or in association with radiotherapy, followed by surgery. Less than 30% of patients achieve a pathological complete response (CR) to nCT, associated with better 5-year overall survival. Our recent multidimensional analysis of pre-treatment tumor biopsies has shown that pre-existing immunity strongly associates with the clinical response of EAC to nCT, suggesting that T cells specific for tumor antigens, particularly tumor neoantigens (TNAs) generated by tumor somatic DNA mutations, may participate in the tumor control. We hence sought to assess TNA-specific T cell response comparing EAC responders vs non-responders to nCT.

We are investigating TNA-specific T cell responses from pre- and post-treatment peripheral blood mononuclear cells (PBMCs) collected from EAC patients. TNAs are identified by computational analysis of tumor whole exome and RNA sequencing data obtained from each pre-treatment tumor biopsy. Patients' PBMCs are stimulated with pools of synthetic peptides corresponding to specific TNAs and assessed for specific antigen recognition by coculture with immortalized patients' autologous B cells that are transduced with minigenes encoding for the same TNAs. T cell activation is verified by flow cytometry determination of CD137, CD107a and CD40L upregulation and ELISA quantification of IFN γ secretion. Similarly, we have set up a protocol to investigate TNAs-specific T lymphocytes responses from tumor-infiltrating lymphocytes (TILs) collected from patients' tumor biopsies. TILs are pre-expanded in vitro polyclonally for 2 weeks to increase their yields, before stimulation with autologous immortalized B expressing TNAs-encoding minigenes and final coculture with the autologous B cells expressing the specific TNAs-encoding minigenes .

Preliminary results obtained from the initial N=3 EAC patients show specific autologous TNA recognition of circulating T cells from N=2, suggesting the potential immunogenicity of one or more of the tested TNAs. Reactive T cells were enriched for future validation experiments and TCR characterization. Recognition tests highlighted also the potential reactivities of TILs against one or more of the tested TNAs.

We have set up a robust platform to investigate TNAs-specific T cell responses from PBMCs and TILs of EAC patients and assess differences between responders versus non responders to nCT.

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SUPPORTING ADOPTIVE CELL THERAPY FOR CANCER BY COMBINATORIAL TREATMENT WITH IMMUNOSTIMULATORY GENE THERAPY THAT ALTERS THE TUMOR MICROENVIRONMENT

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Adoptive cell therapy using tumor-infiltrating lymphocytes (TIL) or natural killer (NK) cells are promising personalized cancer cell therapies, although success has been limited to a proportion of patients with certain cancers; TIL mainly in malignant melanoma (MM) and NK cells mostly in blood cancers. We aim to boost TIL/NK cell therapy by altering the tumor microenvironment (TME) by immunostimulatory gene therapy using adenoviruses as gene vehicles, and thereby make these therapies available for more cancer patients.

The combinatorial effect of the immunostimulatory gene therapy and TIL or NK cells was evaluated in solid tumors using malignant melanoma (MM) as a model system for TIL therapy and soft-tissue sarcoma (STS) as a model system for NK cell therapy. For TIL we have access to matched TIL/MM tumor cell line pairs that allows us to make autologous co-culture setups and investigate the activation of true tumor specific T cells in this setting. However, for initial optimization experiments we have used allogeneic T cells for these co-cultures. For NK cells we focus on STS since these tumors are commonly “cold” with little T cell infiltration possibly due to low mutational burden and, in addition, STS can occur in small children with a less developed T cell repertoire where NK cells may be more important. Taken together this makes NK cell therapy an attractive alternative for STS.

The immunostimulatory gene therapy used are all members of the LOAd (Lokon Oncolytic Adenoviruses) platform. LOAd are adenoviruses of serotype 5, with the fiber of serotype 35 which enables host cell entry via CD46. LOAd-viruses have a E1Delta24 gene deletion restricting replication to cells with a dysregulated retinoblastoma pathway commonly seen in cancer cells. LOAd encodes human immunostimulatory transgenes under the control of a CMV promoter enabling transgene expression in both tumor cells and its stroma. LOAd703 is armed with trimerized, membrane-bound (TMZ)-CD40L and 4-1BBL while LOAd732 also expresses IL2. The transgenes will affect both the innate and the adaptive immune system, CD40L trigger dendritic cell (DC) maturation whereas 4-1BBL can trigger activation of DCs as well as activation and expansion of T and NK cells. LOAd732 further drives T and NK cell expansion by co-expressing IL2.

A panel of 3 MM cell lines and 11 STS cell lines were evaluated for CD46 expression by flow cytometry. The analysis showed that all MM and STS cell lines expressed CD46 before treatment. The cell lines were subsequently infected with LOAd(-) that lacks transgenes, LOAd703 or LOAd732. CD46 expression was retained and LOAd703- and LOAd732-infected cells upregulated the expression of the transgenes TMZ-CD40L and 4-1BBL as measured by flow cytometry, as well as, in the case of LOAd732 IL-2 which was detected by ELISA. In addition, qPCR analysis confirmed LOAd replication in all tested cell lines in a dose and time dependent manner. Replication induced oncolysis of all investigated cell lines, as analyzed by an MTS cell viability assay.

Increased activation was seen in purified allogeneic T cells after co-culture with LOAd-infected compared to uninfected MM cells. Similarly, increased differentiation and activation was found in purified NK cells after co-culture with LOAd-infected compared to uninfected STS cells. In both cases, increased IFN-gamma production was found in the co-cultures with LOAd-infected cells. These immunological effects were strongest with LOAd703 and LOAd732.

In conclusion, LOAd viruses could cause expression of immunostimulatory transgenes in both MM and STS cells and subsequently induce tumor oncolysis due to LOAd replication. The LOAd-infected cells activated T and NK cells, and the strongest effect was seen with LOAd703 and LOAd732 expressing immunostimulatory transgenes. In the MM system, autologous TILs matched with the 3 MM cell lines will be investigated in upcoming co-culture experiments.

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ANTAGONIST ANTIBODIES INHIBITING THE BINDING OF MYELOID CHECKPOINT CLEC-1 TO NOVEL ENDOGENOUS LIGANDS DEMONSTRATE HIGH ANTI-TUMOR EFFICACIES IN HUMANIZED PRECLINICAL MODELS

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CLEC-1 is a c-type lectin receptor belonging to the pattern recognition receptor family [Drouin, Saenz and Chiffolleau, *Front Immunol.*, 2020]. It is a highly conserved protein, expressed by endothelial and myeloid cells in mice, non-human primates, and humans. Genetic deletion of CLEC-1 in mice does not lead to any developmental defect, but CLEC-1 deletion or CLEC-1 targeting through treatment with monoclonal antibodies increases necrotic cell antigen cross-presentation by cDC1 dendritic cells, leading to enhanced T-cell activation and anti-tumor response [Drouin et al., *Science Adv.*, 2022]. However, the identification of CLEC-1 endogenous ligands and their relative involvement in the immune checkpoint activity of CLEC-1 remained to be fully investigated.

Affinity capture assays and LC/MS analysis enabled the identification of CLEC-1 ligand candidates. Ligand candidates were subsequently validated through recombinant protein ELISA binding assays and Biacore affinity measurements. Anti-CLEC-1 monoclonal antibodies were generated through an immunization campaign. Antibodies were assessed for CLEC-1 protein binding and for their abilities to inhibit the binding of CLEC-1 to its novel endogenous ligands. Several clusters of antagonist anti-CLEC-1 antibodies were identified and further evaluated for their anti-tumor efficacies in human CLEC-1 knock-in mice inoculated with hepatocellular carcinoma (Hepa1.6) or colorectal cancer (MC38) cells.

We confirm that CLEC-1 specifically interacts with the intra-cellular Fc receptor and E3 ubiquitin ligase TRIM21 [Drouin et al., *Science Adv.*, 2022] and to the secreted histidine rich glycoprotein (HRG) [Gao et al., *iScience*, 2020]. Furthermore, we identify several additional intra-cellular and cell surface CLEC-1 ligands. CLEC-1 binding to these novel ligands is protein-specific, since deglycosylation does not impact protein/protein interactions. Eventually, we investigate the anti-tumor response levels following treatment with different clusters of antagonist anti-CLEC-1 antibodies: while inhibition of CLEC-1 binding to HRG mildly enhances anti-tumor responses, blocking of CLEC-1 binding to its cell surface ligands significantly decreases MC38 tumor growth kinetics ($p=0.04$, $n=12$) and largely augments the overall survival of Hepa1.6-inoculated mice ($p=0.002$, $n=12$), as compared to mock treatment.

Altogether, by dissecting the relative roles of CLEC-1/CLEC-1 ligand interactions, our results shed new light on the mechanism of action of the myeloid checkpoint CLEC-1 in its ability to impair anti-tumor immunity and highlight this receptor as a highly promising target for cancer immunotherapy.

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CIS-DEMASKING CYTOKINE LINKER TECHNOLOGY ALLOWS SELECTIVE CYTOKINE DELIVERY TO ACTIVATED IMMUNE CELLS WHILE SPARING OTHERS AND PERIPHERAL TOXICITY

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Immunocytokines are promising new therapeutic approaches to enhance T-cell response by delivering interleukins into the tumoral site. Fusion of wild-type or attenuated interleukins to anti-PD-1 antibody has shown some efficacy to preferentially cis-activate PD1-expressing T-cells but on-target/off-tumor activity on PD-1-negative cells is still observed due to the high affinity of the cytokine to its receptor leading a broad systemic effect, toxicity, high clearance and low tumor biodistribution limiting the potential of some immunocytokines (e.g. anti-PD-1/IL-2/IL-15/IL-21). Various strategies have emerged to achieve localized cytokine activation using conditional strategies (e.g. allosteric modulators, MMP-cleavable linkers or pH-dependent binding) that remain challenging due to high dependency to specific TME composition (MMP, acidosis...).

We developed the Cytomask® Platform, a universal and innovative linker technology allowing exclusive cytokine CIS-demasking upon binding of the fused antibody to its target without TRANS-activation associated with undesired effects(e.g. Toxicity).

A series of different linkers in term of length, composition, charge... were screened for cis-activation of cytokine on PD1-expressing vs PD1-negative T-cells using different cytokines fused to a high-affinity anti-PD1. Iterative screening cycles wer performed to select optimal composition. In vivo, pharmacokinetic/pharmacodynamic studies have been performed using humanized PD1KI tumor-bearing mice.

ytomask® linker technology decreases IL-2 or IL-15 cytokine activity (pSTAT5 signaling) on PD1-negative cells while maintaining high activation of PD1-transduced T-cells even in co-culture. On primary naïve (PD1-) vs activated (PD1+) human T-cells, the difference was more striking, since no or very low activation has been observed in naïve T-cells while high potentiation of PD-1+activated T-cells was induced. Importantly, Cytomask® linker technology does not induce TRANS-activation on PD1-negative T-cells illustrating an innovative and strict CIS-demasking mechanism of action. In vivo, Anti-PD-1/IL-15 and/or anti-PD1/IL-2 Cytomask® molecules illustrated better pharmacokinetic compared to conventional linkers, strong reduction of peripheral T-cell proliferation and reduced toxicity after single or multiple injections(2mg/kg). High T-cell proliferation has been observed in tumor-micro-environment where PD1-expressing T cells are located. Low T-cell proliferation in periphery was seen, illustrating the potential of CIS-demasking technology to target the right T-cells at the right place.

The Cytomask® linker technology illustrates specific intrinsic property to mask cytokine on naïve peripheral immune cells not expressing the target of the antibody while allowing selective CIS-demasking of the cytokine and CIS-activation of activated immune cells expressing (e.g. PD-1). This linker technology could be used with a broad range of cytokine to abrogate OFF-tumor cytokine activity associated with toxicity while selectively CIS-activating activated immune cells in the TME.

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ANTI-PD-1/IL-7V IMMUNOCYTOKINE FAVORS PROLIFERATION & SURVIVAL OF TCF1+ STEM LIKE MEMORY T CELLS AND A DURABLE IN VIVO EFFICACY IN MONOTHERAPY OR USING COMBINATORIAL STRATEGY

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Immunocytokines can strengthen anti-PD-(L)1 therapy by promoting T-cell survival, but their shortened half-life and systemic toxicity limit their clinical development. We developed a bifunctional anti-PD-1/IL7v (BICKI@IL7v) to selectively cis-deliver IL-7 to PD-1+tumor-specific T-cells. RNAseq and TILs scRNAseq analyses demonstrate that IL-7R and IL-7R pathway gene expression prior ICI treatment is significantly correlated with better OS and/or PFS across several cancers. IL-7R expression is correlated with higher stemness and lower apoptosis markers, providing a strong rationale of co-targeting IL-7 & PD-1 to sustain durable tumor-specific-T-cell response. The Anti-PD-1/IL-7v aims to cis-deliver IL-7 and sensitizes PD-1+tumor-specific T-cells to antagonizes PD-1 inhibitory signal and provide long-term survival and proliferative signals

Efficacy of anti-PD1/IL7v was evaluated in Ectopic tumor(MC38) or orthotopic HCC (Hepa1.6) in hPD1KI mice. Anti-PD1/IL7v effect was evaluated on chronically-stimulated human T-cells by scRNAseq and FACs.

Anti-PD-1/IL7v was designed using a high-affinity antagonist anti-PD-1, fused to a single point IL-7 mutein (IL7v) having lower affinity to IL-7R complex, to allow an optimal cis-potential of PD-1+T-cells and synergistic activation. Using an in vitro chronic stimulation model of human T cells, we demonstrated that anti-PD-1/IL7v promotes long-term reinvigoration proliferation/survival of stem-like-memory-TCF1+CD8+T-cells (>5 weeks), whereas IL-2/IL-15 promote short-term T-cell survival and differentiation into exhausted phenotype.

Anti-PD1/IL-7v showed significant anti-tumor efficacy in-vivo in responsive and refractory mouse models in monotherapy or combination(8 different orthotopic or ectopic models). In the orthotopic HCC model, anti-PD-1/IL7v induced >60% complete response while anti-PD-1 or IL-7 alone has no effect. Using FTY720 agent blocking ingress of new T-cells within TME, we demonstrated that anti-PD1/IL7v anti-tumor efficacy is mediated by amplification of TILs but also involves migration of fresh T-cells into TME. Further analyses demonstrated that anti-PD1/IL7v enhances quality and biodistribution of T-cells by promoting intratumoral TCF1+stem-like-CD8+T-cell proliferation and T-cell migration into the tumor nest whereas anti-PD-1 induced mostly T-cell exclusion. These data correlate with capacity of anti-PD1/IL7v to induce integrins and adhesion molecules surface expression. Finally, high synergistic efficacy in combination therapy with Sorafenib in HCC model (p=0.023), Oxaliplatin in MC38 model(85%CR) has been observed illustrating the potential of combining chemotherapy with ICI and anti-apoptotic cytokine therapy.

Our data validate the rationale of selective delivery of IL-7 to PD-1+tumor-specific-T-cells to limit risk of I-O/I-O immunotoxicity and sustain long-lasting proliferation and survival of stem-like CD8+T-cells to strengthen PD-(L)1 therapy. Synergistic anti-tumor efficacy with tyrosine-kinase-inhibitor or chemotherapeutics agents was demonstrated highlighting the potential clinical benefit of combination therapy with anti-PD-1/IL7v.

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TROGOCYTOSIS OF THE CAR AND TCR GENERATES CYTOTOXIC TUMOR-SPECIFIC BYSTANDER T CELLS – TEAMWORK OR OVERLOAD?

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The purpose of this study is to shed light on the Chimeric Antigen Receptor (CAR) and T Cell Receptor (TCR) trogocytosis in T cells and dissecting its possible consequences for cancer immunotherapy.

Immune cells are known for their capability to share molecules through a process called trogocytosis. During trogocytosis immune cells nibble pieces of plasma membrane from each other, resulting in the exchange of membrane-associated molecules. Trogocytosis of CD19 from malignant B cells to CD19-directed CAR-T cells has been described as a mechanism of resistance, which leads to antigen escape and fratricide CAR-T cell killing. However, it is not yet known if CAR and TCR molecules are exchanged between T cells in the context of CAR-T cell therapy, and if so, if such an exchange would impact on anti-tumor immunity.

Here, we have used mouse reporter lines and murine tumor models together with flow cytometry and confocal microscopy analyses to observe and characterize the exchange of TCR and CAR molecules between T cells and evaluate its consequences on anti-tumor immunity in glioblastoma.

Our results demonstrate a previously undescribed phenomenon where CAR-T cells actively transfer the CAR molecule to bystander T cells via a trogocytosis-like mechanism, resulting in the formation of “Pseudo CAR-T cells”. We have found that trogocytosis of the CAR molecule occurs during manufacturing of human and mouse CAR-T cells, as well as in vivo following CAR-T cell transfer in murine models of glioblastoma. Although CAR molecules are transferred in large clusters, Pseudo CAR-T cells do not show signs of tonic signaling. Instead, Pseudo CAR-T cells acquire cytotoxic capacities against antigen-expressing tumor cells, resulting in tumor cell killing and cytokine release. Moreover, we observed that the TCR is transferred along with the CAR onto bystander T cells, allowing them to recognize and kill tumor cells expressing either the CAR antigen or the TCR-specific peptide-MHC complex.

Our findings suggest that trogocytosis of CAR and TCR molecules could enhance the anti-tumor response by expanding the tumor-targeted T cell repertoire, through a mechanism that is faster than that of tumor-specific T cell expansion. We are currently investigating if this phenomenon contributes to tumor cell killing, or alternatively enhances immunosuppressive mechanisms such as exhaustion of bystander T cells. Nonetheless, our results suggest that promoting or inhibiting trogocytosis of TCR and CAR molecules during CAR-T cell therapy might affect anti-tumor responses.

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ADOPTIVE CELL THERAPY WITH CYTOKINE-INDUCED KILLER CELLS RETARGETED WITH IMMUNOTOOLS AGAINST HER-2 EXPRESSING BREAST CANCER

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Cytokine-induced killer cells (CIKs) are a heterogeneous population of CD3 and CD56 expressing T lymphocytes, expanded and activated *ex vivo* by peripheral blood mononuclear cells (PBMCs) or umbilical cord blood, with the addition of interferon recombinant human- γ (rhIFN- γ), anti-CD3 monoclonal antibody (mAb), and recombinant human interleukin-2 (rhIL-2). CIK cells are gaining considerable clinical relevance, due to the low risk of acute graft-versus-host disease (GvHD) in both autologous and allogeneic settings, their feasibility and limited production costs. In this study we combined CIK cells with either Trastuzumab (TRS) mAb, due to CD16a expression, or Trastuzumab (TRS) engineered mAb V90Lec13, which carries two amino acid substitutions (S239D/I332E) and lacks Fc fucosylation, or with the bispecific single-chain fragment variable (bscFv) Her2xCD3 and evaluate against Her2+ tumor cells.

CIK cells were obtained from PBMCs from both healthy donors and Her2+ breast cancer patients, under serum-free and a GMP expansion protocol. Effector cell cytotoxicity and dose-dependent activity of immunotools were evaluated by real-time cell assay (xCELLigence) against Her2+ breast cancer cell lines. The concentration of cytokines released was evaluated with a multiplex assay. To evaluate *in vivo* therapeutic efficacy, NSG mice were injected fat pad with Her2+ cell lines or with Her2+ patient derived xenograft and left untreated or treated with TRS, HerxCD3, 10x10⁶ CIK+Isotype, CIK+TRS or CIK+Her2xCD3. The mice were monitored for tumor growth and survival.

CIK cells from patients cultured in GMP condition are able to expand in clinically relevant number. The combination of CIK cells with all immunotools, significantly enhances Her2+ tumor cell killing. In particular at a very low effector/target (E/T) ratio, such as 0.1:1 E/T ratio, CIK cells combined with HER2xCD3 had a remarkable and fast cytotoxicity, that completely kill target cells. Interestingly, TRS-resistant tumor cell lines showed to be sensitive to HER2xCD3-armed CIK cell lytic activity. Moreover, bsAb resulted to be effective also at very low concentrations, and the cytokines released from CIK cells matched with a proinflammatory profile, with no significant concentration of cytokines correlated with Cytokines Release Syndrome. *In vivo* experiments demonstrated that the combination of CIK cells with both TRS or Her2xCD3 delay significantly the tumor growth *in vivo* and prolong the survival.

Taken together, these results highlight the potentiality of using new perspectives for the treatment of Her2+ breast cancer with the adoptive cell therapy by combining non-antigen-specific CIK effector cells with already clinically approved tumor-antigen-specific antibodies or recombinant molecules.

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ATP128 VACCINE WITH EZABENLIMAB PROMOTES ANTIGEN-SPECIFIC IMMUNE RESPONSES IN STAGE IV COLORECTAL CANCER IN THE KISIMA-01 PHASE 1B TRIAL

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KISIMATM is a vaccine platform based on a single chimeric fusion protein, containing a proprietary cell-penetrating peptide (CPP) for antigen delivery, a proprietary Toll-like receptor (TLR)-peptide agonist with self-adjuvant properties and a modulable multi-antigenic domain (Mad). ATP128 vaccine targets 3 antigens: carcinoembryonic antigen (CEA), Survivin and Achaete-scute complex homolog 2 (ASCL2); it is used in combination with a PD-1 inhibitor in the treatment of MSS/MMR proficient stage IV colorectal cancer (CRC) patients, after 4-6 months of first line of standard of care therapy or as perioperative administration in patients with resectable liver metastases.

KISIMA-01 (NCT04046445) is an open-label, multi-center Phase 1b trial to investigate the safety, tolerability and immunogenicity of ATP128 alone or in combination with the anti-PD-1 antibody ezablimab (BI 754091) in patients with stage IV CRC. ATP128 is given subcutaneously every two weeks for the first 3 immunizations (prime) and every 4 weeks for the last 3 immunizations (boost). Ezablimab is administered every 3 weeks starting with the first ATP128 administration. Blood and tissue samples are collected before, during and after ATP128 treatment to monitor the induction of a tumor associated antigen-specific immune response (ELISpot) and immune-related changes in the peripheral blood and in the tumor microenvironment by immunohistochemistry (IHC) and flow cytometry. Personalized, tumor-informed circulating tumor DNA (ctDNA) assay was performed to detect ctDNA in plasma samples.

In more than 45% of evaluated patients treated with ATP128 alone or with ezablimab, a cellular immune response against at least one out of three antigens was observed as determined by IFN- γ ELISpot analyses of patient PBMCs after the 3rd vaccination. Analysis of liver metastases by IHC indicated that 92% of evaluated patients have liver metastasis expressing all the 3 antigens in ATP128. Flow cytometry analysis of tumor infiltrating lymphocytes (TILs) comparing untreated resected liver metastases patients (historical controls) and ATP128/ezablimab-treated patients showed a similar quantity of the different infiltrated subsets but an improved quality of infiltrated T cells, indicated by an increase (more than 2-fold) in proportion of central memory T cells and an impressive decrease of the proportion of cells positive for exhaustion markers expression in KISIMA-01 patients. Importantly, post-operative ctDNA status was 100% concordant with relapse status in resected liver metastases patients.

Altogether, analyses indicate an induction of ATP128-specific immune response in the peripheral circulation of vaccinated patients, an increased infiltration of TILs into liver metastases with an improved quality of T cells and a ctDNA status, of resected liver metastasis patients, that mirrors relapse status.

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RIP3 GENE THERAPY FOR LUNG CANCER VIA IMMUNE CELL RECRUITMENT

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Necroptosis is a form of regulated necrotic and immunogenic cell death mediated by RIP3. However, RIP3 shows low expression in various cancer types and has impact on tumor progression and low chemotherapy. Thus, gene therapy using the RIP3 may be alternative immunotherapy for cancer treatment.

We developed CA-PEI/RIP3 for the delivery of the RIP3 gene, and aminopropyl imidazole was conjugated to chondroitin sulfate (CA) to enhance cancer cell targeting and pH sensitivity. We evaluated the function and mechanism of CA-PEI/RIP3 in human lung cancer cell lines and the antitumor activity using xenograft mouse model. Also, we observed the immune cell activity and recruitment by CA-PEI/RIP3 using lung orthotopic mouse model.

CA-PEI/RIP3 increased cell death in lung cancer cells, compared to PEI/RIP3-treated cells. Also, the cell death induced by CA-PEI/RIP3 was rescued by specific necroptosis inhibitors such as necrostatin-1 (Nec-1). Increased levels of HMGB1, EEA1, and Rab5, and high activation of RIP3/MLKL signaling were observed with CA-PEI/RIP3, indicating improved expression efficiency of RIP3 by efficiently inducing endosome escape. CA-PEI/RIP3 showed significant tumor selectivity and ablation efficacy in xenograft mouse model. Also, necroptotic cell death induced by CA-PEI/RIP3 enhanced the immune response by increasing the phagocytosis of dendritic cells (DCs) in lung orthotopic mouse model, leading to activity of CD4+ and CD8+ T cells and that infiltrate tumor tissues and lymph nodes.

CA-PEI/RIP3 shows significant potential in cancer gene therapy through enhanced transfection efficiency and the tumor-targeting properties of CA, further offering a safe and robust strategy to activate the immune response for cancer immunotherapy.

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IDENTIFICATION AND CHARACTERIZATION OF VACCINE-INDUCED NEOEPITOPE-SPECIFIC T CELL RECEPTORS IN PATIENTS WITH IDH1-MUTANT GLIOMA

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Gliomas are primary brain tumors that are considered to be difficult targets for immune interventions due to their low mutational burden and limited immune infiltration. Interestingly, more than 80% of diffuse astrocytomas and oligodendrogliomas carry the R132H driver mutation in isocitrate dehydrogenase type 1 (IDH1), which forms an attractive target for immunotherapy due to its homogenous expression in tumor tissue. We previously showed that the IDH1R132H neoantigen is presented on major histocompatibility complex class II (MHC II) molecules and induces a T helper cell response. These findings provided the basis for the first-in-human NOA16 clinical trial (NCT02454634), in which both safety and immunogenicity of a long IDH1R132H peptide vaccine was demonstrated.

Based on the success of NOA16, NOA21 (AMPLIFY-NEOVAC, NCT03893903) was initiated as a multicenter phase 1 clinical trial. Patients are vaccinated with the long IDH1R132H peptide vaccine in combination with the anti-PD-L1 (programmed death-ligand 1) antibody Avelumab to assess safety, tolerability and immunogenicity of the treatment. The “window-of-opportunity” trial allows monitoring of both the peripheral as well as intratumoral adaptive immune response, as patients with clinically warranted re-resection of a recurrent IDH1R132H-mutated glioma are recruited. The NOA21 trial stands out as single cell analysis of immune infiltrates from post-treatment brain tumor tissues is possible, allowing to gain deep insights into the mode-of-action of the vaccine-induced anti-IDH1R132H intratumoral immune response.

Here, we focus on characterizing the anti-IDH1R132H T cell response in detail. First, peptide-based expansion assays were used to pre-select TCRs for cloning and testing, leading to the identification of a total of 106 vaccine-induced IDH1R132H reactive T cell receptors (TCRs) from the blood of seven representative patients. Analysis of HLA-restriction of reactive clonotypes showed recognition of the IDH1R132H neoepitope primarily on HLA-DR MHC II alleles, which are known to allow for promiscuous binding of peptides, thereby potentially explaining the success of the vaccine in an HLA-diverse patient population. TCRs had a broad range of affinities against IDH1R132H presented on the same MHC II allele in peptide titration experiments. IDH1-specific T cell responses could be tracked over time in blood as well as in tumor tissue resected after three vaccination doses, showing a strong vaccine-induced increase in clone frequency. Moreover, a distinct signature of IDH1R132H-vaccine induced tumor-infiltrating lymphocytes was observed intratumorally, with an upregulation of T cell activation markers such as CD40LG (CD40 Ligand) in IDH1R132H-reactive clones. While no IDH1R132H-reactive T cells could be detected by IFN γ ELISpot in blood withdrawn before onset of vaccination, for at least one patient, enrichment of a reactive clonotype was found in tumor tissue resected prior to study treatment. This clone was shown to expand in blood after receiving the IDH1R132H long peptide vaccine.

TCRs identified in the scope of this study may be used to build a TCR warehouse of IDH1-reactive TCRs with known HLA-restriction, which could be used to offer patients with IDH1-mutant tumors off-the-shelf adoptive T-cell therapies. Most importantly, the presented data provides evidence that the observed highly beneficial effect of the IDH1R132H peptide vaccine in IDH1-mutant glioma

patients is mediated through a vaccine-induced T cell response, with IDH1R132H-reactive T cells found enriched intratumorally.

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MULTIDIMENSIONAL EXPLORATION OF THE CHONDROSARCOMA MICROENVIRONMENT TO IDENTIFY POTENTIAL RESPONDERS TO IMMUNOTHERAPY

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Sarcomas represent a heterogeneous group of rare malignancies accounting for about 1 % of all tumors. While patients with low-grade, localized sarcomas have a high chance of complete recovery with surgery, the prognosis of patients with advanced and metastatic sarcomas remains poor. The rarity and heterogeneity of sarcomas have slowed down both research and clinical trial recruitment, hindering the approval of new treatments in the last decades. Recently, increasing evidence indicates that some sarcomas, especially soft-tissue sarcomas, might respond to immune checkpoint inhibitors (ICIs). Some rare responses have been also observed in bone sarcomas, mainly in chondrosarcoma (CHS), though the limited number of enrolled patients prevents conclusions on the real efficacy of ICIs in this population.

As high-grade CHS have scarce therapeutic options and are resistant to standard therapies, it is crucial to understand if some of these patients might benefit from immunotherapy. We started a multidimensional characterization of the tumor microenvironment (TME) and matched blood of CHS patients, with a focus on myeloid cells and macrophages that are known to limit T cell function and infiltration in other tumors, to unveil potential predictive biomarkers for the selection of patients as well as new targets for combination immunotherapy.

Cell suspensions from surgical samples of untreated CHS (n=14) have been analysed by multiparametric flow cytometry (FC) to identify the main immune populations of the TME and their phenotype. The blood of CHS patients (n=20, including 14 matched samples) and healthy donors (n=6) has also been analysed by FC using the same antibody panels. Flow cytometry data have been analyzed through unsupervised clustering using Euclidean distance. Formalin-fixed paraffin-embedded slides of the same patients have been used to perform multiplex immunohistochemistry (mIHC) to localize immune cells and cell interactions in the CHS microenvironment. Snap-frozen samples of CHS surgical samples as well as plasma are being collected and stored for subsequent transcriptomic and proteomic analyses.

The analysis of the TME revealed the presence of two main groups of tumors: CD45high CHS (mainly comprising grade 1 tumors), with a strong infiltration of PMNs, PMN progenitors, B cells and T cells, and CD45low CHS (mainly composed of grade 2 and 3 tumors), displaying high infiltration of TAMs and NK cells and reduced B cell numbers. The unsupervised clustering of FC data also confirmed these differences in the immune composition of these CHS. CD45high and CD45low CHS patients also display distinct proportions of circulating leukocytes, although blood features alone do not allow a clear clustering of patients as observed with TME data. Interestingly, not only the abundance but also the phenotype of TAMs and circulating monocytes seems to differ in these groups of patients. In addition, mIHC reveals an enrichment of immune cells within the stromal areas of the TME over the tumor areas in CD45low CHS, reminiscent of “immune excluded” carcinomas, that warrants further investigation.

High-grade CHS show features of “cold” and “excluded” tumors that would benefit from drugs able to increase the recruitment and infiltration of T cells in contact with tumor cells and to inhibit the immunosuppressive TME. Further investigation is needed to better characterize the functional

orientation of tumor-infiltrating myeloid cells, predominant in these tumors, and their interactions with T cells.

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CD4 NEOANTIGEN MRNA VACCINE ENHANCES ENDOGENOUS CD8 RESPONSES AND TUMOR CONTROL

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While conventional type 1 Dendritic Cells (cDC1s) are specialized in antigen (Ag) cross-presentation for CD8 T cell priming, cDC2s are thought to present exogenous Ag to CD4 T cells. For effective CD8 T cell priming, cDC1s are licensed (or “helped”) by CD4 T cells that recognize Ag on MHC class II (MHC-II) molecules presumably presented by the same cDC1. CD4 help is in part mediated by CD40 signaling, which acts to enhance DC maturation, Ag presentation and survival, but whose mechanisms and spatiotemporal regulation remain incompletely understood.

As a new approach to understanding the mechanistic basis of CD4 help, we compared two mRNA-Lipoplex (mRNA-LPX) vaccines that express a single MHC-II-restricted Ag either tumor-specific (neoAg) or tumor-irrelevant. mRNA-LPX is injected intravenously and is taken up by professional antigen-presenting cells, including cDCs, in the spleen and other lymphoid organs.

As expected, both vaccines only elicited a systemic CD4 T cell response and not a direct CD8 T cell response. However, the anti-tumor CD4 T cell response uniquely elicited the accumulation of tumor-infiltrating CD8 lymphocytes (CD8 TILs), local tumor cDC1 expansion and tumor regression in a CD8 mediated manner. In contrast, and maybe not surprisingly, the irrelevant CD4 T cell response had little impact on tumor growth. We further examined the role of cDC1s and cDC2s in the response to the vaccines.

We found that both mRNA-LPX vaccines induced better maturation, including CD40 upregulation, of cDC1s than cDC2s in the spleen. Using *Batf3*^{-/-} mice lacking cDC1s, we also found that cDC1s, rather than cDC2s, were required for vaccine-specific CD4 T cell priming. Although cDC1-depletion in mice vaccinated with the tumor-specific vaccine had no impact on total CD8 T cells in spleen and tumor-draining lymph nodes, it reduced CD8 TIL accumulation and suppressed vaccine efficacy.

These results show that MHC-II-restricted neoAg mRNA-LPX vaccine can indirectly enhance endogenous CD8 responses responsible of tumor control. cDC1s are required for CD8 TIL accumulation and for vaccine efficacy and suggest that neoAg-specific CD4 T cells only license tumor Ag-presenting cDC1s for inducing CD8-mediated tumor rejection.

Moreover, we found that vaccine-expanded neoAg-specific CD4 T cells are mostly primed by cDC1s in the setting of mRNA-LPX vaccine expressing a single MHC-II-restricted Ag.

Taken together, our data emphasize the importance of both cDC1s and tumor-specific CD4 T cells for improving in situ anti-tumor cytotoxic CD8 responses and tumor control and suggest a novel mechanism whereby immunizing with exogenous CD4 T cell epitopes can produce tumor immunity by generating an endogenous CD8 responses.

We are currently investigating the mechanisms and location of the MHC-II-restricted neoAg dependent-cDC1 licensing using both in vivo and cell culture approaches.

This model provides fundamental insight for the development of cancer vaccines and highlights the need of tumor-specific CD4 T cells and mature tumor-Ag loaded cDC1s. Finally, this strategy may also identify new therapeutic opportunities designed to enhance endogenous anti-tumor immune responses.

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SPATIAL TRANSCRIPTOMICS OF ANTIGEN RECEPTORS MAPS B AND T CELL RECEPTORS IN HUMAN TISSUE

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Spatially restricted B and T cell clonal responses are critical to the initiation, regulation, and longevity of immune responses. Unbiased and high-throughput detection of human B and T cell clonal sequences within their tissue niches has been technically challenging.

To address this knowledge gap, we have developed a spatial transcriptomics-based protocol (Spatial VDJ) that maps full-length B and T cell receptor sequences in human tissue sections. Spatial VDJ is an extension of a commercially available and widely used spatial transcriptomics protocol starting from frozen tissue specimens.

From the same tissue section, Spatial VDJ maps B and T cell clones alongside the whole transcriptome and tissue microanatomy. The B and T cell clonal spatial distribution captured by Spatial VDJ matched canonical B cell, T cell, and plasma cell distributions and amplified clonal sequences were confirmed by orthogonal methods. In breast tumor tissue, we found spatial congruency between paired receptor chains and develop a computational framework to predict receptor pairs. We further linked the expansion of distinct B cell clones to different tumor-associated gene expression programs. In human lymphoid tissue, Spatial VDJ captured B cell clonal diversity and receptor evolution within and between germinal centers.

Thus, Spatial VDJ captured B and T cell spatial clonal architecture in human tissues, providing a platform to harness clonal sequences for therapeutic applications in cancer and beyond.

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MOLECULAR PROFILING OF ADVANCED NON-SMALL CELL LUNG CANCER (ADVNSCLC) REVEALS ANCESTRY-INFORMED PERSONALIZED CANCER VACCINE STRATEGIES

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Tumor-specific peptides, such as neoantigens, are primarily derived from somatic short variants (SV) and play a critical role in eliciting an anti-tumor immune response. Recent advancements in evaluating the clinical benefit of personalized neoantigen vaccines have been promising. Despite these breakthroughs, further optimization of neoantigen selection for vaccines to better identify responders is needed. By combining characteristics of HLA-I (human leukocyte antigen class I) peptide binding affinity, neoantigen clonality, patient genomic ancestry, and patterns of HLA-I loss of heterozygosity (HLA-I LOH), we sought to investigate the landscape of neoantigens in advanced non-small cell lung cancer (advNSCLC) to identify and prioritize potential immunotherapy targets.

Comprehensive genomic profiling (FoundationOne®) on 1.1Mb of sequenced DNA was performed as part of routine clinical care for 14,450 patients with advNSCLC. For every patient, genomic ancestry was inferred using a single nucleotide polymorphism-based approach and HLA-I genotyping was performed by OptiType v1.3.1 to a four-digit resolution. SVs (including base substitutions and small insertions and deletions) were assessed within all coding exons of 315 cancer-associated genes and neoantigen prediction was performed for evaluable missense, frameshift, and non-frameshift alterations by NetMHCpan v4.1. SVs with an HLA type-peptide binding affinity <500 nM were classified as neoantigens. Evaluable SVs were considered clonal if they had a cancer cell fraction of at least 50%.

63,674 unique neoantigens were identified, with a median of 4 (IQR 2-7) neoantigens per tumor specimen. Only 12% of neoantigens were generated from alterations known or likely to be pathogenic and only 9% of neoantigens were recurrently identified in more than one patient. Of these neoantigens, 51% were considered suboptimal by being either subclonal or exhibiting HLA-I LOH of the presenting allele. Amongst the remaining 31,063 unique clonal neoantigens in patients with an intact corresponding HLA-I presenting allele, a median of 2 (IQR 1-3) neoantigens were identified per tumor specimen.

In an independent assessment of the 30 most frequently observed SVs amongst all 14,450 patients, 25 SVs were predicted to be neoantigenic, of which 16 exhibited very strong binding affinity (<100 nM). Of note, only 8% of the cohort was found to have at least one of those very strong binders. Importantly, neoantigen prevalence differed by genomic ancestry, with only 7 of the top 25 advNSCLC neoantigens being observed amongst all five superpopulations (Africans (AFR), Admixed Americans (AMR), East Asians (EAS), Europeans (EUR), and South Asians (SAS)). These included KRAS G12C (9%, 8%, 3%, 12%, 7%), KRAS G12V (5%, 3%, 2%, 5%, 6%), KRAS G12D (4%, 4%, 2%, 4%, 5%), EGFR L858R (2%, 7%, 21%, 3%, 6%), TERT promoter -124C>T (2%, 2%, 1%, 3%, 5%), PIK3CA E545K (2%, 2%, 1%, 1%, 2%), and EGFR T790M (1%, 3%, 9%, 2%, 8%). Amongst the remaining 18, the most prevalent in each ancestry group were TP53 R273L (2% in AFR), TP53 V157F (2% in AMR), ERBB2 A775_G776insYVMA (2% in EAS), KRAS G12A (2% in EUR), and MLL3 M305L (6% in SAS).

Taken together, the findings in this study highlight that neoantigen quality and genomic ancestry should be an important consideration when prioritizing neoantigen target selection to improve patient outcomes and health equity.

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DECIPHERING IMMUNE MODULATORY EFFECTS OF IONIZING RADIATION AND IMMUNE CHECKPOINT BLOCKADE IN EXPERIMENTAL BRAIN METASTASIS TO DEVELOP STRATEGIES FOR IMPROVED RADIO-IMMUNOTHERAPY

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Brain metastasis (BrM) represent a major clinical issue with limited effective therapies for patients. Those therapies are classically surgery and radiation as well as systemic therapies such as novel immunotherapies. Nevertheless, the median survival of patients with BrM accounts only a few months and even multimodality intervention strategies show no long-term efficacy. This poor patient prognosis emphasizes the urgent need for novel and effective treatment options. Of special interest is a detailed understanding of the brain tumor microenvironment (TME), particularly immune cell infiltration and a detailed mechanistic insight in T cell biology to understand mechanisms of therapy resistance and finally develop effective treatment options to combat this lethal disease. Therefore, TME-targeted therapies, such as immune checkpoint inhibition (ICI) and different radiotherapy regimens are emerging to interfere with immune suppressive signals by tumor and myeloid cells to enhance the anti-tumoral T cell activity.

Our work is mainly focussing on experimental mouse models to analyze direct effects of immune checkpoint inhibition and radiotherapy *in vivo*. Together with histological and FACS analyses as well as transcriptomics and spatial transcriptomics, it forms our main focus to investigate the TME in BrM.

Using RNA sequencing approaches, we analyzed transcriptomic profiles of BrM-associated tumor infiltrating lymphocytes (TILs) and observed an exhausted phenotype that prevents the T cell anti-tumor activity. Using a breast-to-brain metastasis model, we demonstrated improved survival in response to radio-immunotherapy applied as whole brain radiotherapy (WBRT) which was associated with increased TIL recruitment [1]. In this project, we investigated effects of fractionated WBRT applied as 5x2 Gy and stereotactic radiosurgery (SRS) on BrM progression and immune cell infiltration. Likewise, efficacy of different immune checkpoint inhibitors with and without combination of radiotherapy was analyzed. We found that immune checkpoint therapy with α CTLA-4 in combination with fractionated WBRT significantly improved overall survival of mice compared to single treatment. Immunophenotyping by FACS analyses revealed no differences in immune cell infiltration in WBRT+ α CTLA4 treated mice compared to WBRT as monotherapy. In contrast, treatment with α CD96 or α VISTA demonstrated no significant benefit in combination with fractionated WBRT in the murine breast-to-brain metastasis model. Interestingly, fractionated WBRT combined with α PD-1 and α CTLA-4 did not further improve the overall survival of mice compared to the WBRT+ α CTLA-4 group. Moreover, SRS, which more closely reflects current clinical radiation regimens, in combination with α PD-1 inhibition results in significantly improved survival and further enhancement of T cell infiltration.

Taken together, our data demonstrate that alteration of the TME by radiotherapy in combination with immune checkpoint blockade could alter T cell-mediated anti-tumor activity and modulates the immune suppressive brain TME towards a more favorable milieu for immune checkpoint therapy. Further combinatorial *in vivo* trials, bulk and single cell RNA sequencing as well as spatial transcriptomic analyses will shed light on individual T cell subgroups and T cell activation states. We believe that such insight will provide scientific rationale for improved radio-immunotherapy treatment schedules to overcome therapy resistance, mitigate the risk of neurotoxicity and improve the

therapeutic efficacy for brain metastasis patients.

Niesel, K., Schulz, M., Anthes, J., Alekseeva, T., Macas, J., Salamero-Boix, A., Möckl, A., Oberwahrenbrock, T., Lohies, M., Stein, S., et al. (2021). The immune suppressive microenvironment affects efficacy of radio-immunotherapy in brain metastasis. *EMBO Mol Med* 13, e13412. [10.15252/emmm.202013412](https://doi.org/10.15252/emmm.202013412).

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REPROGRAMMING OF THE TUMOR MICROENVIRONMENT IN GLIOBLASTOMA MULTIFORME BY TRANSPLANTATION OF GENETICALLY ENGINEERED HEMATOPOIETIC STEM CELLS: A FIRST-IN-MAN STUDY

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Glioblastoma multiforme (GBM) is an incurable, highly aggressive tumor affecting the central nervous system, with a reported median survival of only 12.7 months in the unfavorable subgroup of patients with unmethylated (u)MGMT promoter. Immunotherapy has largely failed, due to low T cell infiltration and a highly immunosuppressive myeloid tumor microenvironment (TME)

We are conducting a phase 1/2a study in newly diagnosed patients presenting with (u)MGMT GBM (NCT03866109) evaluating safety, and biological activity of Temferon, a hematopoietic stem cell (HSC)-based gene therapy acting on the myeloid GBM TME. We hypothesized that stably engrafted, genetically engineered HSC produce myeloid cell progeny that home to the tumor and integrate into the TME, where they selectively release alpha-interferon (IFN- α) and stimulate anti-tumor immunity. We performed scRNAseq and scTCRseq analysis of second surgery GBM tumors from n=5 patients enrolled in the Temferon study (post gene therapy) and n=6 control patients treated according to best standard-of-care. Findings were validated on an extended control group encompassing >100 GBM patients from publicly available scRNAseq datasets.

As of April 30th, 2023, 19 uMGMT GBM patients in 4 cohorts received incremental doses of Temferon. With a median follow-up of 15 months, all patients show long-term engraftment of genetically engineered cells, with no dose-limiting toxicities. The interim survival rate at 2 years is 28% (5 out of 18 patients; 1 patient excluded – alive with a follow-up below 1 year). One patient is alive for >3 years after diagnosis, without further therapy. Genetically engineered cells were locally detected within the tumor lesion at levels >3% in 3/6 patients at second surgery, and all tumors showed evidence of increased IFN α -induced gene expression compared to the pre-treatment sample. These data indicate recruitment and integration of Temferon progeny into the TME, where they locally release IFN- α . Single cell RNA seq analysis unveiled reprogramming of the myeloid TME, with an increase in pro-inflammatory (M1-like) and a decrease in hypoxic (M2-like) macrophages following Temferon exposure. Of note, this population shift was particularly evident when comparing a stable with a progressive tumor lesion biopsied contemporaneously in 1 GBM patient, reproducing preclinical findings from a GBM mouse model (PMID: 35857642). Analysis of the T cell compartment highlighted an overall increase of CD8+ T cells (mainly effector T cell subsets) and a decrease in CD4+ T cells in Temferon patients. By mapping published signatures of antitumor neoantigen-reactive T cells (NeoTCR; PMID: 35113651) onto the GBM T cell landscape and integrating this data with clonal frequency metrics from concurrent scTCRseq data, we observed an approximately 3-fold increase of predicted tumor-reactive CD8+ T cells bearing expanded clonotypes (>1% within each patient) in Temferon vs. control patients. Intra-population differential gene expression analysis, followed by Gene Set Enrichment Analysis showed strong up-regulation of IFN- α and inflammatory responses in most of the clusters from myeloid and T cells, but also CD45-

tumor and stromal cell compartments from Temferon patients suggesting diffuse IFN- α payload delivery into the GBM TME

Temferon, a first-in-class gene therapy targeting the myeloid TME in GBM patients is safe and biologically-active at the tumor site favoring anti-tumor immunity. Preliminary survival data are promising, but longer follow-up is needed

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UNRAVELING THE TUMOR MICROENVIRONMENT WITH SPATIAL PROTEOMICS: IN SITU DETECTION OF IMMUNE CHECKPOINT INTERACTIONS IN CANCER PATIENT TISSUES

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Immunotherapy and the use of immune checkpoint inhibitors (ICIs) have shown promise in enhancing the immune response against cancer. However, cancer cells can exploit immune escape routes through interactions involving PD-1, PD-L1, LAG3, and MHCII receptors and ligands. Blockading these interactions has emerged as an attractive immunotherapeutic approach. Nevertheless, the efficacy of ICIs is limited to a poorly defined subset of patients, as traditional stratification based on single immune checkpoint protein expression often proves inconclusive. This study aims to investigate the utility of new potential biomarkers.

Formalin-fixed, paraffin-embedded tissue sections and tissue microarrays (TMAs) were used to assess the activation of immunosuppressive pathways. Staining with specific antibody pairs targeting PD1-PD-L1 and LAG3-MHCII interactions was performed. The Naveni proximity ligation method, a sensitive technique for detecting protein-protein interactions, was employed to examine the presence of these pathways in cancer tissues. The technology allows for the formation of a detectable signal only when two target proteins are in close proximity and is thus a sensitive tool for the detection of protein-protein interactions.

In healthy tonsil tissue, overlapping PD1-PDL1 and LAG3-MHCII interactions were observed within germinal centers, known as sites of communication between antigen-presenting cells and T cells. Positive staining of PD1-PDL1 interactions was found in various TMAs, including tumor tissues from patients with NSCLC, malignant melanoma, colon cancer, and pancreatic cancer. Additionally, PD1-PDL1 interactions were detected in draining lymph nodes of NSCLC and malignant melanoma patients.

This study demonstrates the activation of immunosuppressive pathways mediated by immune checkpoint interactions, particularly PD1-PDL1 and LAG3-MHCII, in healthy and cancerous tissues. Quantifying immune checkpoint interactions indicative of pathway activation may be a more reliable predictor of treatment response and patient outcomes than traditional single protein expression-based stratification.

The overlapping interactions between PD1-PDL1 and LAG3-MHCII in germinal centers highlight their critical role in the communication between antigen-presenting cells and T cells. The widespread detection of PD1-PDL1 interactions across different tumor types suggests their involvement in immune evasion mechanisms.

Understanding the dynamics of immune checkpoint interactions within the tumor microenvironment can enhance the development of effective strategies to overcome immune escape mechanisms employed by cancer cells. Further research is needed to validate the utility of immune checkpoint interactions as biomarkers and explore their therapeutic potential, advancing cancer immunotherapy and improving patient outcomes.

P285

SELECTIVE DELIVERY OF TGF β “TRAP” TO CD39-EXPRESSING IMMUNE AND STROMA CELLS RESHAPES TUMOR MICROENVIRONMENT AND REJUVENATES ANTITUMOR IMMUNITY

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CD39-Adenosine and transforming growth factor- β (TGF β) are two key immune suppressive pathways within the tumor microenvironment (TME) that cause broad immune suppression resulting in resistance to current CPI immunotherapies. Given that TGF β effector cells (immune cells and stroma cells) and CD39 expressing cells are highly correlated in localized microenvironment of tumors, it is thus possible to design an inhibitory molecule to enrich inhibit TGF β function in location where it matters by using CD39 targeting. We have since developed a bifunctional antibody–ligand trap called ES014 which comprises an antibody targeting CD39 fused to a TGF β receptor II ectodomain, resulting in inhibition of TGF β activity in the vicinity of CD39 expressing stroma cells and immune cells.

The immunological function of ES014 was studied in series of in vitro immuno-assays including RNAseq analysis. The in vivo efficacy of ES014 was investigated in human PBMC engrafted models. The effects of ES014 on immune cells were also analyzed using malignant pleural effusions (MPE) collected from lung cancer patients. The pharmacokinetic (PK), pharmacodynamics (PD) and safety profile were assessed in cynomolgus monkeys after intravenous administration of ES014. Single cell RNAseq analysis was used to analyze CD39 and TGF β expression across different tumor types and cell types. The pharmacokinetics (PK), plasma TGF β , CD39 target occupancy and safety profile were assessed in cynomolgus monkeys after intravenous administration of ES014.

The single cell RNAseq and IHC result indicated that CD39 mainly expressed in intratumoral immune cells (macrophages, MDSCs, DCs, B cells, Tregs and exhausted T cells), cancer associated fibroblasts (CAFs) and endothelial cells, but not on tumor cells. CD39 and TGF β are highly elevated in several tumors including SCLC, breast cancer and HNSCC, indicating these indications are ideal intervention candidates for ES014 bispecific antibody. The bispecific antibody, ES014 exhibited synergistic effects in T cell activation and suppression of Treg differentiation, much better than what was observed with the TGF β receptor-trap and anti-CD39 antibody combined. ES014 also activates dendritic cells, macrophages and natural killer cells by maintaining extracellular ATP level and neutralizing the immune-suppressive effects of TGF β . Interestingly, we found ES014 molecule demonstrated a unique mechanism by protecting effector T cells from activation induced cell death (AICD), which was not observed with TGF β receptor or anti-CD39 antibody alone, or the combination of the two. The ES014 molecule is very effective in inhibiting tumor progression in a PD-1 antibody-unresponsive in vivo model. Treatment of ES014 in malignant pleural effusions (MPE) collected from lung cancer patients led to the switch of M2 to M1 macrophage as indicated by increased CD86 expression and decreased CD163 expression on CD11b⁺ cells. Importantly, more CD8⁺ T cells were maintained with ES014 treatment in malignant pleural effusions as compared to control, which leads to significant cancer number decrease. In cynomolgus monkeys, TGF β neutralization in the periphery and full CD39 target occupancy on peripheral blood CD11b⁺ granulocytes were observed after dosing, and the drug duration time increased along with doses. ES014 was well tolerated in cynomolgus monkeys, no severe adverse effects were observed up to 90 mg/kg.

ES014 bispecific antibody is a first-in-class bi-specific antibody that demonstrated strong immune-promoting effect, resulting in killing of tumor cells. It inhibits both CD39-adenosine and TGF β pathway within the TME. ES014 has a favorable preclinical PK and safety profile in monkey study.

A phase 1 study is ongoing to investigate the safety, tolerability, pharmacokinetics, pharmacodynamics, and preliminary clinical activity of ES014 in patients with advanced solid tumors.

P286**TUMOR SIZE-DEPENDENT ENDOTHELIAL SWITCH MODULATES CD8+ T CELL IMMUNE SURVEILLANCE IN LIVER CANCER**

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Hepatocellular carcinoma (HCC), the most common form of liver cancer, is the third leading cause of cancer-related death worldwide. Surgical resection, liver transplant or local ablation can ameliorate the prognosis of HCC patients at early stages while they are not a feasible solution at later stages where only few therapeutic approaches are available. For this reason, patients diagnosed with advanced HCC have a survival expectancy of few months. CD8+ T cells play a crucial role in controlling tumors including HCC. Their potential anti-tumor activity relies on their recruitment to the tumor site, on antigen recognition and on the exertion of effector functions. We previously demonstrated that CD8+ effector T cells (Teff) have a unique dynamic behavior inside the liver. Particularly, they adhere and crawl along liver sinusoids where they recognize hepatocellular antigens by extending cytoplasmic protrusions that bridge the sinusoidal wall at the level of endothelial fenestrations. This behavior was so far described in healthy conditions, however, liver immune surveillance can be altered in HCC, since endothelial cells gradually undergo loss of fenestration and a fully formed basal membrane is generated. In this project, we aim at elucidating and characterizing the precise dynamics of Teff interactions with the tumor vasculature and possible strategies to boost Teff efficacy against HCC.

We generated a novel mouse model spontaneously developing HCC, in which the expression of a nominal antigen and a fluorescent protein are restricted to transformed hepatocytes. This model develops multiple lesions of different sizes, while preserving an abundant portion of normal liver. Then, we adoptively transferred tumor-specific effector CD8+ T cells (Teff) in HCC mice and we evaluated their potential therapeutic activity. Taking advantage of magnetic resonant imaging we monitored the tumor growth overtime upon Teff adoptive transfer. By confocal microscopy we studied the capability of Teff to home and get activated within single HCC lesions. Subsequently, we studied the transcriptomic profile of endothelial cells present within HCC lesions.

We found that upon adoptive transfer of tumor-specific Teff in HCC mice, only some lesions responded to the cytotoxic activity of Teff and this was dependent on tumor volume. More in details, responder lesions (Rs, vol <10 mm³) were sensitive to Teff cytotoxic activity, which rapidly infiltrated the tumor parenchyma, became activated and displayed an optimal antigen scanning rate. On the contrary, in non-responders (NRs, vol >100 mm³) the Teff functions were impaired, leading to tumor immune-escape. Interestingly, liver blood vessels changed dramatically both phenotypically and transcriptionally between Rs and NRs. HCC volume positively correlated with a strong reduction in the amount of liver sinusoidal endothelial cells (LSECs) in favor of capillarized (cECs) ones. Moreover, single cell RNA-seq of isolated ECs revealed an upregulation of endothelial immunomodulatory genes and prognostic markers for human HCC such as Cxcr4, Esm1, Fabp4, Ccdn1 and Cav1, Pparg, while typical markers of LSECs, such as Gata4, Lyve1 and Maf, and of leukocyte adhesion molecules, such as Vcam1 and Icam1, were significantly downregulated in NR-derived endothelium.

These results suggest that ECs can modulate the Teff anti-tumoral activity by providing (or not) their access to transformed hepatocytes. Reprogramming NR ECs gene expression may therefore provide an efficient tool for Teff delivery to HCC and guarantee their anti-tumoral cytotoxic activity.

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MESENCHYMAL STEM CELLS ENGINEERED WITH CD40L/4-1BBL-ENCODING ADENOVIRUSES ENABLE DENDRITIC CELL ACTIVATION

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Immunostimulatory gene therapy using viral vectors with selective replication competence in tumor cells is a promising tool for cancer treatment as they can inflame the tumor microenvironment to trigger an anti-tumor immune response, while replicating and infecting surrounding tumor and stroma to continuously drive the inflammatory response.

However, the administration of viral gene vehicles is largely limited to intratumoral injections due to an otherwise rapid viral clearance by neutralizing antibodies circulating in blood post repeated treatment cycles. Hence, strategies are being developed to mask viral gene vehicles from the immune system to facilitate systemic administrations, which may be required to efficiently target metastatic sites or in patients where intratumoral injections are not feasible. Mesenchymal stem cells (MSCs) have been proposed as carrier cells for replication competent viral gene vehicles as they naturally migrate via chemotaxis to sites of inflammation, such as the tumor microenvironment, and as the viruses replicate, they will be released in the tumor microenvironment. MSCs have low immunogenicity due to the lack of HLA class II expression and co-stimulatory molecules and can evade the immune system. However, they normally have pro-tumoral properties by promoting angiogenesis, by inducing the infiltration of immunosuppressive myeloid cells and macrophages and by hampering dendritic cell (DC) maturation, thereby impairing tumor-specific Th1 responses.

Herein, we hypothesized that loading MSCs with selective replication competent adenoviral gene vehicles derived from the LOAd virus platform (LOAd703, LOAd732) encoding the immunostimulatory transgenes (CD40L + 4-1BBL +/- IL-2), would reverse their immunosuppressive properties.

MSCs derived from adipose tissue (AT-MSC) or from bone marrow (BM-MSC) were infected with LOAd viruses and analyzed for transgene expression with flow cytometry and ELISA, and for oncolysis with MTS viability assay. Viral replication was determined with quantitative PCR. Cell lysates were evaluated for the proteomic profile with Olink® Target 96 Immuno-Oncology panel. Infected MSCs were co-cultured with immature monocyte-derived DCs for 48 hours before DCs were evaluated for their maturation status with flow cytometry and cell culture supernatants were investigated with Olink proteomics.

AT-MSCs and BM-MSCs both expressed the transgenes 48 hours post infection and were killed by viral replication-mediated oncolysis. Dendritic cells co-cultured with LOAd703 or LOAd732-armed AT-MSC or BM-MSC displayed an activated profile and significantly upregulated the expression of the maturation marker CD83, co-stimulatory molecules CD80 and CD86, as well as important molecules for lymph node homing (ICAM-1, CCR7). PD-L1, which is expressed on DCs post activation, was also significantly increased. Moreover, LOAd703/LOAd732-MSC-stimulated DCs secreted higher levels of pro-inflammatory cytokines and chemokines, including IL-12, IL-15, IL-18, IFN γ , TNF, CCL19, CXCL9, CXCL10 and CXCL11.

In conclusion, it is feasible to arm MSCs of different origin with LOAd viruses and the expression of the immunostimulatory transgenes alters the immunosuppressive profile of the MSCs and allows for DC maturation, thereby likely facilitating anti-tumor immune responses at the tumor site in the presence of LOAd-engineered MSCs.

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UNRAVELLING THE CHARACTERISTICS OF TUMOR-DRAINING LYMPH NODES IN GASTRIC CANCER BY SINGLE-CELL SEQUENCING AND A “SEC-MEM” METHOD

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Lymph node metastasis occurs frequently in all stages of gastric cancer and represents one of the most common ways of metastasis.

Here we report a comprehensive transcriptome atlas of 46,040 cells using single-cell RNA sequencing (scRNAseq) to unravel the tumor microenvironments (TMEs) of tumor-draining lymph nodes (TDLNs) in gastric cancer.

We discover a group of metastasis-related cancerous cells enriched in TDLNs with a high epithelial-to-mesenchymal (EMT) ability regulated by autocrine TGF- β ; We find an increased number of cycling cancer-associated fibroblasts (CAFs) in TDLNs, and strong adhesion between these cells and metastasis-related cancerous cells mediated by integrins, suggesting a CAF-driven invasion process in TDLNs; We also reveal a suppressed immune microenvironment in TDLNs featured by a monocyte-to-M2-macrophage differentiation process mediated by transcription factor MAFB, enrichment of immunosuppressive components such as myeloid-derived-suppressor cells (MDSCs), dysfunctional CD4+ cells, regulatory T cells, and IL10+ B cells, depletion of immune components such as mast cells, cytotoxic CD8+ cells, and plasma B cells. Notably, we develop a “Sec-Mem” method to screen for abnormal communications between different components using scRNAseq data and depict a metastasis-related regulatory network including many known interactions revealed for the first time in TDLNs of gastric cancer.

Our study provides a valuable resource for the exploration of lymph node metastasis in gastric cancer and has implications in developing novel therapies for the inhibition of gastric cancer metastasis.

P289

CD73 IMMUNOPET IMAGING FOR NON-INVASIVE EVALUATION OF TUMOR CD73 EXPRESSION IN VIVO

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The reactivation of tumor-specific T cells by immune checkpoint inhibitors (ICIs) has recently demonstrated remarkable clinical efficacy in various tumor types. However, the response to ICIs treatment has its limits. Tumor-mediated immunosuppressive microenvironment contributes to immune tolerance and reduced CD8+ CTL induction, infiltration and cytolytic function. CD73 is highly expressed by most types of immunosuppressive cells and plays an important role in adenosine-mediated immunosuppressive pathways. Therefore, CD73 imaging biomarkers could be imaged positron emission tomography with computed tomography (PET/CT), which can provide in vivo, real-time and non-invasive imaging of CD73 expression level in tumor and lymph nodes and predict immune responses to combining ICIs and/or novel therapeutic regimens.

Here we prepared the immunoPET imaging agent which mouse CD73 (mCD73) specific antibody was conjugated p-SCN-Bn-3,6,9,15-Tetraazabicyclo-pentadeca-triene-(4-isothiocyanatobenzyl)-3,6,9-triacetic acid (PCTA) and radiolabeled Cu-64, and performed cell binding assay in 4T1 (CD73 positive) and Pan02 (CD73 negative) mouse cancer cells, the biodistribution and microPET imaging in naïve BALB/c mice and 4T1 syngeneic tumor model.

Cu-64-labeled mCD73 antibody specifically bound to CD73 expressing 4T1 cells, not Pan02 cells. In biodistribution and microPET imaging study, the uptake of Cu-64 labeled mCD73 antibody showed similar pattern in most organs between naïve BALB/c mice and 4T1 syngeneic tumor model and Cu-64 labeled mCD73 antibody was selectively localized in 4T1 tumor and tumor-draining lymph nodes.

Cu-64 labeled mCD73 immunoPET imaging could be useful for the evaluation of the CD73 expression level in tumor and lymph nodes and may be used as the predictive imaging biomarker for monitoring immune response to ICIs treatment and/or alternative therapeutic strategy.

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EXPLOITING TUMOR RNA-SEQUENCING DATA FOR PREDICTION OF IMMUNE CHECKPOINT INHIBITION RESPONSE

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Immune checkpoint inhibitors (ICI) have become the standard of care as a first- or second-line systemic treatment for patients with various cancer types. Unfortunately, many patients do not respond to this treatment and the occurrence of immune-related adverse events varies widely. There is an urgent need for robust predictive biomarkers for ICI response to select patients with likely clinical benefit from this costly treatment. Several biomarkers, aimed at predicting response to ICI, have been proposed, including PD-L1 expression, tumor mutation burden (TMB), infiltration of cytotoxic T-cells in the tumor, Microsatellite Instability (MSI) and expression of various immune gene signatures. However, these individual biomarkers have suboptimal performance and multiple complementary omics technologies are required to properly quantify them

Integration of these multi-omic biomarkers has proven valuable in increasing the accuracy and robustness of ICI response prediction. We have developed a computational pipeline that determines the expressed mutation burden (eTMB), MSI status, fraction of infiltrating immune cells and various immune gene expression signatures directly from the RNA-sequencing profile of the tumor. Each biomarker is quantified using a dedicated algorithm that applies machine learning (eTMB and MSI) or computational deconvolution (infiltrating immune cells) and integrates external data sources to maximize performance.

Algorithm performance has been validated on large cohorts of tumor RNA-sequencing data with matching gold standard quantification of said biomarkers. Moreover, integrating these biomarkers improves prediction of response to checkpoint inhibition therapy.

Taken together, our approach enables the quantification of various ICI response biomarkers from a single omics layer (RNA-seq) and can contribute to ICI response prediction.

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DISCOVERY OF ANTIGEN-SPECIFIC B-CELL AND T-CELL CLONOTYPES USING A MULTIPLEXED dCODE DEXTRAMER®-BASED WORKFLOW

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Understanding the functional status of the antigen-specific B and T cell responses and identification of the BCR/TCR clonotypes are key for developing novel cancer vaccines and immunotherapies. The DNA barcoded dCODE Dextramer® and dCODE Klickmer® reagents allow detection of antigen-specific cells through their corresponding B and T cell receptors (BCR/TCR), providing access to full-length paired receptor sequences, gene expression profile, and cell surface proteins from the same cell when used in combination with single cell technologies. Obtaining full-length, paired BCR sequences provides a resource for efficient antibody discovery for therapeutic and prophylactic use. Likewise, accessing full-length, paired TCR sequences enables investigation of receptor potential in adoptive cell therapies like CAR T-cell therapy.

Here, we established a workflow for simultaneous discovery of antigen-specific B-cell and T-cell clonotypes using a multiplexed dCODE Dextramer®-based approach combined with the 10x Chromium Single-Cell Analysis System.

A virus-specific model was used to establish the workflow. The workflow contained a panel of dCODE MHC Dextramer® and dCODE Klickmer® reagents covering 21 specificities for detection of antigen-specific B and T cells, respectively, recognizing CMV, EBV, flu, SARS-CoV-2 antigens and controls. The workflow was demonstrated by assessing the virus specific cells and their BCR/TCR clonotypes after a SARS-CoV-2 infection. PBMC samples were collected from a donor immediately after (TP1) and six weeks after (TP2) infection. Each of the PBMC samples was stained with the panel of dCODE® reagents in the same tube and subjected to (1) Sorting of antigen-specific cells by flow cytometry, (2) 10x Chromium Single-Cell Analysis System, (3) Library preparation and (4) Sequencing.

A total of 3408 (TP1) and 5680 (TP2) T cells and 4686 (TP1) and 4765 (TP2) B cells were called in the VDJ pipeline with a high frequency of productive V-J spanning pairs (60-92%). We detected BCR and TCR clonotypes towards the tested virus specificities and identified clonal expansion of the SARS-CoV-2-specific B and T cell populations. Several of the identified paired V(D)J sequences were matched with publicly available COVID-19 BCR and TCR datasets, confirming the accuracy of the hits from the workflow.

Here we show a multiplexed workflow for simultaneous detection of antigen-specific B and T cells in combination with their corresponding BCR and TCR clonotypes, gene expression and surface marker profiles thereby investigating the nature of induced antigen-specific B and T cell responses. We included a total of 21 antigen specificities using a virus as model system. However, this workflow can be tailored to cancer-specific responses by using dCODE reagents displaying cancer antigens. The workflow can provide information with potential to facilitate diagnostic approaches, prediction of disease progression and development of novel cancer vaccines and therapeutics.

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COMBINATION THERAPY OF PADELIPORFIN VTP WITH CYCLOPHOSPHAMIDE (CTX) IN MICE BEARING ORTHOTOPIC KPC PANCREATIC TUMORS.

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Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal human malignancies. Early detection and surgical removal of PDAC, when the cancer is localized with no clinical evidence for systemic spread, may be curative but when tumor spreads into the vicinity of major blood vessels, e.g. superior mesenteric artery (SMA), a surgery can be lethal and therefore will be avoided. The principle of WST11 VTP comprises systemic injection of the photodynamic substance WST11, shortly followed by photosensitization in the tumor vasculature at 755nm using light delivering system adjusted to the tumor bearing organ. The underlying mechanism involves primarily vascular occlusion that initiates an ischemia/reperfusion like cascade of events resulting in vascular collapse, paraptosis of endothelial cells, propagating wave of cell death from the tumor vasculature into the tumor core, release of danger associated molecular patterns (DAMPs) and activation of adaptive antitumor immunity which was shown by us and other to eradicate remote micro metastases. Here we show that Vascular Targeted Photodynamic Therapy (VTP) with WST11 in combination with cyclophosphamide (CTX), allows PDAC tumors ablation while preserving large normal vessels and tissues in animal models. New endovascular illumination system recently developed by our group (1) provides the light needed for such ablation with no damage to the SMA and complete remodeling of the surrounding normal tissue.

Orthotopic model of pancreatic cancer, non-labeled KPC tumors (NL-KPC) in C57B mice was subjected to VTP, alongside intraperitoneal CTX treatment. Multiplex immunohistochemistry (mIHC) and single-cell analysis using 10X Genomics platform of tumor were performed for resolving the key factors in the therapeutic process. Final Results of a Phase I Trial of WST-11 VTP.

WST11 VTP in combination with CTX primarily ablates only 65-80% of the orthotopic PDAC in mice at 24-72h following 10 min interstitial illumination. However, it conveys prolonged anti-tumor immune response in correlation with the observed delayed disease progression and a significant higher prolongation of animal survival. At 72 h post treatment, the ablated tumor volume by the combined therapy is smaller than by VTP alone, tumor growth rate is significantly delayed compared to VTP alone or CTX alone. Significant trafficking of cytotoxic T cells to the tumor rim confirms tumor conversion from immunogenically "cold" to immunogenically "Hot." The increased exposure to the anti-tumor immune system appears to result in a longer median survival of animals that underwent combined treatment for 50 days compared to 33 days in mice treated with 6 doses of CTX alone. VTP only treated mice presented significantly prolonged survival in comparison to non-treated animals (average survival time is 17 days for VTP-treated mice compared with an average 13 days of non-treated mice).

The administration of CTX amplified and prolonged the VTP oxidative stress. WST11 VTP combined with immune modulating chemotherapeutic agents administrations, activated by endovascular illumination through the SMA, may provide a solution to the unmet need for early stage diagnosed PDAC patients.

(1) Franz E. Boas et al, "Downstaging Locally Advanced Pancreatic Cancer To Resectability: Perivascular Ablation Using An Intra-arterial Balloon Laser Catheter In Pigs", Abstract Archives of

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DECODING THE ANTIGEN PRESENTING STROMA LANDSCAPE OF LUNG TUMORS.

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We recently discovered universal antigen presenting cancer associated fibroblasts (CAFs) across human and murine lung tumors and showed that they directly stimulate cancer-specific CD4 T cells, creating immune hotspots that support immune rejection. These studies achieved a breakthrough on the role of in situ cancer antigen presentation and proposed a novel model whereby lung tumors can sustain T cells independently of lymph nodes. For the immunotherapeutic exploitation of antigen presenting fibroblasts a significant bottleneck must be overcome: our incomplete understanding of their heterogeneity, origins and configurations. Our laboratory works to address the following questions: i) how do diverse antigen presenting stroma states emerge and evolve in lung tumors? ii) which gene regulatory networks drive specificity of these states? iii) which are the functional modules that drive

In this work, we analyzed an integrated paired lung tumor/healthy lung scRNAseq dataset from 37 non-small cell lung cancer (NSCLC) patients, 2 multiplexed imaging datasets and 2 publicly available spatial molecular imaging (CosMx SMI) datasets. State-of-the art single cell and spatial transcriptomic analysis tools were applied to identify and deconvolute the antigen presenting stroma, infer their origins and gene regulatory networks and decode their organization in immune hot neighborhoods. To experimentally validate our computational findings, we employed orthotopic murine lung cancer models and utilized ex vivo human/murine co-culture systems.

2 distinct human lung cell states co-expressed fibroblastic and antigen-presenting signatures: an epithelial cell-like with an alveolar-type II signature (epiFibros) and a fibrocyte-like with a hematopoietic cell/monocyte signature (Fibrocytes). EpiFibros and fibrocytes were found enriched in human lung tumor versus unaffected lung specimens and shared a common adaptive immune gene expression program. Cell-cell-communication analysis inferred novel heterotypic interactions between epiFibros, fibrocytes, B cells and T cells via paracrine signaling. In vivo lineage tracing of murine fibrocytes using fluorescent reporter bone marrow chimeras confirmed that a subset of bona fide lung fibroblasts originates from the bone marrow and effectively presents MHCII cancer antigens to CD4 T cells ex-vivo. Trajectory inference uncovered a dynamic molecular network of ETS family members that underlines the transition of alveolar type II cells to epiFibros. SMI and multiplexed phenotyping (CODEX) show that epiCAFs and fibrocytes are organized close to/within lymphoid aggregates of primary human lung tumors. We are currently working to decode these spatial patterns.

In summary, integrated computational and experimental approaches uncover diverse antigen presenting states and their organizing principles in lung tumors.

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SYSTEMATIC IDENTIFICATION OF CANCER NEOANTIGENS DERIVED FROM NEO-OPEN READING FRAMES

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Immunotherapy has provided a major leap forward in the treatment of cancer. Major efforts focus on development of personalized neoantigen-directed immunotherapies, such as cancer vaccines and T-cell therapies. However, identification of highly immunogenic neoantigens as target for therapy from a tumor sample is challenging. We integrated cancer whole genome and long-read transcript sequencing to identify the entire collection of novel open reading frame peptides (NOPs) expressed in tumors. As opposed to neoantigens derived from missense mutations, NOPs represent tumor-specific peptides that are completely different from wild-type proteins and thus have the potential to be strongly immunogenic.

We applied combination of whole genome sequencing, and short- and long RNA sequencing to identify NOPs expressed by tumor cells in 61 tumor samples of various origin. This approach detects full-length transcripts encoding NOPs at a single-molecule resolution, thereby accounting for isoform diversity. RiboSeq and proteomic analysis were used to confirm translation of NOPs into proteins. Model cell lines were used to analyze presentation of peptides on MHC class I by immunopeptidomic analysis. The immunogenicity of NOP-derived peptides was tested in in vitro immunogenicity assay using blood of healthy individuals where production of IFN gamma and TNF alpha by antigen specific CD8+ T cells was measured by flow cytometry. Blood of patients with non-small cell lung cancer was used to detect antigen specific CD8+ T cells recognizing NOP-peptide tetramer complexes and their phenotype (naïve versus memory) was determined by flow cytometry.

We describe an uncharacterized class of so-called hidden NOPs, which is derived from structural genomic variants involving an upstream known protein coding gene that directly drives expression and translation of non-coding regions of the genome downstream of a rearrangement breakpoint. NOPs are prevalent in major cancer types and represent a vast amount of possible neoantigens particularly in tumors with many (complex) structural genomic variants and a low number of missense mutations. We show that NOPs are being translated and can be detected on protein level. The epitopes derived from NOPs can bind to MHC class I molecules and are inducing in vitro CD8+ T

cell responses. We additionally provide evidence for the presence of memory T-cells specific for hidden NOPs in a lung cancer patient peripheral blood.

Our work highlights a major source of neoantigens for personalized immunotherapies and provides a workflow for analyzing the complete cancer genome and transcriptome as a basis for systematic detection of NOPs.

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SELECTIVE INCREASE OF NA⁺ INTRACELLULAR KILLS MURINE HEPATOCARCINOMA CELLS: A NOVEL DIAGNOSTIC CANCER BIOMARKER AND A NEW ANTICANCER STRATEGY BESIDE IMMUNOTHERAPY?

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Limited or lack of responses to chemotherapy, biological therapy or immunotherapy make necessary exploring novel anticancer targets and diagnostic biomarkers.

Intracellular alkaline pH is a hallmark of cancer cells and relies on the increased activity or expression of pH regulatory proteins and, among them, of Na⁺ transporters. In primary non-cancerous hepatocytes, following ATP depletion, an irreversible increase of intracellular Na⁺ is the final cause of the osmotic lysis and death of the cells. In cancer research, the significance of Na⁺ intracellular variations is under investigated. Early X ray microanalysis of rodent cells and recent MRI studies on human cancers, however, evidence an augmented intracellular concentration of Na⁺ in cancer cells in comparison to healthy cells. Moreover, "Field-Cycling Relaxometry" studies show that there is a correlation between cellular water molecule efflux rate constant and the related intracellular water mole fraction, with cancer aggressiveness.

This study explores the hypothesis that cancer cells, owing to a constitutively high intracellular Na⁺ concentration and high energy-consuming metabolism might be, unlike non-cancerous cells, energetically unable to compensate and survive to a further Na⁺ load induced by the cation ionophore Monensin.

The effects of Monensin treatment were evaluated in vitro and in vivo employing murine and human HCC cell lines (C1C7 and HepG2 cells) and primary mouse hepatocytes (cultured in DMEM and Krebs or Krebs without Na⁺ buffers) or HCC (C1C7 cells) implanted allografts in male NSG mice. Cell death, ATP, levels and glycolysis were evaluated with standard protocols. Mitochondrial O₂ consumption rate fluxes were analyzed with an Oxygraph-2 K high-resolution respirometer. Na⁺ levels were monitored by fluorimetry, ICP-MS, ²³Na-MRI acquisition on a 7 T Bruker Biospin Pharmascan 70/16 scanner equipped with a ¹H/²³Na transmit-receive surface coil and confocal or time-lapse imaging analysis with the THUNDER Imager 3D Live Cell. Water fluxes across cell membrane were determined by ¹H NMR relaxometry. Hemopoiesis was evaluated by analysing bone marrow cells acquired on a Attune NxT Acoustic Focusing Cytometer. Tissue damage and proliferation were estimated by histological and immuno-histochemical analyses (H&E and Ki67 staining).

Na⁺ levels of healthy hepatocytes and liver was lower compared to mouse and human HCC cells and tumour tissue. Monensin further increased Na⁺ levels in HCC cells and in HCC allograft but not in primary hepatocytes and in normal hepatic and extrahepatic tissue. Sodium increase was associated to ATP depletion, mitochondrial Na⁺ load, inhibition of mitochondrial O₂ consumption and decrease of the water efflux rate constant and preceded the death of HCC cells. Prevention of intracellular Na⁺ increase by maintaining HCC cells in a Na⁺ free media protected energy and osmotic alterations and appearance of cell death. Monensin systemic treatment induced shrinkage and necrosis of the allograft tumor but did not display damaging effects nor affected the integrity of normal organs. Monensin did not evidence cytostatic effects in either healthy or transformed proliferating tissues nor altered hemopoiesis.

These results show that a forced sodium influx selectively kills hepatocarcinoma cells without affecting normal cells or inhibiting proliferation of normal or transformed tissues. Such observation enlightens Na⁺ homeostasis as novel druggable target for HCC therapy and cancer in general. The capacity of Monensin to selectively increase Na⁺ in transformed tissue thereby inducing

specific cancer cell death reveals a novel anticancer mechanism of Na⁺ ionophores and, additionally, suggests their potentiality as diagnostic tools to magnify the detection of the increased sodium levels associated with malignancy.

P296**ANTI-TUMOUR ACTIVITY OF HUMAN NATURAL KILLER CELLS ENGINEERED WITH A CHIMERIC ILT RECEPTOR**Duong M., Park J., Ognar R., Wain--Hobson S.*, Bayle H.*NKILT Therapeutics, Inc ~ Houston ~ United States of America*

Design of CAR-based cell therapies that target neoplastic tissue is challenged by a lack of cell surface target proteins that are discretely expressed on tumor tissue and not normal tissue. The MHC Class 1B protein HLA-G is highly expressed in cytotrophoblasts of the human placenta where it directs the immune evasion of haploidentical tissue. HLA-G is broadly expressed by approximately 50% of solid tumors and leukemias including Acute Myeloid Leukemias (AML) as a mechanism to evade immune attack. HLA-G has very little expression outside the placenta. HLA-G directs immunosuppression by binding to ILT receptors (ILT2 in lymphoid cells and ILT4 in myeloid cells). ILT2 and ILT4 have a similar protein architecture with four extracellular immunoglobulin domains and 2 to 4 ITIM signaling domains that recruit SHP phosphatases to block immune cell activation. HLA-G is expressed as 7 isoforms produced by alternative mRNA splicing that can evade epitope-specific targeting by an antibody/scFv-based CAR approach, but functional HLA-G isoforms necessary to direct immune evasion of tumor tissue must bind with ILT2 and ILT4 receptors. We report the engineering of ILT2 and ILT4 proteins into chimeric signaling agents (Chimeric ILT Receptors, or CIRs) that drive activation of Natural Killer (NK) cell upon engagement with HLA-G and targeting of HLA-G expressing AML cells.

CD56+ NK cells were selected from peripheral blood mononuclear cells (PBMC), incubated with IL-15 and activated with a feeder free cocktail of an immobilized cytokine and activating ligand. Activated NK cells were then mock transduced or transduced with gamma-retroviruses (rv) directing expression of CIR proteins, soluble IL-15 and a del-CD19 marker protein (ILT2D1-D4.CD8alpha.4-1BB.CD3zeta-IL15-delCD19, ILT4D1-D4.CD8alpha.4-1BB.zeta-IL15-delCD19, similar constructs containing only the D1 and D2 domains of ILT2 and ILT4 or control constructs expressing scFv-based CARs directed to HLA-G or CD33). In comparative experiments, T cells from PBMCs were prepared transduced to produce CIR-T cells by standard methods. After 8 days of expansion, CIR- or CAR-NK cells were cocultured with human GFP-FfLuc-expressing AML target cell lines with or without exogenous HLA-G isoforms. Short-term (2-day) and long-term (7-9 day) cytotoxicity of CIR-NK cells were determined by IncuCyte or Celigo imaging and luciferase activity. NK cell phenotype was determined by flow cytometry and release of cytokines measured by ELISA.

NK cells were transduced efficiently (up to 70%) with gamma-rv driving CIR or CAR expression. In cocultures with THP-1 AML cells that do not express endogenous HLA-G, mock-transduced NK cells displayed innate killing activity that was not augmented by CIR expression. In cocultures against THP-1-GFPffluc target cells expressing exogenous HLA-G1, G2 or G5 isoforms, CIR-NK cells or CIR-T cells displayed augmented target cell killing relative to mock-transduced effector cells in 2-day and 7-day assays. CIR constructs with deleted D3 and D4 immunoglobulin domains showed enhanced HLA-G targeting. In these assays, ILT4 CIR-T cells recognized target cells expressing HLA-G2 that were not recognized by HLA-G CAR-T cells. MOLM13 and Kasumi1 AML cells express low endogenous levels of HLA-G1 and G5. In stress-test cocultures against these targets, CIR-NK cells were displayed potent serial killing activity as well as enhanced secretion of IFN-gamma at an effector to target (E:T) ratio of 1:10 and 1:20. This anti-tumor efficacy and IFN-g production were further enhanced in a screen for potent coactivation domains other than 4-1BB that naturally activate innate immunity.

Engineered CIR-NK cells can convert HLA-G expression in AML from a potent inhibitor of lymphocyte activity into a target for anti-tumor control. This technology takes advantage of the

physiological pairing of the ILT receptor and HLA-G ligand thus permitting targeting of multiple HLA-G isoforms.

P297**DEFINING AND THERAPEUTICALLY TARGETING A FUSION-DERIVED PUBLIC NEOANTIGEN IN DESMOPLASTIC SMALL ROUND CELL TUMOR USING T CELL RECEPTOR GENE THERAPY**

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Sarcomas are a heterogeneous group of malignancies of mesenchymal cell origin that are difficult to treat with often poor prognoses. Approximately 30% of sarcomas are characterized by expression of oncogenic fusion proteins. Desmoplastic small round cell tumor (DSRCT) is a prototypical fusion-driven sarcoma defined by a pathognomonic EWSR1-WT1 fusion event. The resultant EWSR1-WT1 oncogenic fusion protein contains a shared junctional amino acid sequence divergent from normal self-proteins. Such clonally conserved oncogenic fusion proteins might yield a particularly immunogenic subset of shared, or public, neoantigens (NeoAgs) serving as potential targets for novel immunotherapeutic approaches.

To determine if fusion-derived NeoAgs are processed and presented in the context of prevalent HLA alleles, we performed a functional HLA-immunoprecipitation/mass spectrometry (HLA-IP/MS) screen. Next, using fluorophore-conjugated HLA-multimers (dextramers) loaded with HLA-IP/MS-derived fusion NeoAgs, we labeled peripheral blood mononuclear cells (PBMCs) from HLA+ DSRCT patients to identify circulating NeoAg-specific CD8+ T cells. We then used in vitro antigen-directed clonal expansion of fusion NeoAg-specific T cells to identify fusion NeoAg-reactive T cell clones. Using 10x single cell sequencing, we retrieved the TCR α gene sequences of the T cell receptors (TCRs) expressed by these T cells and cloned these into retroviral expression vectors. Polyclonal CD8+ T cells were then transduced with candidate TCR genes for functional validation via three assays: i) the ability to bind fusion-NeoAg loaded dextramers, ii) effector cytokine production after co-culture with EWSR1-WT1+ antigen presenting cells (APCs), and iii) direct lysis of DSRCT cells in vitro.

Using our functional HLA-IP/MS screen, we detected a 9-amino acid peptide sequence (SSYGQQSEK) derived from the junction of the EWSR1-WT1 fusion protein and presented in the context of the prevalent HLA-A*03 and -A*11 alleles. We confirmed by HLA-IP/MS that HLA-A*03+ DSRCT cells physiologically present the same peptide sequence. Furthermore, we identified a rare population of T cells that bind dextramers loaded with this NeoAg in PBMCs from DSRCT patients, confirming immunogenicity. We subsequently discovered and sequenced n=3 HLA-A*03-restricted and n=1 HLA-A*11-restricted fusion NeoAg-specific TCRs. All candidate TCRs bound fusion NeoAg-loaded dextramers, but not viral-peptide loaded control dextramers. Additionally, CD8+ T cells expressing candidate TCRs robustly upregulated TNF α expression after co-culture with APCs expressing the requisite HLA allele and the EWSR1-WT1 fusion (HLA+/Fusion+), but not APCs that were either HLA-/Fusion+ or HLA+/Fusion-. Lastly, CD8+ T cells expressing candidate TCRs specifically lysed HLA+ DSRCT cells in vitro. DSRCT cell killing was attenuated in the presence of an HLA blocking antibody and HLA- DSRCT cells. CD8+ T cells expressing viral protein-specific TCRs did not lyse DSRCT cells. Interestingly, one HLA-A*03-restricted TCR engaged fusion NeoAg in the context of both HLA-A*03 and -A*11, but not HLA-A*02. This TCR mediated HLA-A*03+ and -A*11+ DSRCT cell lysis, implying that a single TCR therapeutic could cover >36% of all North American DSRCT patients.

Our data establishes that the junction of the recurrent EWSR1-WT1 fusion is physiologically processed and presented by DSRCT cells in the context of two prevalent HLA alleles. Illustrating that fusion-derived public NeoAgs are indeed actionable, we have successfully cloned a library fusion NeoAg-specific TCRs that confer specific cytolysis of DSRCTs, laying the foundation for first-in-human clinical translation of a T cell-based therapy targeting EWSR1-WT1. More broadly, our work establishes proof of principle that fusion proteins are a viable source of immunogenic public NeoAgs that can facilitate the generation of targeted immunotherapies for fusion-driven malignancies.

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CO-TARGETING AUTOPHAGY, MACROPHAGES, AND VASCULATURE IN GLIOMA TUMORS TRIGGERS IMMUNOGENICITY

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Glioblastoma (GBM) is the most common primary tumor arising in the central nervous system (CNS). The currently approved standard of care, consisting of radiotherapy combined with chemotherapy, has transient clinical benefit as GBM tends to be exceedingly aggressive. The VEGF-blocking antibody bevacizumab is also approved, despite its lack of survival benefit, primarily due to its benefits in reducing edema. Other therapeutic agents have been tested, including inhibitors targeting aberrantly activated pathways in GBM, such as EGFR, PDGFR, or c-Met, and immune checkpoint inhibitors, all largely to no avail. As such, given the dearth of effective treatments for this devastating cancer, new treatment strategies are urgently needed. One approach would be to co-target and thereby disrupt distinct hallmarks of cancer, aiming to elicit sustained therapeutic responses. We chose to focus on the intense angiogenic tumor vasculature of GBM, reasoning that perhaps the failed therapeutic efficacy of the anti-VEGF antibody bevacizumab and several small molecule receptor tyrosine kinase inhibitors of the VEGF receptors might nevertheless prove to have benefit if combined with drugs targeting other mechanistic vulnerabilities.

To facilitate critical assessment of the hypothesized benefits of co-targeting strategies, we established a mouse model of focal, de novo development of GBM, involving stereotactic inoculation into the hippocampus of a lentivirus conditionally expressing an activated HRasV12 oncogene and an shRNA that knocks down expression of the p53 tumor suppressor, along with a luciferase reporter. This immunocompetent transgenic mouse model resembles the human disease in its development, histopathology, and response to therapy. Using state-of-art technologies, and ex vivo analyses, we have extensively profiled and assessed the role of innate and adaptive immune cells, vascular modulation, and cancer cell-intrinsic mechanisms in orchestrating a potent anti-tumoral response.

We have assessed the concept of co-targeting distinctive mechanistic vulnerabilities of the disease by combining a tricyclic antidepressant -imipramine - with drugs targeting VEGF-A ligand or VEGF-Receptor in mice bearing de novo GBM. All monotherapies were ineffective. In notable contradistinction, we found that combinatorial regimens significantly increased survival benefit and regressed established tumors. Investigation of the basis for the therapeutic efficacy revealed that combining the VEGF pathway inhibitor with imipramine accentuated immunostimulatory autophagy in cancer cells while modifying the angiogenic tumor vasculature to be more normal-like with induction of high endothelial venules. In addition, imipramine downregulated an M2-like phenotype of tumor-associated macrophages via histamine receptor and reprogrammed them to express chemokines attracting otherwise rare CD8 T cells, which demonstrably contributed to the observed efficacy. As such, these hallmark co-targeting combinations reprogram the GBM microenvironment from immunosuppressive to pro-inflammatory, thereby rendering it immunogenic and sensitizing the tumors to immune checkpoint blockade, as evidenced by enhanced responses when an anti-PD-L1 therapy was included in the mix.

In conclusion, the mechanism-guided combination of three classes of clinically-approved drugs described herein has remarkable therapeutic efficacy in a mouse model of GBM, by virtue of concordantly modifying multiple features of the otherwise immuno-suppressive glioma tumor microenvironment, thereby rendering it immunogenic and susceptible to efficacious immune attack. The results to be presented will elaborate on a provocative new therapeutic approach for glioblastoma that has the prospect of motivating clinical evaluation in this daunting form of human cancer.

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ANALYSIS OF ANTIANGIOGENIC AND IMMUNE CHECKPOINT INHIBITOR COMBINATION TREATMENT IN PRIMARY RENAL CELL CARCINOMA EXPLANT MODEL

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Renal cell carcinoma (RCC) is an immunogenic cancer, with high immune cell infiltration. It is characterized by the overproduction of vascular endothelial growth factors, leading to a hyper angiogenic state. While immunotherapy and antiangiogenic treatments show promise, targeted inhibitors of angiogenesis often yield only temporary responses. Furthermore, the immunomodulatory effects of currently used inhibitors remain poorly understood.

In this study, our objective was to characterise the immunological effects of antiangiogenic targeted therapy in combination with immune checkpoint inhibition therapy in RCC tumor samples.

We collected tumor, adjacent healthy tissue, and peripheral blood from fifteen immuno-oncology (IO) treatment naïve RCC patients undergoing nephrectomy. The immune phenotypes of tumor and healthy tissue infiltrating lymphocytes were characterized using flow cytometry. We established an ex vivo renal tumor model allowing the testing of early immunological responses to targeted therapy and immunotherapy. To maintain the tumor immune contexture and assess immune cell functionality, we utilized matrix-embedded tumor cell dissociates.

Our study included five different conditions: negative control, tyrosine kinase inhibitor axitinib, anti-PD1, combination treatment and immunocult as a positive control.

Immune cell activation was measured using mass cytometry for seven patients and single-cell sequencing for additional eight patients. As a validation, cytokine profiling may be performed from the culture media samples.

Our study integrated a comprehensive analysis of the immune microenvironment in RCC samples using mass cytometry and single-cell sequencing. We uncovered a diverse set of tumor-infiltrating lymphocytes (TILs), including T- and B-cells as part of adaptive immunity, as well as NK- and myeloid cells representing innate immunity.

We further analyzed the T lymphocyte population to identify a distinct subtype characterized by cytotoxic and exhausted features. This subset exhibited unique marker expression patterns, such as PDCD1, LAG3, HAVCR2, PRF1, GNLY and various granzymes. These findings provide valuable insights into the functional state of TILs and potential immune responses. Additionally within this subset, we observed the presence of the most clonally expanded T-cells.

Up to date, our study yielded preliminary results showing modest, but reproducible effects of axitinib at the transcriptional level. However, when axitinib was combined with an anti-PD1 drug, no significant additive effects were observed. It is important to note that these changes were not restricted to T-cell clusters, but were also apparent in other immune cell subtypes, including NK-cells. These findings suggest a broader effect of axitinib on the immune response in patients with RCC.

The present study aimed to understand the immunological effects of combining antiangiogenic targeted therapy with immune checkpoint inhibition in RCC patients. The observed effects of axitinib on T- and NK-cell subsets contribute to a deeper comprehension of the interaction between the therapy and immune cells, advancing our knowledge of immune responses in RCC. Furthermore, our results have the potential to aid in optimizing treatment strategies in the future.

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COMPLEMENT ACTIVATION SUSTAINS TUMOR PROGRESSION AND INVASIVENESS IN HUMAN AND MOUSE CRC

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The complement system emerges as a major player of tumor-promoting inflammation (1, 2). Here, we investigated its contribution to colorectal cancer (CRC) development, where its role is still debated. It has been reported that complement activation may exert pro or anti-tumoral function by regulating tumor cell functions or the tumor microenvironment (TME) through several mechanisms (3, 4). These different effects may depend on sites of complement activation, composition of the TME, tumor cell sensitivity to complement attack and tumor model. For example, in genetic and chemically-induced models, complement targeting appears to mainly impact on immune cell recruitment and functions, while in transplantable models it modulates TME or cancer cell functions (2-5).

First, we interrogated the human TCGA dataset in an effort to obtain indications as the possible correlation between complement elements expression and CRC patient outcome. Then, we investigated C5aR1 expression on a cohort of CRC patient-derived tissues. We evaluated the role and mechanisms of complement in CRC by exploiting transplantable primary and metastatic MC38 CRC models in mice deficient of complement components. Finally, in vitro invasion assays have been used to evaluate the responsiveness of MC38 cells to C5a.

We observed a negative correlation between the expression of C3 or C5aR1, but not C3aR nor C5aR2, and survival of CRC patients from the human TCGA dataset. In a cohort of CRC patient-derived tissues, we found that C5aR1 was mainly expressed by tumor cells and its expression was associated with lymph node metastasis, stage II to stage III transition and poor clinical outcome. In agreement with these results, C5aR1 expression by tumor cells and its association with tumor progression and poor prognosis have been also reported in lung, gastric and breast cancer (2). In preclinical mouse models of CRC, MBL1/2^{-/-}, C4^{-/-} and C3^{-/-} mice showed reduced susceptibility to primary tumor growth, as well as to CRC liver metastasis development, while C1q and factor B deficiency had no impact, indicating the importance of C3 activation through the lectin pathway. We also investigated C3-downstream events and observed that C5aR1 deficiency did not affect MC38 growth, suggesting that its role in the TME was irrelevant in this model. In contrast, in vitro experiments showed that MC38 cells express C5aR1 and respond to C5a in invasion assays.

Overall, these results confirm that the role and target of complement in CRC depend on the pathogenesis of the tumor, and highlight that lectin pathway activation leading to C3 activation and are components of CRC progression and metastatic process, suggesting C5AR1 as a potential therapeutic target in this disease.

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ANTI-HPSCA CAR-NK92 CELL IMMUNOTHERAPY FOR THE TREATMENT OF PANCREATIC CANCER

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Pancreatic cancer (PC) is one of the leading causes of malignancy-related death, with almost half-million new diagnoses every year. Aberrant over-expression of the human prostate-stem cell antigen (hPSCA) has been detected in the vast majority of patients diagnosed with PC. Indeed, this molecule can be used as a potential therapeutic target, in particular in the context of adoptive cell therapy (ACT). In this regard, the innovative employment of NK92 as novel ACT cells is a new strategy. In fact, this cell line is characterized by well-defined expansion kinetics and high transduction efficiency, able to avoid the immunosuppressive tumor microenvironment, due to the absence of most inhibitory killer-cell immunoglobulin-like receptors (KIRs). Moreover, NK92 cells have been already approved for several clinical trials against different tumor types, showing high cytotoxic activity, even after irradiation required by authorities.

We designed a new CAR anti-hPSCA to transduce NK92 cells, representing a ready-to-use therapy, avoiding problems related to other ACTs, such as the donor-dependent variability of the transduction efficiency and the expansion kinetics. We analyzed the expression of the CAR molecule and the phenotype of NK92 cells, before and after the transduction. We tested anti-hPSCA CAR transduced NK92 cells for specific cytotoxic activity, cytokine release ability, and degranulation in the presence of target cells both before and after irradiation, to assess the effect of irradiation itself on the CAR-NK92. Moreover, we have evaluated the therapeutic activity of CAR-NK92 in vivo in disseminated and orthotopic xenograft mouse models of human pancreatic cancer.

After 72h from the transduction of NK92 cells, the CAR anti-hPSCA was already detectable, together with the eGFP, used as a reporter gene, both under the control of a bi-directional promoter. Their expression was stable over several weeks, even after freeze and thawing. The phenotypic profile of activated NK cells was stable before and after the transduction. Cytotoxic activity of CAR-NK92 cells was evaluated after 4h-co-incubation with target cells, showing high and specific lysis of the antigen-expressing tumor cells, as well as specific cytokines release ability and the activation of degranulation after antigen engagement. In vivo, in disseminated and orthotopic tumor mouse models, the CAR-NK92 cell therapy showed control of the tumor growth and a significant improvement in survival compared to control mice.

Pancreatic cancer is still a hurdle in terms of effective treatments. NK92 cells are a promising strategy in the Adoptive cell therapy landscape, concerning the economic aspect, the high-scalable manufacturing, and the manageable handling procedure. Overall, the preliminary data of this work demonstrated that this CAR-NK92 therapy paves the way for a new therapeutic approach for PC, based on the development of an Off-the-Shelf, renewable, effective, and low-cost product that can be used as a ready-to-use drug.

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EXPLOITING MICROBIOTA COMMENSALS TO BOOST IMMUNOTHERAPY AND SWITCH MULTIPLE MYELOMA TRAJECTORY

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Clonal proliferation of neoplastic plasma cells in the bone marrow (BM) and organ dysfunction characterize multiple myeloma (MM), a treatable but incurable disease. Although asymptomatic smoldering multiple myeloma (SMM) often precedes symptomatic MM, most patients affected by SMM are only offered active observation, which increases their frustration and anxiety.

We recently demonstrated a direct link between the gut microbiota and T helper 17 (Th17) lymphocytes, which migrated from the gut to the BM in transgenic Vk*MYC mice developing de novo asymptomatic MM (Early-MM), and favored the expansion of neoplastic plasma cells, thus fueling progression to symptomatic disease. Similarly, in SMM patients, higher levels of BM IL-17 predicted accelerated disease. Interestingly, *Prevotella melaninogenica* (P.m.), a Gram negative human commensal, limited MM aggressiveness by restraining Th17 cell expansion. Because the gut microbiota also contributes to the therapeutic efficacy of immune checkpoint blockade (ICB) and neoplastic plasma cells express PD-L1, we hypothesized that modulation of the gut microbiota by P.m. would limit the expansion of Th17 cells in mice affected by Early-MM, thus fully exploiting the therapeutic potential of anti-PD-L1 against MM.

C57BL/6J mice challenged with MM cells and Vk*MYC mice affected by asymptomatic MM were treated with P.m. and/or anti-PD-L1 and disease progression was monitored by paraprotein quantification in blood. Anemia provided clinical evidence of symptomatic disease. Modification of the gut microbiota composition after the treatment were assessed by 16S rRNASeq. At sacrifice, gut, spleen and BM were analyzed by flow cytometry. To clarify the mechanism of Th17 induction by the gut microbiota, we conducted in vitro assays.

Administration of P.m. to mice challenged with Vk*MYC-derived MM cells increased the therapeutic efficacy of anti-PD-L1 antibodies limiting the expansion of Th17 cells. In the context of SMM-to-MM evolution, treatment of transgenic Early-MM Vk*MYC mice with P.m. significantly delayed the progression to symptomatic (Late)-MM. When P.m. was combined with anti-PD-L1 antibodies, evolution from Early-MM to Late-MM was further delayed or blocked. Mechanistically, P.m. restrained the expansion of gut-born Th17 cells in the BM of treated mice without dampening the antitumor cytotoxic response elicited by the ICB. Thus, combination of P.m. and anti-PD-L1 resulted in more favorable Th17/T regulatory cell ratio and CD8/Th17 ratio in the BM. Ex vivo, P.m.-conditioned DCs showed a limited propensity to produce Th17-polarizing cytokines. In vitro stimulation of both human and mouse DCs with P.m. reduced polarization of naïve T cells toward a Th17 phenotype.

Taken together, our data support the development of microbiota-based strategies in combination with ICB to treat full-blown MM and to prevent progression of asymptomatic SMM to full-blown disease.

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PROSTATE-CONFINED RADIOTHERAPY EMPOWERS TCR/CAR T CELLS AGAINST PRIMARY AND METASTATIC PROSTATE ADENOCARCINOMA

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Prostate Cancer (PC) is the most frequently diagnosed male cancer worldwide, and the second leading cause of cancer death. Radiotherapy (RT) and surgery can control the disease in the prostate, but expression of metastatic spreading emerges in a consistent fraction of patients. Adoptive T cell therapy (ACT) can transfer anti-tumor immunity and yet to date has shown poor efficacy against prostate cancer, due to its intrinsic characteristic: relatively low mutational load, scarcity of tumor-infiltrating lymphocytes, and the presence of an immunosuppressive tumor microenvironment. Given the immunogenic potential of fractionated RT, we postulated it could unlock PC to T-cell mediated immunorejection.

To test these possibilities, we adopted syngeneic (TRAMP, autochthonous Transgenic Adenocarcinoma of the Mouse Prostate model) and xenogeneic mouse prostate cancer models and set up a multiparametric flow cytometry analyses of prostate cancer patients undergoing radiotherapy. In preclinical settings, focal RT (3x8Gy RT) was delivered by a dedicated small animal micro-irradiator. ACT was performed by pre-conditioning mice with cyclophosphamide and infusing TCR-transduced T-cells. Magnetic resonance imaging was employed to image RT-induced effects within the tumor. Multiparametric flow cytometry, multiplex immunofluorescence, qPCR and histological analysis were used to define therapeutic effects and subtending mechanisms. Patients with low-grade prostate cancer patient minimally invasive disease were enrolled in an observational study (RIPRO-19). Peripheral blood mononuclear cells were bio-banked and analyzed by multiparametric flow cytometry.

We report that RT alone fails to confer anti-tumor effects, and yet it empowers ACT. Indeed, the combination of RT and ACT achieved acute and complete debulking of well-established primary adenocarcinomas and significantly prolonged mouse survival. Mechanistically, 3x8Gy RT exerted tumor-cell intrinsic and extrinsic effects. It increased MHC-I surface levels promoting T cell activation and proliferation both in vitro and in vivo. It also increased tumor vessel permeability, the expression of type I-IFN-responsive genes and caused remodeling of the tumor stroma, marked by an enrichment in MHC-II+/CD11c+ antigen presenting dendritic cells and decrease in myeloid-derived suppressor cells (MDSCs) in vivo. These events accompanied RT-dependent intra-tumoral proliferation of transferred T cells and the accumulation of IFN-gamma+ effector T cells of endogenous origin. In xenogeneic settings, the combination of local 3x8Gy RT with Prostate Specific Membrane Antigen (PSMA)-specific CAR T cells proved able to control primary and metastatic PC3-PSMA tumor lesions. Preliminary analysis of PC patients undergoing hypofractionated RT indicate RT-induced lymphopenia and the enrichment of T cells with memory features.

Data indicate that the sequential administration of prostate-confined hypo-fractionated RT and ACT can cure mice of established mouse and human PC. Radiotherapy promoted local inflammation,

immune cell infiltration and remodeling of the tumor microenvironment. This resulted in a significant survival advantage. In patients we found evidence of immune modulation, which paves the way for the combination of RT and immunotherapy.

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ELUCIDATION OF NOVEL EXTRACELLULAR MATRIX DERIVED IMMUNOMODULATORY MECHANISMS OF MACROPHAGES IN 3D MODELS

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Tissue architecture changes are common to solid tumors, resulting in dense, stiff tissue which is exploited by cancer cells to gain a niche for immune evasion and survival. It is well understood how this can benefit intrinsic cancer cell survival strategies, concurrently with poor penetration of immune cells and drugs due to occlusion at the tumor periphery. Emerging evidence indicates that beyond the structural difficulties this altered tissue architecture poses, there are inherent immunomodulatory features of the extracellular matrix (ECM) which can influence tumor microenvironment (TME) signaling. Successfully targeting the ECM has been a research area of great interest to further exploit the efficacy of immune checkpoint blockade (ICB) therapies in solid tumors. To elucidate novel ECM-derived immunomodulatory mechanisms, cell derived matrices from multiple different fibroblast cell types were assessed for their capacities to influence the phenotype of bone marrow-derived macrophages (BMDMs).

Cell-derived matrices (CDMs) were produced from multiple fibroblast/fibroblast-like cells including dermal fibroblasts, Mouse Embryonic Fibroblasts (MEFs) and MCA205 fibrosarcoma cells, all derived from a C57BL/6 background. Fibroblast RNA was isolated following 7 days of ascorbic acid-induced ECM deposition and gene expression was assessed for ECM associated genes. Alternatively, ECMs were decellularized to produce cell derived matrices (dCDMs) and characterized for area coverage, protein staining intensity, porosity, fibril diameter and fibril alignment. Bone marrow cells were isolated from C57BL/6 mice and cultured on dCDMs for 7 days followed by extraction of RNA from BMDMs and FACS analysis of cells for viability and phenotype. Alternatively, dCDMs were mechanically disrupted and added to BMDM's, with RNA isolated following 24 hours of stimulation. Gene expression analysis of key macrophage polarization genes was assessed by qRT-PCR.

Each fibroblast cell type produced dCDM's covering greater than 95% of the total area of wells, although protein quantity, porosity, fibril diameter and fibril alignment varied between cell types. qPCR analysis of fibroblasts mRNA expression suggested the CDM composition to differ greatly between cell types, with differences observed in key ECM genes including type I collagens and several Small Leucine Rich Proteoglycans. . Gene expression analysis of BMDMs revealed a strong inhibition of genes associated with classical activation of macrophages, suggesting the presence of these ECM influenced cells toward a 'wound-healing' phenotype. BMDMs cultured on these dCDMs showed varying phenotypes, with dCDM from several cell types capable of inducing protein expression of multiple macrophage polarization markers, including MHCII and PD-L1. Gene transcript analysis of BMDMs in response to mechanically disrupted dCDMs reflected this, with an inhibition of M1-associated genes concurrent with an induction of M2 markers, indicating the compositional make-up of these CDMs is influential in altering the transcriptome of BMDMs.

This work indicates that the ECM composition can play an influential role in the TME by directly influencing the transcriptome of key infiltrating immune populations and may contribute to tumor progression. These initial findings warrant further studies investigating the mechanisms and key components of the ECM which may be targetable to reduce the immunosuppressive effects of this dynamic tumor architecture.

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SYMPATHETIC NERVES ASSIST IN GROWTH OF EXPERIMENTAL LIVER METASTASES INDEPENDENTLY OF B-ADRENERGIC RECEPTORS

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The liver is a major site for metastasis, potentially due to its naturally tolerogenic immune environment. The immune system is suppressed in the tumor microenvironment (TME) of the liver, however treatment options that attempt to prevent or reverse the suppression of the immune system, like checkpoint inhibitors, have poor efficacy in patients with liver metastasis. This suggests that other mechanisms suppress immunity in the TME. Interestingly, high densities of nerve fibers have been reported in many cancer settings, and inhibition of nerve signaling (with β -blockers) was associated with reduced rates of metastasis and better responses to cancer immunotherapy. As the liver is innervated by the autonomic nervous system, activation of these nerves may contribute to immune suppression in the TME. However, the localization and distribution of autonomic nerves in the liver, the growth of nerves during liver metastasis, and the influence of tumor-associated autonomic nerves on immune function is not well characterized. In this study, we aim to 1) map the anatomical location and determine the type of autonomic nerves innervating the liver (sympathetic vs parasympathetic), 2) investigate the changes to hepatic neuroanatomy following liver metastasis, and 3) determine whether interfering with neuronal signaling alters growth of liver metastasis.

To study this, mouse liver tissue was stained with anti-PGP9.5, anti-tyrosine hydroxylase (TH), and anti-choline acetyltransferase (ChAT) antibodies to label all nerves, sympathetic nerves, and parasympathetic nerves, respectively. Furthermore, we generated a TH-cre-tdTomato reporter mouse to allow for in vivo visualization of TH-expressing nerves via intravital spinning disk confocal microscopy. As an experimental model of liver metastasis, the spleen of anesthetized mice was surgically exteriorized to allow for injection of fluorescently labelled B16F10 tumor cells (B16F10-iRFP) which seed and grow in the liver. Tumor burden in the liver was assessed using intravital microscopy at experimental endpoints. For sympathetic denervation, 6-hydroxydopamine (6-OHDA) was intraperitoneally injected 3 days prior to liver metastasis surgery, and tumor burden was assessed at experimental endpoints. Inhibition of β -adrenergic receptors (sympathetic neurotransmitter receptor) was achieved by intraperitoneal injection of propranolol twice a day for the duration of the experiment.

We found that PGP9.5+ nerve branches enter the liver from the portal vein and hepatic artery and follow bifurcations of larger blood vessels. These nerves almost fully colocalized with TH-staining, suggesting that the liver is primarily innervated by sympathetic nerves (and not parasympathetic nerves). However, PGP9.5+/TH+ nerves were not visible in the sinusoids (capillaries of the liver) and central veins as confirmed using tissue clearing and intravital microscopy. The location and distribution of sympathetic nerves was not altered at D10 following liver metastasis with B16F10 tumor cells. Despite this, sympathetic denervation with 6-OHDA could reduce tumor burden in the liver at D10. This protection was not mediated through β -adrenergic signaling as propranolol treatment did not improve tumor burden at D10 following liver metastasis. Interestingly, using the TH-reporter mouse, round TH+ cells, which did not have the morphology of nerve fibers, were identified within metastases.

In this study, we show that the liver is primarily innervated by sympathetic nerves and promote tumor growth following metastasis. Propranolol treatment did not reduce tumor burden suggesting that sympathetic nerves promote tumor growth through α -adrenergic receptors. We visualized TH+ cells within TME which may play an undiscovered role in promoting tumor growth. Future studies will characterize the TH+ cells within the TME and determine whether sympathetic nerves control response rates to immunotherapies.

P306

SPATIAL SIGNATURES OF IMMUNE CELLS IN THE TUMOUR MICROENVIRONMENT PREDICTS OUTCOMES IN STAGE III MELANOMA

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Current clinical practice to assess and treat patients with stage III melanoma relies largely on clinicopathological features. There is therefore an urgent need for reproducible and sensitive biomarkers to inform clinical treatment decisions. This project aimed to map immune cells in the tumour microenvironment (TME) of patients with high-risk stage III melanoma to determine markers of relapse and survival.

Imaging Mass Cytometry (IMC) employing 40 markers was performed on a tissue microarray with 101 node dissections of patients participating in a phase 3 randomised controlled trial using adjuvant radiotherapy for high-risk stage III nodal melanoma in an era before routine use of adjuvant systemic therapies (ANZMTG 01.02/TROG 02.01 trial). This cohort affords a unique opportunity to accurately map the pre-treatment phenotype of immune cells in the TME and associate immunological differences with clinical outcomes. Samples underwent cell segmentation and phenotypic marker analysis to produce a detailed and robust profile of the TME in melanoma.

Tumours from patients with better relapse and survival profiles had significantly more T cells (CD4+ and regulatory T cells), B cells, natural killer cells and neutrophils compared to tumours from patients with poorer outcomes. Unbiased clustering followed by spatial analysis revealed distinct tissue regions and cellular interactions within the TME linked to outcomes (relapse and survival).

This high-dimensional spatial mapping of the TME in treatment-naive patients with advanced melanoma not only provides important insight into immune cell interactions but also may indicate spatial signatures predictive of outcomes.

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IMMUNOGENETICS, CANCER AND IL-6 GENE REGULATION BY A LONG NON-CODING RNA

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Interleukin-6 (IL-6) is a crucial cytokine involved in a diverse array of physiological functions, including the regulation of immunity and inflammation, and the development of cancer. Dysregulation of IL-6 signaling, particularly through polymorphisms in its regulatory long non-coding RNA (lncRNA), IL6-AS1, can potentially lead to susceptibility to various diseases, including infection-related cancers. Our prior work identified an elevated susceptibility to systemic fungal infections in the Hmong population of Wisconsin, which we have linked to polymorphisms in the IL6-AS1 gene. Interestingly, the Hmong population also presents a distinctive pattern of cancer susceptibility, with a higher incidence of infection-related cancers. Conversely, there are lower rates of non-infection-related cancers, which could also be explained by low IL-6 levels because this cytokine is known to play diverse roles in cancer pathogenesis. We hypothesize that IL6-AS1 augments IL-6 gene expression by interacting with miRNA, RNA-binding protein, or transcription factor binding sites, and Hmong polymorphisms modify these actions.

Induced pluripotent stem cells (iPSCs) from patients and healthy subjects were created to investigate three polymorphisms in and around the lncRNA IL6-AS1 hypothesized to contribute to the defect in IL-6 expression in the Hmong – positions -572, 1400 and 1430. CRISPR was used to edit iPSCs from a Hmong donor to revert candidate single nucleotide polymorphisms from the susceptible “Hmong” genotype to the resistant “European” genotype, and vice-versa for the European donor iPSCs. The parental and edited cell lines were then differentiated into endothelium for functional study. Subcellular fractionation and RT-qPCR analysis were performed to localize expression of IL6-AS1 in the cytosol vs. the nucleus. To investigate the role of IL6-AS1 on IL6 expression, we targeted the lncRNA using siRNA and antisense oligonucleotides. We also transiently transfect cells to overexpress the lncRNA.

Our data suggest that Hmong-specific IL6-AS1 polymorphisms lead to reduced IL6-AS1 and IL-6 expression, consequently affecting resistance to infections and potentially precipitating infection-related cancers. Our findings suggest differential subcellular localization of IL6-AS1 in endothelial and cancer cells, indicating the potential for distinct functional roles of IL6-AS1 within these cellular contexts. IL6-AS1 depletion was found to decrease IL-6 expression, while its overexpression resulted in the opposite effect in both cancer and stromal cells upon IL-1 β treatment. Evaluating the co-expression dynamics and cellular localization of IL6 and IL6-AS1, our preliminary findings suggest a dual role of IL6-AS1 in both promoting IL6 transcription and potentially stabilizing IL6 mRNA.

Our ongoing research seeks to clarify the mechanism by which IL6-AS1 regulates IL-6 gene expression and how IL6-AS1 polymorphisms within the Hmong population alter IL6-AS1 function and IL-6 expression. Our findings demonstrate a positive regulatory influence of IL6-AS1 on IL-6 expression, suggesting a dual role for IL6-AS1 in promoting IL6 transcription and potentially enhancing IL6 mRNA stability. Metabolomic labeling and ActD transcription inhibition assays are currently in use to further probe the role of IL6-AS1 in IL6 transcription and/or RNA stability. A better understanding of the regulatory mechanisms of IL-6 expression and the functional impact of IL6-AS1 polymorphisms could inform the development of personalized immunotherapies. By modulating IL-6 expression, we could potentially fine-tune the immune response to cancer, facilitating precise therapeutic interventions and propelling the advancement of precision medicine in cancer treatment. Additionally, our research could pave the way for the development of reliable predictive biomarkers

for cancer therapy, offering invaluable insights into the susceptibility basis of infection and non-infection related cancers.

P308

ORGANIZATION, FUNCTION AND GENE EXPRESSION OF TERTIARY LYMPHOID STRUCTURES IN PANCREATIC CANCER RESEMBLES LYMPHOID FOLLICLES IN SECONDARY LYMPHOID ORGANS AND THEIR ABUNDANCE IS RELATED TO SUPERIOR SURVIVAL

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Tertiary lymphoid structures (TLS) have been described in close proximity to tumor areas in a variety of cancer types. Abundance of TLS is related to cancer-specific survival and susceptibility to immune-checkpoint inhibition. TLS in the tumor microenvironment are assumed to represent hotspots for T cell and B-cell activation leading to tumor-specific humoral and cellular immune responses.

To identify shared and TLS/secondary lymphoid organ (SLO)-specific features, we performed immunohistochemistry staining (CD20, CD3, CD8, FOXP3 and FDCM1) of previously untreated pancreatic ductal adenocarcinoma (PDAC) samples. 5-color immunofluorescence microscopy was used to elucidate the spatial organization of TLS/SLO. Tissue extraction by laser microdissection and NanoString-based RNA expression analysis was conducted for 12 patients to compare gene expression in TLS, PDAC, SLOs and normal pancreatic tissue.

TLS were found in 95% of analyzed samples from 168 patients and abundance of TLS was highly variable. TLS were mainly localized in the 2000 µm invasive tumor margin with few TLS also occurring in the center of the tumor. Patients with a high density of TLS inside and surrounding the tumor showed a higher overall survival and we observed a correlation between abundance of TLS and infiltration by CD8+ T cells in the tumor. Five-color Immunofluorescence revealed structural homologies between TLS and SLO with a similar distribution of B cells, T cells, follicular dendritic cells and lymphatic vessels. In addition to the structural similarities of TLS and SLOs, we found largely overlapping expression patterns of a variety of immune related gene clusters. However, especially expression levels of T-cell and complement-associated genes were distinct. Most TLS expressed Ki-67, Pax5, AID and IgG, proving proliferation, class switching and affinity maturation of B cells in close proximity to the tumor. B-cell receptor sequencing of microdissected TLS and SLO, identified clonal expansion and an overlap of expanded sequences.

Our analyses of organization, function and gene expression patterns revealed a high overlap between SLOs and TLS in PDAC. Our results indicate a role of TLS in cancer immunosurveillance of PDAC, which may be susceptible to therapeutic targeting in this highly aggressive and immunotherapy-resistant disease.

P309**ORGAN-SPECIFIC DETERMINANTS OF LYMPHOID NEOGENESIS IN HUMAN LUNG AND KIDNEY CANCER**

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Tertiary lymphoid structures (TLS) are ectopic lymphoid organs in inflamed peripheral tissues that potentially mediate similar functions to lymph nodes. TLS develop via the process of lymphoid neogenesis initiated by inflammatory signals but depend on activated lymphocytes for their maturation from early unorganised aggregates to organised structures containing an active germinal centre reaction (GC). TLS, especially when harbouring a GC, correlate with improved survival and increased adaptive immune responses in most solid tumours including lung squamous cell carcinoma (LSCC). In contrast, untreated renal cell carcinoma (RCC) is among the few exceptions where TLS are mainly immature and are associated with reduced survival. Factors orchestrating TLS development in human cancer are poorly understood. Here we employed spatially resolved transcriptomics and quantitative pathology to explore the determinants of lymphoid neogenesis and the differential prognostic associations of TLS in LSCC and RCC.

We performed unbiased gene expression profiling of >200 TLS in different maturation stages obtained from 6 LSCC and 6 RCC patients using the GeoMX Whole Transcriptome Atlas (Nanostring) as well as analysed the transcriptome across whole tissues using the Visium platform (10X Genomics). We compared gene expression profiles (1) between early and mature TLS to identify factors involved in TLS development in each tumour type, as well as (2) in maturation stage-matched TLS between LSCC and RCC to define tumour type-specific TLS characteristics. We employed multiplex immunofluorescence to analyse the composition of TLS and the tumour immune-microenvironment in a cohort of 75 RCC patients and to validate the expression of novel TLS-associated genes in RCC and LSCC samples.

We identified gene signatures characterising TLS from early lymphocytic aggregates to GC-containing TLS. Besides several canonical factors of lymphoid neogenesis (i.e. CCL19, LTB), we found novel genes (such as CORO1A, LIMD2, JAK3) expressed across all TLS maturation stages in both tumour types. The early TLS stage was characterised by upregulation of chemokine CCL21 and factors involved in actin cytoskeleton regulation. In contrast, canonical GC markers (like CD23, FDCSP), chemokine CXCL13, various B cell-specific molecules (P2RX5, BAFFR), and poorly understood factors including CLU and LTF were upregulated in mature TLS. Our immunofluorescence analysis confirmed the expression of CLU and LTF in mature TLS as novel follicular dendritic cell associated proteins. We found reduced expression of mature TLS-associated genes (including CD37, CXCL13, CLU) and increased expression of early TLS genes (such as CCL21, TAGLN) in TLS from RCC in comparison to TLS from LSCC independently of the TLS maturation stage, suggesting that TLS functions in RCC are hampered. Quantitative pathology analysis further demonstrated that the overall TLS number as well as the fraction of mature TLS were significantly lower in RCC than LSCC samples. RCC patients with high TLS counts showed reduced survival; however, we did not observe any correlation between TLS counts and tumour-infiltrating immunosuppressive myeloid cells or T regulatory cells in RCC patients, suggesting that TLS do not directly contribute to immunosuppression in untreated RCC.

In summary, we identified multiple novel factors associated with lymphoid neogenesis in human cancer and demonstrated that the process is impaired in RCC compared to LSCC. Since chronic inflammatory stimuli initiate TLS development, we propose that TLS in RCC indicate increased inflammation, while impaired TLS maturation in RCC indicates reduced activation of adaptive immunity. Increased inflammation and reduced adaptive immunity are associated with disease

progression in multiple tumours and may explain the association between increased early TLS and poor prognosis in untreated RCC.

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HARNESSING T CELL DYSFUNCTION IN SOLID CANCERS TO OVERCOME IMMUNOTHERAPY RESISTANCE

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Immunotherapy has revolutionized the treatment landscape across cancer types; however, a minority of patients respond (~30%) and resistance is common. The highly immunosuppressive tumor microenvironment, and chronic TCR activation from high antigen loads, results in transcriptional, epigenetic, and metabolic changes that drive functional T cells into a hyporesponsive, dysfunctional state. This is a major contributor to poor immunotherapy response. Understanding the biology underpinning T cell dysfunction and development of therapeutic strategies to modulate this hypofunctional state is crucial for overcoming resistance and improving immunotherapy efficacy.

We have established a robust in vitro assay using repetitive stimulations with soluble anti-CD3 and anti-CD28 to exhaust antigen specific NY-ESO-1 TCR transduced PBMCs (NY-ESO PBMCs) as well as isolated un-transduced CD4+ and CD8+ T cells. We tested the efficacy of existing immunotherapies to restore T cell cytotoxicity, assessing the killing capacity and cytokine secretion of exhausted NY-ESO PBMCs following co-culture with a melanoma cancer cell line. Finally, we successfully applied targeted RNP CRISPR to unstimulated human CD4+ and CD8+ T cells, prior to exhaustion and in exhausted T cells, to assess the potential of prospective targets to block and reverse T cell exhaustion respectively.

Our in vitro generated exhausted T cells show changes in key exhaustion markers (PD-1, Tim3, 4-1BB), significant reductions in cytokine secretion (IL-2, IFN γ , TNF α , and Granzyme B), and impaired killing capacity. These findings are consistent with T cell exhaustion, phenocopy patient-derived TILs, and are reproducible across T cell compartments and between donors. Of note, we observed accumulation of a heterogenous population with various exhaustion states in our in vitro assay.

We then assessed and compared the ability of existing immunotherapies to restore the cytotoxicity of our in vitro generated exhausted T cells. Anti-PD-1 therapies (Nivolumab and Pembrolizumab) were unable to increase exhausted T cell killing whilst only Nivolumab moderately increased cytokine secretion, compared to isotype treated controls. In contrast, initial results with Urelumab (4-1BB agonist) showed markedly increased cytokine secretion and partial restoration of killing capacity. Our readouts demonstrate potential differences in mechanism of action of novel immune modulators in cancer and highlight the requirement of a better understanding of different dysfunctional states for improved immunotherapy response. These data also indicate the reprogramming potential of our in vitro generated exhausted T cells. Thus, we applied our in vitro platform to interrogate novel targets for the restoration of exhausted T cell function.

We have successfully edited unstimulated CD4+ and CD8+ T cells (70%-90% reduction in expression) for several targets, one of which was RASA2, a RAS GTPase-activating protein. Depletion of RASA2 has previously been shown to increase T cell activation, cytokine secretion, and killing capacity using RNP CRISPR in activated CD8+ T cells (Carnevale et al., 2022). In our assay setup, we have validated that RASA2 can block T cell exhaustion in CD8+, and for the first time in CD4+ cells, shown by significant increases in 4-1BB expression and increased cytokine secretion (IFN γ and Granzyme B). This data validates our in-house exhaustion pipeline, enabling us to interrogate the different biology of novel targets in CD8+ but also CD4+ dysfunction where comprehensive understanding is lacking.

T cell dysfunction is a driver of immunotherapy resistance in cancer. We have developed a robust in vitro T cell exhaustion protocol that phenocopies patient-derived TILs. Using RNP CRISPR we have

developed a pipeline to interrogate the targets' role in T cell dysfunction, which could improve clinical responses to immunotherapies by restoring exhausted T cell function.

P311

ENGINEERING CAR T CELLS TO LIMIT ON-TARGET, OFF-TUMOR TOXICITY IN SOLID CANCERS

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Targeting tumor-associated antigens with CAR T cells is an attractive idea because of the potential to treat a large population of individuals with cancer. Unlike an engineered TCR that targets a cancer-specific antigen (e.g. KRASG12D), CAR-T cells do not require specific HLA matching. Furthermore, selection of a tumor-associated antigen as the T cell target would make a large majority of individuals with certain cancers eligible for such a cell therapy (e.g. CEA in colorectal cancer). However, a major barrier to adoption of CAR T cells that target tumor-associated antigens is on-target, off-tumor toxicity. On-target, off-tumor toxicity occurs when the engineered T cell appropriately recognizes healthy cells, in addition to malignant cells. This on-target, off-tumor toxicity has been responsible for death and disability in several clinical trials. It is a problem that must be addressed to move forward with targeted tumor-associated antigens in solid tumors.

On-target, off-tumor toxicity can only be studied in systems where the host has both expression of the CAR target and a fully functional immune system; immunocompromised mice combined with CAR T cells that target a human version of the protein are not adequate. We therefore designed a murine CAR that binds murine EpCAM in order to test interventions to limit on-target, off-tumor toxicity. EpCAM was chosen as a representative model for a tumor-associated antigen because it is overexpressed in many cancers, and also is present in various organs including lung and pancreas.

First, we established that T cells transduced with our EpCAM CAR were activated in vitro when EpCAM was present, and had in vivo anti-tumor efficacy. As expected, EpCAM CAR+ T cells caused tissue destruction in the normal lung and pancreas, where EpCAM is expressed, and were lethal at higher doses. To overcome the problem of on-target, off-tumor toxicity, we devised a system to direct and retain T cells at the desired site of activity, and away from tissues with the potential for undesired activity. Our first type of "tissue residency programmed" T cells were designed to treat liver metastases. In this case, the desired site of CAR T cell activity is the liver. We engineered our EpCAM CAR T cells such that they express a transcriptional program for liver tissue homing and residency. We term CAR T cells that have this program "liver tissue residency programmed (Liver TRP)". Using a competitive homing assay, we found that liver TRP EpCAM CAR+ T cells were preferentially trafficked to the liver and away from the lung and pancreas. Critically, the liver TRP EpCAM CAR T cells were significantly less toxic than the original EpCAM CAR T cells. This superior tolerability did not compromise efficacy, as the liver TRP EpCAM CAR T cells exerted anti-tumor activity in mice bearing intrahepatic KPC tumors.

In conclusion, we find that a loco-regional approach to limit on-target off-tumor toxicity of CAR T cells is effective and warrants further investigation. Specifically, our liver tissue residency programmed CAR T cells to traffic to the liver, positioning this approach as applicable in the setting of liver metastatic disease.

P312

SPATIAL DISTRIBUTION ANALYSES OF PD-L1 EXPRESSION, MACROPHAGE POLARISATION AND CD8+ T CELLS STATES IN IMMUNOLOGICALLY HOT AND COLD CARCINOMAS

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Immune checkpoint inhibitors (ICIs) have revolutionized the clinical management of various oncological indications. Along similar lines, ICIs appear to be particularly active in patients bearing tumors with genetic, functional and/or immunological features that a priori are supportive of anticancer immunity. Similar findings have been obtained for the expression levels of PD-L1, an immunosuppressive ligand expressed by malignant and myeloid cells in response to ongoing anticancer immunity. At odds to non-small cell lung carcinoma (NSCLC), epithelial ovarian carcinoma is poorly sensitive to ICIs employed as standalone immunotherapeutic agents, most likely due to a relatively low tumor mutational burden (TMB) and indolent anticancer immunity. Thus, studying the interaction between malignant cells and immune components within tumor microenvironment (TME) is essential to identify possible factors of tumor progression and clinical outcome.

Multispectral immunofluorescence microscopy was employed on 15 chemotherapy-naive high-grade serous carcinomas (HGSCs) and 15 NSCLC samples. Flow cytometry was used for functional assessments on freshly resected HGSC/NSCLC samples.

We demonstrate that PD-L1 expression distributed within myeloid and malignant cells is tumor-type dependent. In addition, the PD-L1 expression within tumor compartments impact the clinically relevant spatial distribution of T cells subsets, including progenitor TCF1+PD-1+CD8+ and terminally exhausted TIM-3+PD-1+CD8+ T cells.

Combining the cellular distribution patterns with cellular distances, we have identified clinically relevant tumor-specific distribution patterns within immunologically hot and cold tumors. Spatial distribution provides a unique tool to improve our understanding of tumor microenvironment with potential implication in cancer immunotherapy.

P314

IDENTIFICATION OF POTENTIAL NOVEL TARGETS FOR CANCER IMMUNOTHERAPY USING IMMUNOCOMPROMISED MOUSE MODELS

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The concept of cancer immunoediting describes how clinically detectable tumors can arise despite cancer immunosurveillance, where small accumulations of malignant cells provoke an immunological reaction that can result in elimination. Malignant cells achieve this escape through different immunosuppressive mechanisms. Cancer immunotherapies targeting some of these escape mechanisms, especially immune checkpoint inhibitors, have improved treatment options for patients with cancer over recent years. However, objective response rates remain low. Identifying novel immunosuppressive molecules can lead to the discovery of new treatment targets improving cancer immunotherapy.

The immunogenic cancer cell lines MC38 and CT26 were inoculated in B6.129S6-Rag2tm1Fwa N12 mice and BALB/c nu/nu mice or the corresponding wildtype mice, respectively. Tumor-associated macrophages (TAMs) were FACS-sorted from the tumors. Bulk RNA sequencing was performed on RNA isolated from tumors and from sorted TAMs to identify differentially expressed genes (DEGs). The STRING database was used to visualize protein-protein interactions and perform gene ontology (GO) analysis of DEGs. Gene Expression Profiling Interactive Analysis (GEPIA) was used to analyze the expression of DEGs in tumor tissue of patients with clear cell renal cell carcinoma (ccRCC), an immunologically hot tumor, and corresponding healthy tissue. The expression of DEGs in ccRCC was correlated with the expression of genes associated with immune suppression using cBioPortal. Additionally, the effect of DEGs on overall survival (OS) of patients with ccRCC was assessed using Kaplan-Meier plotter.

The immunogenic cancer cell lines MC38 and CT26 formed faster growing tumors in T cell deficient mice compared to mice with a fully functional immune system. 28 and 16 genes were upregulated across both models in tumor bulk and sorted TAMs, respectively. Numerous genes associated with antigen-processing and presentation, as well as established immunosuppressive molecules, were among the upregulated genes in wildtype mice. To identify novel immunosuppressive genes, DEGs with minimal functional annotations were pursued, yielding two final DEGs for further investigation. Expression of candidate genes was shown to be higher in tumor tissue of patients with ccRCC compared to matched healthy tissue. In ccRCC, expression of one of these candidates was highly correlated with the expression of several genes linked to immune suppression in cancer. Similar to other immune checkpoint molecules, higher expression of either of the genes correlated with decreased OS of patients with ccRCC.

Using immunocompromised and immunocompetent mouse models, we were able to identify two potential novel immunosuppressive molecules upregulated in response to T cell effector mechanisms. Functional analyses of the candidate molecules could reveal unknown mechanisms involved in tumor immune escape leading to the identification of new immunotherapeutic treatment targets.

P315

TERTIARY LYMPHOID STRUCTURES AND B CELLS SIGNATURES DETERMINE CLINICALLY RELEVANT T CELL PHENOTYPE IN OVARIAN CANCER

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Tertiary lymphoid structures (TLSs) are ectopic lymphoid formations that arise at sites with persistent inflammatory conditions, including tumors. TLSs are composed predominantly of B cells, T cells, and CD21+ CD23+ follicular dendritic cells and exhibit different levels of the organization, ranging from locally concentrated aggregates of immune cells to well-defined B cell follicles to mature follicles with germinal center formation. Supporting key relevance for TLSs in natural and immunotherapy-driven immunosurveillance, intratumoral TLS density has been associated with improved disease outcome and response to cancer immunotherapy, mainly check-point inhibitors (ICIs) in solid carcinomas.

We harnessed a retrospective cohort of 180 chemotherapy-naive HGSC patients to investigate TLS abundance in relation with prognosis and function orientation of the tumor microenvironment. Transcriptomic, spatial transcriptomic and functional studies complemented the data obtained by immunofluorescence analysis. An in silico analysis of publicly available RNA expression data from 308 HGSC samples was performed as a confirmatory approach. The second retrospective cohort was used for the analysis of the relationship between tumor mutation burden and TLS abundance.

Here, we demonstrate that compared to the immunologically hot tumors like non-small cell lung carcinoma (NSCLC), human high-grade serous ovarian carcinoma (HGSOC) contains only a limited number of TLS with germinal center formation. In parallel, TLS frequency and maturation positively correlate with progenitor stem-like CD8+ T cell phenotype. In line with this notion, B cell depletion in HGSOC biopsies promoted terminally exhausted, poorly ICI-sensitive CD8+ T cells, suggesting a critical role of intratumoral B cells in maintaining T cell effector functions.

These findings point to key numerical and functional differences between mature TLSs in ICI-responsive vs ICI-irresponsive neoplasms that may guide the development of alternative ICI-based immunotherapies for patients with HGSOC.

P316

THE LONG-TERM ANTITUMOR IMMUNE RESPONSE OF OVA MRNA-BASED VACCINE COMBINED WITH ANTI-PD-1 BLOCKING ANTIBODY IN AN OVA-EXPRESSING MURINE TUMOR MODEL

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The development of personalized therapies and their combination with antitumor treatments already used clinically are crucial to fight the therapeutic resistance of cancer patients. We have shown that lipid nanoparticles (LNP Lipidots®) are able to vector protein antigens and induce humoral and cellular immune responses¹⁻². Here, we tested Lipidots® for the delivery of mRNA encoding antigen and eliciting an antitumor immune response. We also assessed whether the combination with an anti-PD-1 blocking antibody could improve the memory anticancer immune response by preventing tumor recurrence.

For all the experiments, an mRNA encoding Ovalbumin (OVA) or an irrelevant protein was used. We tested the effectiveness of our vaccine strategy with a curative therapeutic regimen in mice bearing subcutaneous B16OVA or E.G7-OVA tumors. Three injections of 3 µg of vectorized mRNA were performed. The intratumoral immune cell response was analyzed by flow cytometry and ELISA after ex vivo restimulation with OVA peptides and histology. Experiments blocking CD8+ T cells, NK cells, and the PD-1/PD-L1 axis were carried out by injecting intraperitoneally blocking monoclonal antibodies (anti-CD8β, anti-NK1.1, anti-PD-1). The surviving mice were challenged with the engraftment of a new tumor expressing or not OVA (B16OVA or B16F10). The tumor growth was assessed and the peripheral immune response was evaluated by flow cytometry.

In a curative scheme, vaccination slows B16OVA and EG.7-OVA tumor growth drastically for short-term experiments (17 days). This is associated with an increased intratumoral frequency of CD45+ cells in live cells and CD8+ T cells in CD45+ cells as well as a higher number of tumor-infiltrating CD45+ and CD8+ cells compared with the LNP-control mRNA-treated mice. In addition, we noted higher frequencies of intratumoral activated CD8+ T cells expressing PD-1, CD69, and coproducing IFNγ and TNFα or IFNγ and GzB in the LNP-Ova mRNA group. In B16OVA tumor-bearing mice, blocking CD8+ T cells mainly prevents the antitumor effect of the Ova mRNA-based vaccination while blocking NK cells did not. Promisingly, combining the vaccination with an anti-PD-1 improves significantly the survival of B16OVA tumor-bearing mice vs. vaccination alone. Although the median survival was increased with the vaccination alone (34 days vs. 21 days in the control group), 100 days after tumor cell inoculation 3/12 mice survived in the LNP Ova mRNA + IgG2a group while 9/12 mice in the LNP Ova mRNA + anti-PD-1 group were still alive. Moreover, Ova mRNA-based vaccination renders immunologically active and sensitive to anti-PD-1 the immunologically inactive and anti-PD-1 resistant B16OVA tumor model with which no mice survived in the LNP-control mRNA + anti-PD-1 group. This combinatory treatment leads to a long-lasting antitumoral immune response by completely preventing the growth of a new B16OVA tumor engrafted in the contralateral site of surviving mice at day 103 and reducing that of a B16F10 tumor. In the B16OVA challenged mice, 15 days after tumor cell inoculation, ex vivo restimulation of splenocytes showed a higher frequency of CD8+ T cells coproducing IFNγ and TNFα, an increased percentage of OVA-specific CD8+ T cells identified by dextramer staining, a higher percentage of activated B cells (CD86+MHC+) and an enhanced activation of dendritic cells (↑ MFI of CD86) when compared with the control mice.

Collectively, these data demonstrate that Lipidots® are able to vectorize mRNA and are usable for the elaboration of mRNA-based anticancer vaccines. Our vaccinal strategy allows the elaboration of a CD8+ T cell-dependent antitumor immune response that benefits from blocking the PD-1/PD-L1

axis to improve survival. PD-1/PL-1 axis-mediated immunosuppression is a mechanism of tumor cell escape from mRNA-based vaccination that needs to be blocked to increase the chances of establishing a long-term memory immune response and thus prevent tumor recurrence.

P317

CARDIOVASCULAR EVENTS AFTER IMMUNE CHECKPOINT INHIBITORS AS ONCOLOGIC EMERGENCIES

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Immune-related adverse events (irAEs) from Immune Checkpoint Inhibitors (ICI) can be clinically significant. In cancer patients, cardiovascular irAEs (CV-irAEs) can be life-threatening and require emergency intervention and care. While clinical guidelines for diagnosis, grading, and management of irAEs include CV-irAEs, data are lacking to support more comprehensive coverage of all types of CV-irAEs. In this study, we described reported cardiovascular events in lung cancer patients who received ICI, and estimated their incidence and time to diagnosis.

A cohort study of lung cancer patients 18 years and older who received ICI at MD Anderson Cancer Center between April 1st, 2016, and March 31st, 2020. Demographics and clinical data were collected from the institution's data warehouse. The billing database was queried for all cardiovascular-related diagnoses (International Classification of Disease-version 10) before and after ICI initiation up to 01/01/2022. Cumulative incidence for different cardiovascular events using competing risk models, with death as a competing event were estimated and reported.

A total of 1,803 patients were included, with a median age of 66 years (interquartile range= 59-72 years), who were mostly white (75.4%) and males (55.4%). The majority of the patients (77.8%) had single ICI therapy during the study period. Almost one-quarter (26.0%) of the patients had cardiovascular disease documented before ICI initiation. Of the remaining 1334 patients, 522 (39.1%) patients had new diagnosis of at least one cardiovascular disease documented after ICI therapy.

New diagnoses of tachyarrhythmias (20.4%), diseases of the pericardium (9.4%), and heart failure (5.2%) were among the topmost frequent cardiovascular-related diagnosis after ICI. Median time to new diagnosis was earliest for myocarditis (2.8 [Interquartile range= 2.1-5.1] months); followed by tachyarrhythmias (4.3 months); endocarditis (5.0 months); diseases of the pericardium (5.4 months); and cardiomyopathy (5.8 months). When compared to single ICI therapy during the study period, lung cancer patients with multiple ICI had significantly higher cumulative incidence of diseases of the pericardium ($P=0.017$); conduction disorders ($P<0.001$); myocardial infarction ($P=0.015$); and tachyarrhythmias ($P=0.004$).

The use of ICPI in lung cancer is still expanding and early and delayed manifestations of CV irAEs are yet to be defined in lung cancer survivors. Our study is the first to characterize the spectrum of potential CV-irAEs in lung cancer patients on ICI therapy and CV-related ED visits. Given the heterogeneity among lung cancer patients with respect to cardiotoxicity risk, the timing of onset, clinical presentation, and outcome, efforts for personalized risk stratification, early detection, prevention, monitoring, and management of CV-irAEs during treatment and survivorship is of utmost importance.

P318**ADOPTIVE CELL THERAPY OF ACUTE LEUKEMIA ACROSS MHC-BARRIERS BY T AND INKT CELLS ENGINEERED WITH A CD1C-RESTRICTED TCR**

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Disease recurrence is the major unmet clinical need of acute leukemia following treatment by chemotherapy and allogeneic stem cell transplantation. Adoptive cell therapy (ACT) with allogeneic donor-derived T cells can control disease recurrence by inducing a beneficial Graft-versus-Leukemia (GvL) reaction. However, grafted alloreactive T cells can cause a detrimental graft-versus-host disease (GvHD), prompting the search for new donor-unrestricted strategies targeting malignant cells. Primary acute myelogenous (AML) and B-lymphoblastic (B-ALL) leukemia blasts express CD1c antigen-presenting molecules, which are recognized by T cell clones specific for the CD1c-restricted leukemia-associated methyl-lysophosphatidic acid (mLPA) lipid antigen. Because CD1c molecules are identical in all individuals and expressed only by mature leukocytes, a donor-unrestricted ACT strategy leveraging conventional T or CD1d-restricted iNKT cells redirected against CD1c-expressing acute leukemia by mLPA-specific TCR transfer may reduce the risk of GvHD induction. Furthermore, iNKT cells restrict immunosuppressive myeloid cells in the tumor microenvironment (TME) through their TCR, allowing the generation of bi-specific effectors that can simultaneously modulate intratumoral suppressive myeloid populations and kill malignant cells.

By interrogating a library of human mLPA-specific TCRs cloned in lentiviral vectors, we identified a lead mLPA-specific TCR (DN4.99) which could be efficiently transduced and expressed into primary T lymphocytes from any donor and displayed the strongest reactivity against CD1c+ leukemia cells. T and iNKT cells transduced with the DN4.99 TCR were assessed for the recognition of leukemia in vitro and in vivo in immunodeficient mouse xenografts.

DN4.99-transduced T cells efficiently kill in vitro several different CD1c+ acute leukemia cells, but not normal circulating CD1c+ B cells, monocytes and DCs. The adoptive transfer of DN4.99 TCR-transduced T cells substantially delay the progression of 3 models of leukemia xenograft in NSG mice, an effect that is boosted by mLPA-loaded DC vaccination. Human primary iNKT cells can also be efficiently isolated, expanded and transduced with the mLPA-specific TCR. These cells kill leukemia targets expressing CD1c in vitro and, interestingly, show enhanced recognition of targets co-expressing CD1c and CD1d, which is frequent in acute leukemia. We are now assessing in mouse xenograft models whether DN4.99 TCR-transduced iNKT cells can further improve efficacy and safety of this adoptive immunotherapy strategy.

Our results highlight a novel approach for ACT of acute leukemia with T or iNKT cells engineered to recognize malignant cells by the transfer of a lipid-specific TCR that works across MHC-barriers like a CAR.

P319

A PRECISION ADOPTIVE CELL THERAPY PROCESS BASED ON THE EXPANSION OF CLONAL NEOANTIGEN-REACTIVE T CELLS FROM THE BLOOD OF PATIENTS WITH CANCER

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Tumor neoantigens are considered a key determinant in the immune response to cancer. Amongst the different types of neoantigens, clonal neoantigen burden shows the strongest correlation with patient survival and response to immune checkpoint inhibitors pointing to the potential critical role of this target class in cancer immunotherapy. At Achilles Therapeutics we have developed a clinical protocol for the generation of personalized clonal neoantigen reactive T cells (cNeT) products from a patients' tumor and initiated clinical trials to evaluate their activity against melanoma and non-small cell lung cancer (NSCLC) (NCT03997474, NCT04032847). However, not all cancer patients can undergo surgery or have accessible tumor for generation of cNeT. Importantly, neoantigen-reactive T cells have been identified in the blood of cancer patients pointing to the blood as a potential non-invasive source of clonal neoantigen-reactive cells for therapeutic development. Here, we describe a novel process for the generation of cNeT from the blood of cancer patients and in-depth characterization supporting the potential therapeutic value of blood-derived cNeT.

Matched tumor and peripheral blood from patients undergoing routine surgery were obtained from patients through the Achilles Therapeutics trial NCT03517917. Using our proprietary PELEUS™ bioinformatics platform and our NeoRanker immunogenicity tool we generate a list of the most immunogenic clonal neoantigens in a patient's tumor. Immunogenic clonal neoantigens are then included in co-cultures of blood T cells and professional antigen presenting cells to expand cNeT. We perform in-depth characterization of blood-derived cNeT products to assess their neoantigen-reactive potential using our proprietary potency assay, immunological fitness and clonal composition.

T cells were successfully expanded across several solid tumor indications in 37 of 45 patient samples (success rate 82.2%). T cell products had enriched clonal neoantigen reactivity (median 6.4%, range 0.08-81%, n=37). Extensive phenotyping showed that cNeT express markers of activation, cytotoxicity and migration following activation with neoantigen peptides and are capable of killing tumor targets.

Our data demonstrate the feasibility for the expansion of large numbers of cNeT from the blood of cancer patients. Furthermore, product characterization underscores the fitness and anti-tumor activity of blood-derived cNeT supporting further clinical development of this novel approach.

P320

MAdCAM-1+ TUMOR-ASSOCIATED HIGH ENDOTHELIAL VENULES ARE KEY FOR LIGHT-MEDIATED ANTI-GLIOMA IMMUNITY

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Glioblastoma is the most common and aggressive variant of primary brain cancer in adults, with an extremely poor 5-year overall survival of only 7%. There are a number of treatment barriers for this type of cancer, including the extensive abnormal vasculature and immunosuppressive microenvironment that lead to low immune cell infiltration. Tertiary lymphoid structures (TLS) are ectopic clusters of lymphoid cells that can form in chronic inflammatory conditions, and have been associated with positive prognosis in various cancer types. These clusters often form around high endothelial venules (HEVs), specialized blood vessels that are involved in immune cell recruitment and express either MAdCAM-1 or PNA_d.

Here, we pre-screened a panel of lymphoneogenic cytokines by orthotopically injecting mice with CT-2A tumors that overexpressed each cytokine of interest (CXCL13, CCL21, LT $\alpha\beta$ or LIGHT) to study whether they could induce HEVs and TLS in murine glioma. In addition to following the survival of the mice, we used immunofluorescence staining to study HEV and TLS formation, and flow cytometry to analyse the T cell compartment. From this, LIGHT was selected as the most promising therapeutic candidate for further study. We next utilized a MAdCAM-1 knockout mouse model in order to elucidate the interplay between HEVs, TLS and the stem-like CD8⁺ T cell population in the LIGHT-mediated anti-tumor response.

Overexpression of CXCL13 or CCL21 did not provide any survival benefit compared to control, whereas mice bearing LT $\alpha\beta$ - or LIGHT-overexpressing tumors exhibited prolonged survival and, in a proportion of cases, completely cleared their tumors. Upon immunofluorescence staining, clusters of CD45⁺ immune cells resembling TLS were found in the meninges and ventricles, which had a higher T to B cell ratio in the LT $\alpha\beta$ and LIGHT groups. Furthermore, LT $\alpha\beta$ and LIGHT increased the formation of functional MAdCAM-1⁺ tumor-associated HEVs (TA-HEVs). Interestingly, expression of LIGHT but not LT $\alpha\beta$ boosted the stem-like CD8⁺ T cell population (T_{stem-like}), which are known to be responsible for tumor control following checkpoint blockade. As such, LIGHT was selected as the most promising therapeutic candidate for further study.

Next, we utilized a MAdCAM-1^{-/-} mouse line to abolish the formation of TA-HEVs, aiming to elucidate their role in the LIGHT-mediated anti-tumor response in relation to both TLS and T_{stem-like} cells. Interestingly, while LIGHT overexpression still prolonged survival in the absence of MAdCAM-1, the survival benefit in MAdCAM-1^{-/-} mice was reduced compared to their MAdCAM-1^{+/+} counterparts, suggesting that MAdCAM-1⁺ TA-HEVs have an important role in LIGHT-mediated tumor clearance but are not the only mechanism at play. Knockout of MAdCAM-1 completely prevented the LIGHT-induced boost in T_{stem-like} cells, indicating that MAdCAM-1⁺ TA-HEVs have a critical role in the recruitment of these cells, but had no effect on TLS formation.

This study demonstrates that MAdCAM-1⁺ TA-HEVs have a central role in LIGHT-mediated anti-tumor immunity. Knockout of MAdCAM-1 and the consequent reduction in T_{stem-like} cells reduces the survival benefit provided by LIGHT. However, this benefit is not completely abolished and TLS formation is unaffected, suggesting that these structures may also play an important role. Further elucidating the mechanisms behind the effects of LIGHT in the glioblastoma setting will pave the way for its future use as an immune and vascular co-targeting therapy.

P321

GENERATION AND ANALYSIS OF ANTIBODIES TARGETING GANGLIOSIDES FOR THE IMMUNOTHERAPEUTIC TREATMENT OF NEUROBLASTOMA

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Neuroblastoma is a type of cancer that originates from early neural crest cells and is the most prevalent solid tumor outside the brain in children. It has a high clinical heterogeneity and accounts for 6-10% of pediatric malignancies, being fatal in 15% of cases. Treatment options for neuroblastoma include surgery, chemotherapy, radiotherapy, autologous hematopoietic stem cell transplantation, and immunotherapy using the chimeric monoclonal antibody dinutuximab, which targets ganglioside GD2.

Gangliosides are a class of glycosphingolipids located on the outer surface of the plasma membrane and exposed to the extracellular milieu. They are known to be overexpressed in various solid tumors, including melanoma, small cell lung cancer, and neuroblastoma, making them potential targets for immunotherapeutic approaches.

Despite the advancements achieved through antibody-based immunotherapy with dinutuximab, the 5-year survival rate for high-risk patients remains below 60%, and many patients still relapse. Consequently, there is an urgent need to develop novel therapeutic strategies to improve prognosis and outcomes for neuroblastoma patients. Our objective was to identify novel targets on neuroblastoma cells and explore potential antibodies for an immunotherapeutic approach.

In this study, we aimed to generate recombinant antibodies by utilizing five different available B cell receptor sequences. The objective was to specifically target gangliosides that were highly expressed on the surface of neuroblastoma cells. The antibodies were evaluated and compared on the basis of their stability, antigen binding properties, and cytotoxic activity to gain insight into their potential as candidates for the treatment of neuroblastoma.

The antibody clones used in this study had different degrees of stability, resulting in different behavior with respect to their binding specificities. Interestingly, some antibody clones failed to recognize the intended target, while others demonstrated successful target detection, as confirmed through FACS analysis performed with neuroblastoma cell lines. Among the antibody clones tested, one particular clone showed superior recognition of the target antigen. Additionally, this clone also demonstrated strong and specific complement-dependent cytotoxicity (CDC) and antibody-dependent cell-mediated cytotoxicity (ADCC) against neuroblastoma cells, making it the most promising candidate in this study.

In conclusion, our research highlighted gangliosides as promising targets for the treatment of neuroblastoma patients. Notably, one specific antibody clone exhibited remarkable efficacy in eliminating neuroblastoma cell lines that expressed the target antigen at high levels. The results proofed the feasibility of utilizing this antibody clone for immunotherapeutic treatment of neuroblastoma.

P322**T CELLS EXPRESSING CAR EQUIPPED WITH EXTRACELLULAR DOMAIN OF BTLA ARE EFFECTIVE AGAINST HVEM-OVEREXPRESSING MELANOMA CELL LINES**

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Chimeric antigen receptor (CAR) T cell therapy has revolutionized recent cancer therapies. It has shown overwhelming clinical efficacy in haemato-oncology. Several CAR T cells have been used to treat melanoma in clinical trials, but have not shown favorable results. Here, we developed a unique melanoma-targeting CAR construct using HVEM-binding proteins including BTLA and CD160 as HVEM recognition domains. HVEM (Herpesvirus entry mediator) is known to be overexpressed in melanoma. In this paper, CAR (Chimeric antigen receptor) construct with BTLA extracellular domain for HVEM recognition, (BTLA-28z) is developed and tested. Jurkat cells transduced with BTLA-28z exhibited enhanced IL-2 secretion when incubated with HVEM-overexpressing melanoma cells. KHYG-1 transduced BTLA-28z also lysed melanoma cell lines. Using primary T cells, we generated CAR T cells that targeted the HVEM. BTLA-28z CAR T cells exhibited excellent lytic activities against melanoma cell lines. Taken together, these results suggest that HVEM-targeting CAR T cells may be useful for the treatment of melanoma.

1. IL-2 release assay: To measure the amount of IL-2 secreted by effector cells, melanoma cells were co-cultured with CAR Jurkat or CAR T cells at an E: T ratio of 10:1 in 96-well plates. After 24 hours, the cell culture medium containing IL-2 was harvested by centrifugation at 13,000 rpm for 10 minutes. The IL-2 levels were determined using a human IL-2 ELISA kit. 2. Cytotoxicity assay: The cytotoxic activity of the effector CAR KHYG-1 or CAR T cells was determined using the Bright-Glo luciferase assay system. Melanoma cells were transduced with a luciferase lentivirus and selected using puromycin. Melanoma cells expressing luciferase were co-cultured with Effector CAR KHYG-1 or CAR T cells at an E: T ratio of 10:1. The luminescence signals were measured after 4 and 24 h of co-incubation. To detect the luminescent signal, BrightGlo reagent was added to each well of a 96-well plate in an amount equal to that of the mixture of target and effector cells. The cells were lysed by shaking in the 96-well plate at room temperature for 5 minutes. The luminescence was measured using an EnVision reader.

1. We tested the BTLA-28z and CD160-28z constructs using Jurkat cells, and the amount of secreted IL-2 was detected using IL-2 ELISA. The BTLA-28z and CD160-28z constructs were transferred into Jurkat cells by electroporation, and each CAR Jurkat cell line was co-cultured with an HVEM-positive or negative cell line. The ability of BTLA-28z CAR Jurkat cells to secrete IL-2 was superior to that of CD160-28z CAR Jurkat cells.

2. BTLA-28z CAR KHYG-1 cells efficiently and specifically lysed HVEM-positive cells. These results imply that our BTLA-28z CAR is sufficient for CAR T cell development.

3. We generated BTLA-28z CAR T cells by lentivirus infection and tested their ability to secrete IL-2 using IL-2 ELISA. As expected, BTLA-28z CAR T cells secreted significant amounts of IL-2 when co-cultured with HVEM-positive cells. FITC-28z CAR T cells were used as negative controls and secreted much lower amounts of IL-2 than the BTLA-28z CAR T cells. This result indicates that BTLA-28z CAR T cells selectively recognize the HVEM. Cell cytotoxicity assays were performed using BTLA-28z CAR T cells against HVEM-positive cells (SK-MEL-28(luciferase) and MALME-3M(luciferase)) and HVEM-negative cells (293T(luciferase)). Non-transduced T cells and ZsGreen-expressing T cells were used as negative controls. BTLA-28z CAR T cells sufficiently and selectively lysed HVEM-positive cells.

We developed BTLA-CAR T cells targeting HVEM and they showed effective anticancer activity against melanoma cell lines. We suggest that BTLA-CAR T cell therapy could be a novel strategy for melanoma treatment.

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VASCULAR CD93 REGULATES INFILTRATION AND FORMATION OF PERIVASCULAR IMMUNE CELL NICHES ON THE GLIOMA-ASSOCIATED VASCULATURE

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Glioblastoma is a rare but lethal primary malignancy of the central nervous system in the adult. Despite recent advances, glioblastoma remains a challenging tumor to treat with no curative therapy. This is in part due to poor lymphocytic infiltration, which stems from the tumor's highly dysfunctional vasculature. Indeed, the abnormal glioblastoma vessels preclude infiltration of immune cells and impair their function, limiting not only the host's immune response but also the efficacy of T cell-reactivating immunotherapies. CD93 is a transmembrane glycoprotein that is upregulated in the vasculature of human high-grade gliomas, where its expression correlates with poor prognosis. In vivo studies in orthotopic murine glioma revealed that CD93 deficiency delays tumor growth and improves survival. However, the role of CD93 in the context of immune cell infiltration in glioma remains poorly understood.

In this work, we used human primary blood endothelial cells and siRNA-based methods in order to downregulate CD93 expression and assess its effects (as well as the underlying mechanism) in vitro. In order to study the roles of CD93 in glioma, we employed the GL261 murine glioma model, in which GL261 glioma cells are injected orthotopically into the brain of mice. The effect of CD93 deficiency was assessed both at endpoint and in the context of immune checkpoint blockade using two mouse strains – constitutive or inducible endothelial-specific CD93 knockout.

Here, we show that siRNA-mediated CD93 knockdown in human endothelial cells significantly increased the expression of the adhesion molecules ICAM-1, E-selectin and VCAM-1, key components for immune cell recruitment, through activation of the NF- κ B pathway. In line with this, in vivo studies using murine models of constitutive or inducible endothelial-specific CD93 knockout showed enhanced expression of these adhesion molecules in the glioma vessels and a significant increase in immune cell infiltration within the tumor tissue. In addition, CD93 deficiency promoted the organization of infiltrating immune cells into niches containing B cells, T cells and antigen presenting cells around the glioma-associated vasculature. Notably, the absence of CD93 skewed the composition of these niches towards a T cell-rich phenotype, which has been shown to be beneficial for the immune response in glioma models. In line with these data, CD93 deletion improved the survival of glioma-bearing mice after PD-1 checkpoint blockade therapy.

Altogether, these findings suggest a central role of CD93 in regulating the expression of vascular adhesion molecules and immune cell infiltration in glioma. This contributes to a better understanding of how CD93 modulates the immune-vascular interface in the glioma microenvironment, paving the way for the development of new treatment strategies to improve the response to immunotherapy in glioma.

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AN MHC CLASS II TARGETED IMMUNOTHERAPY FOR CD33-POSITIVE PEDIATRIC ACUTE MYELOID LEUKEMIA

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The treatment of pediatric acute myeloid leukemia (pAML) continues to be a challenge due to multiple subtypes and a wide variety of mutations. This heterogeneity undermines efforts to develop targeted therapeutics and conventional chemotherapy remains the standard of care (SOC). Sialic acid binding Ig-like lectin 3 (CD33) is a myeloid lineage cell surface glycoprotein that has emerged as a target for immunotherapy in AML. Gemtuzumab ozogamicin (GO), a CD33 specific antibody-drug conjugate (ADC), is FDA approved for the treatment of childhood AML. However, premature cleavage of the linker that connects the gemtuzumab monoclonal antibody to the cytotoxic calicheamicin payload can lead to undesirable off-target effects. Furthermore, while ~90% of AML cases exhibit CD33 expression, 50% of these patients express a single nucleotide polymorphism (SNP) in CD33 that eliminates the antibody binding epitope for GO, and clinical studies have failed to demonstrate an increase in overall survival in pAML patients treated with GO compared to SOC. These drawbacks highlight the critical need for a safer and more effective therapeutic that targets CD33-positive AML.

Our lab has developed a novel immunotherapy platform that targets tumor associated antigens (TAAs) to MHC class II molecules on antigen presenting cells for enhanced presentation to immune effector cells. This MHC class II targeted platform, or M2T for short, consists of an optimized high affinity MHC class II binder linked to a TAA. CD33-M2T uses in silico optimized variants of full length and truncated versions of the CD33 protein to direct specific immune cell responses against full length and alternatively spliced CD33.

Our preliminary data show that CD33-M2T induces a robust polyclonal anti-CD33 humoral response that induces the full antibody repertoire in vivo, including all IgG subtypes and IgA. Additionally, CD33-M2T increases overall survival in a syngeneic mouse model of AML. CD33-M2T has demonstrated clear advantages over anti-CD33 monoclonal antibodies (e.g., gemtuzumab, lintuzumab), including the ability to bind to the truncated alternatively spliced version of CD33, long duration of action, and no overt toxicities in mice.

These experiments demonstrate the preclinical potential of an innovative immunotherapy targeting CD33-positive childhood AML.

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THE JANUS FACED IMMUNE RESPONSE IN MELANOMA

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The adaptive immune system plays a vital role in eliminating cancer cells. It has been suggested that immunogenic tumors generally exhibit a better response to immune checkpoint blockade (ICB) immunotherapy. However, is a strong immune response always associated with improved survival?

In our study, we examined the survival of ICB-treated and ICB-naive melanoma patients. We also analyzed the genomic, transcriptomic and immune-related data of each melanoma sample.

We found that the HLA class I and II genotypes significantly influence patient survival in both ICB-treated and ICB-naive settings. However, when we assessed the specificity of survival-associated HLA variants, we encountered controversial findings at first sight: both detrimental and beneficial combinations of HLA class I and II were likely to bind melanoma-associated mutations. At the same time, these two groups of combinations presented distinct amino acid substitution types, with detrimental combinations more likely to present radical amino acid changes. We observed signs of active immune response in primary samples from both HLA genotype groups. However, immune evasion mechanisms were pervasive in the metastatic samples of the detrimental combination group, potentially explaining the poor survival of patients.

In conclusion, we propose that the combined effect of HLA class I and II molecules determine the strength of the antitumor immune response, and an excessively strong response can trigger immune evasion mechanisms and lead to poor patient survival.

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RETARGETED CIK CELLS AS AN ALTERNATIVE TO CD19 CAR-T LYMPHOCYTES

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Patients affected by aggressive B-cell malignancies who are resistant to chemo-immunotherapy have an extremely poor prognosis and limited therapeutic options. We investigated the combination of Cytokine Induced Killer (CIK) cells with anti-CD20 Obinutuzumab (Obi) or anti-CD19 Tafasitamab (Tafa) mAbs against B-cell malignancies, comparing this strategy with CD19-CAR T cells.

CIK cells were expanded from healthy donors and stimulated with rhIFN- γ , and after 24 hours of incubation, with anti-CD3 mAb and rhIL-2; T cells from the same donors were activated for 48 hours on anti-CD3 and anti-CD28-coated plates and further stimulated by adding hIL-7 and hIL-15, to be subsequently transduced with CD19-CAR viral vectors. The ability of Obi and Tafa to enhance CIK cell-mediated ADCC was assessed using CD19+CD20+ B-cell lines, and compared with CD19 CAR-T cell activity. To assess in vivo therapeutic efficacy, NSG mice were injected i.v. with luciferase-expressing Raji and Granta-519 cells, were divided into experimental groups according to the treatment (untreated, CIK+Tafa, CIK+Obi, CD19 CAR-T cells, untransduced T cells) and were monitored for tumor growth and survival.

CIK cells exhibited a strongly increased lysis of Raji, Granta-519, RCK-8, SU-DHL-4, EHEB and Nalm-6 target cells when combined with anti-CD19 Tafa or anti-CD20 Obi, which was comparable to CD19 CAR-T cells cytotoxicity. Preliminary in vivo data demonstrated a remarkable therapeutic activity of the CIK+mAbs combination. Mice receiving CD19 CAR-T cells showed a significant response to CIK+Obi and CIK+Tafa combination therapies, which restrained tumor growth even more effectively than CAR-T cells. At the end of the experiment, four out of the eight mice treated with CIK+Tafa were in complete remission, showing a statistically significant increase in survival compared to CAR-T cells.

The combination of the easily expandable CIK cell effector population with mAbs already in clinical use, establishes a tumor antigen-specific strategy that could be rapidly translated into clinical practice, providing an effective therapeutic alternative for those patients who are not eligible for CAR-T therapy and do not have any further treatment perspective.

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PANCREATIC CANCER MICROENVIRONMENT FOSTERS PRO-TUMORAL T FOLLICULAR HELPER2 CELLS WITH B-CELL HELPER ACTIVITY INDUCING DETRIMENTAL IGG4 AND PROSTAGLANDIN E2-DEPENDENT IGE INHIBITION

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The highly immunosuppressive microenvironment has a crucial role in determining the dismal prognosis of pancreatic ductal adenocarcinoma (PDAC). In different solid tumors T follicular helper (Tfh) cells act as immunoregulators and usually correlate with better survival. Aim of this study was to evaluate Tfh cell subset distribution in blood, tumor and tumor draining lymph-nodes (TDLNs) and their clinical relevance in PDAC patients.

A total of 145 PDAC patients either chemo-naïve or treated with neoadjuvant chemotherapy were included in this study. High-dimensional flow cytometry, T-cell receptor- and RNA-sequencing, bioinformatics, immunohistochemistry and in vitro mechanistic studies were performed.

High-dimensional flow cytometry identified clusters of Tfh1 (CD4+CD45RO+CXCR5+CCR6-CXCR3+), Tfh2 (CD4+CD45RO+ CXCR5+CCR6-CXCR3-) and Tfh17 (CD4+CD45RO+CXCR5+CCR6+CXCR3-) cells in the blood, tumor infiltrating lymphocytes (TILs) and TDLNs of chemo-naïve PDAC patients. The proportion of the Tfh2 cluster, but not of the Tfh1 and Tfh17, significantly correlated with the cluster of plasmacells/plasmablasts. Tfh cells recirculated within the three anatomical sites, and high percentage of Tfh2 in TILs and TDLNs correlated with reduced patients' survival. Only Tfh2 cells were highly activated (ICOS+PD1+), and decreased in patients who responded to neoadjuvant chemotherapy. Tumor and TDLN samples expressed all immunoglobulin (IGH) isotypes with the exception of IGHE, remarkably low/absent. B cell receptor analyses revealed higher clonal expansion but reduced somatic hypermutation for IGHG4 than IGHG1. Spatial distribution showed coexistence of IgG1 and IgG4 within tumor cell nests, suggesting potential competitive interaction. Mechanistically, predominant Tfh2 cells, differentiated in vitro by tumor microenvironment conditioned-dendritic cells, exerted IL-13-dependent B cell help for anti-inflammatory IgG4. Unexpectedly, secretion of pro-inflammatory IgE was instead inhibited; this was due to Tfh2 cell-derived prostaglandin E2 (PGE2), and restored with treatment with PGE2 receptor 4 (EP4) antagonist. The PGE2-EP4-mediated inhibition of IgE secretion should be regarded as a novel Tfh2-dependent immune evasion mechanism.

Tfh2 cells are relevant negative immunomodulators in PDAC and thus targeting type 2 inflammation that drives their differentiation or re-directing B cells to secrete pro-inflammatory IgE should be

pursued in combination with chemotherapy and/or immunotherapy to improve survival of PDAC patients.

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SYNTHETIC DNA VACCINE IN PERSONALIZED CANCER THERAPY

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In our previous work, we have demonstrated the effectiveness of a neoantigen-based plasmid DNA (pDNA) vaccine when delivered through electroporation (EP) in combination with immune checkpoint inhibitors (ICI) across various animal models. To assess the feasibility and efficacy of this vaccination approach, we conducted a phase I-II clinical trial using the COVID vaccine as a model. Our findings confirmed the induction of a robust T-cell response, including both CD8+ and CD4+ T-cell responses. However, when it comes to personalized cancer vaccines, the time interval from biopsy to treatment (commonly known as "needle to needle" time) is a critical factor. Recognizing the need to improve this aspect, we explored alternative genetic vaccine platforms.

Linear DNA as well as completely synthetic DNA were tested in preclinical animal models.

In preliminary studies, we evaluated the use of PCR-produced linear DNA vaccines, which yielded results consistent with previous publications in preclinical animal models. Unfortunately, the scalability and GMP production of this method did not meet the requirements for clinical trials. To overcome this limitation, we turned to 4basebio's synthetic DNA technology, leveraging its GMP production facility. Our investigations revealed that when administered through electroporation, synthetic DNA vaccines exhibited comparable or even superior efficacy compared to traditional pDNA vaccines. Additionally, this approach allowed for dose sparing, which is particularly advantageous. To support the transition of this novel vaccine platform to human clinical trials, we developed a new neoantigen prediction pipeline. This pipeline enables the identification of relevant neoantigens for personalized vaccines, thus tailoring the treatment to each individual patient. Furthermore, we are currently conducting toxicology studies to ensure the safety profile of the vaccine.

Our ultimate goal is to bring this personalized vaccine approach to the clinic, specifically for the treatment of non-small-cell lung cancer (NSCLC), by 2024. The data obtained from our research underscores the tremendous potential of entirely synthetic neoantigen-based DNA vaccines in combination with ICI for effectively targeting and treating NSCLC.

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IDENTIFICATION OF A NEW TARGET ON TUMOR-ASSOCIATED REGULATORY T CELLS INVOLVED IN THE INHIBITION OF MEMORY CD8 T CELL FUNCTIONS

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The deleterious role of regulatory T cells (Treg) in tumor environment (TE) contrasts with their key role in the control of immune homeostasis in the periphery, and highlights the need to identify specific therapeutic targets of tumor-associated (TA) Treg. Using public scRNAseq data we demonstrated the specific expression of CD177 on TA-Treg. We also highlighted that PECAM1 (CD31), a member of the ITIM-bearing immune co-receptors recognized as CD177 receptor, is expressed on memory TA-CD8 T cells.

We hypothesize that CD177 identifies an activated and highly suppressive population of TA-Treg which, via the engagement of CD31, would block the function of TA CD31+ CD8 T cells.

We performed flow cytometry, multi-immunofluorescence analyses and phosphorylation on healthy donor blood samples and tumor samples.

We confirmed, at the protein level by flow cytometry, the selective expression of CD177 on a subset of TA-Treg and demonstrated their highly activated phenotype. In TE, CD31 is expressed on polyfunctional memory CD8 T cells that secrete high cytokines levels and express markers of residency. Multi-Immuno-fluorescence analyses helped us to demonstrate that CD177+ Treg and CD31+ CD8 T cells colocalized in the tumor stroma. In vitro, TCR stimulation induces higher activation of CD31+ CD8 T cells than their CD31neg counterpart, as shown by the phosphorylation of ZAP-70, downstream TCR signaling, leading to an increased proliferation. The addition of an anti-CD31 mAb targeting the juxta-membrane domain, site of CD177 binding, reduced ZAP-70 phosphorylation and proliferation of CD31+ CD8 T cells. Ongoing experiments aim to investigate the impact of sorted CD177+ TA-Treg and CD177-transfected T cell line on CD31+ CD8 T cell functions (pZAP-70, proliferation, cytokines).

This project describes the first receptor/membrane ligand pair involved in the selective inhibition of CD8 T cells by Treg and the potential of CD177 as a promising target for lifting Treg-mediated suppression in the TE without inducing peripheral autoimmune reactions.

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FIRST-LINE DURVALUMAB AND TREMELIMUMAB WITH CHEMOTHERAPY IN RAS-MUTATED METASTATIC COLORECTAL CANCER: A PHASE 1B/2 TRIAL

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Metastatic colorectal cancer (mCRC) remains a difficult-to-treat disease. While patients with microsatellite instable metastatic colorectal cancer (mCRC) benefit from immune checkpoint blockade, chemotherapy with targeted therapies remains the only therapeutic option for microsatellite stable (MSS) tumors. However, signal of efficacy was shown using combination of anti-PD-L1 and anti-CTLA-4 in multitreated patients and the combination with FOLFOX regimen could lead to a potential positive effect on antitumor immune response. We report here the immune response observed following immunomonitoring of patients before and during MEDITREME trial.

The single arm, phase IB/II MEDITREME trial evaluated the safety and efficacy of durvalumab plus tremelimumab combined with mFOLFOX6 chemotherapy in first line, in 57 patients with RAS-mutant unresectable mCRC. We studied the phenotypic and functional immune response at inclusion and after 15 days (C2), 5 (C5) and 12 (C12) weeks of treatment by flow cytometry and by immunohistochemistry and the specific T response by ELISPOT. These immune parameters could be linked to survival data (RECIST criteria, progression free-survival) to highlight factors that may be prognostic of treatment response. One patient with a complete response allowed further a more detailed analysis of antitumoral immune response by single cell (sc) RNA- and TCR-sequencing, ELISPOT and flow cytometry analyses of the blood and tumor infiltrated immune cells.

Safety was the primary objective of phase IB; no safety issue was observed. The phase II primary objective of efficacy in terms of 3-month PFS in MSS patients was met, with 3-month PFS of 90.7% [95% CI: 79.2-96%]. For secondary objectives, response rate was 64.5%, median PFS was 8.2 months [95% CI: 5.9–8.6] and overall survival was not reached in MSS patients. We observed higher tumor mutational burden and lower genomic instability in responders. Integrated transcriptomic analysis underlined that high immune signature, and low epithelio-mesenchymal transition were associated with better outcome. Immunomonitoring showed induction of neoantigen and NY-ESO1 and TERT blood tumor specific T cell response, associated with better PFS. Deep immune and single cell RNA sequencing analysis of a complete responder showed induction of polyfunctional CD8 T cells at the tumor site, which reacted against tumor neoantigens.

Concurrent durvalumab-tremelimumab with oxaliplatin-based chemotherapy shows promising clinical activity in MSS mCRC and induces tumor specific immune response, driven by the immune and genomic contexture. Finally, the combination of durvalumab-tremelimumab with mFOLFOX6 was tolerable with promising clinical activity in MSS mCRC. Clinicaltrials.gov identifier: NCT03202758.

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THE CELL-BASED CANCER VACCINE VIDIDENCEL TRIGGERS ANTIGEN-SPECIFIC T CELL RESPONSES VIA INDIRECT PRIMING MECHANISMS.

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Cancer vaccines may provide for novel cancer maintenance therapy options, aimed at the prevention or delay of tumor recurrence. Vididence is a whole cell-based cancer vaccine derived from the DCOne human leukemic cell line. During manufacturing, DCOne cells are shifted towards a mature dendritic cell (mDC) phenotype, combining endogenous tumor antigen repertoire with a mDC costimulatory profile and providing the basis for vididence as a highly immunogenic vaccine.

Indirect priming mechanisms have been implied to play a crucial role in the triggering of anti-tumor immunity [1]. It was previously shown that upon intradermal administration, vididence is phagocytosed by skin-resident antigen-presenting cells (APC), which concomitantly become activated [2]. In this in vitro study we further investigated the potential of vididence to activate antigen-specific T cells through indirect priming mechanisms via host APC, using non-GMP grade DCOne-derived mature dendritic cells (DCOne mDCs); equivalent to vididence) and monocyte-derived DC (moDC) as model APC. To study cross-presentation, Far-RED-dye labelled-DCOne mDC ingested antigen containing PLGA-particles, washed and subsequently co-cultured with monocyte-derived dendritic cells for 24 hours. After 24 hours moDC positive for Far RED dye were sorted and co-cultured with antigen-specific T cells. INF- γ production by cocultured antigen-specific T cells was used as cross-presentation readout.

We observed that DCOne mDCs can effectively process and present endogenously expressed tumour-associated antigen such as WT-1. This specifically leads to secretion of effector cytokine IFN- γ by activation of co-cultured WT-1-reactive T cells, but not by NY-ESO-1-reactive T cells, as NY-ESO-1 is not expressed by DCOne mDC (Mendus AB, internal data). Moreover, DCOne mDC were found to be efficiently phagocytosed by donor-derived "bystander" moDC. This resulted in antigen transfer from DCOne mDCs to moDC and subsequent cross-presentation of antigens to antigen-specific T cells.

DCOne mDCs to moDC and subsequent cross-presentation of antigens to antigen-specific T cells. In conclusion, the in vitro data support a mechanism of action whereby vididence activates tumor

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BLOCKADE OF BETA ADRENERGIC SIGNALING REDUCES CANCER METASTASIS BY MODULATING THE TUMOR MICROENVIRONMENT

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Cancer metastasis contributes to most of the cancer-related deaths. Metastasis is a complex process involving various cellular and molecular interactions. In recent years, beta-adrenergic (ADRB) signaling has been identified as a potential player in the metastatic cascade. Retrospective studies indicate that the usage of ADRB blockade correlates with reduced cancer metastases, recurrence, and mortality. However, the mechanism of action of ADRB on cancer metastasis remains unclear. In this study, we investigated the immune modulatory effect of beta adrenergic receptor (ADRB) signaling in the context of cancer metastasis.

We utilized murine models of experimental lung metastasis, where C57BL/6 mice were injected with B16F10 melanoma cells, MC38-GFP colon adenocarcinoma cells, or T241-tdtomato fibrosarcoma cells via the lateral tail vein. Mice were offered a non-selective ADRB blockade propranolol in drinking water (0.5g/L) or regular drinking water and monitored for lung metastases formation. We performed blood sampling on metastasis bearing mice to quantify the circulating immune cell population using flow cytometry. At experimental end point, metastatic burdens in lung lobes of all mice were quantified by manual counting of macrometastasis nodules, flow cytometry analysis of lung derived single cell suspensions, and qRT-PCR of cancer cell specific genes. Immunohistochemistry stainings on formalin fixed paraffin embedded lung sections were also performed. Additionally, lung infiltrating, extravascular immune cells were quantified and phenotyped by flow cytometry.

We demonstrated that pharmacological blockade of ADRB significantly reduced metastasis formation in all three cancer types, supported by a decrease in total cancer cell number and cancer cell specific genes in the lung tissue. Hematoxylin and Eosin staining of lung sections further corroborated this finding. Flow cytometry analysis revealed that ADRB blockade led to a substantial decrease of myeloid derived suppressor cells ((CD11b⁺ Ly6Chi⁺ Ly6G⁻) in lung tissue and the peripheral blood of metastases bearing mice. This reduction was accompanied by an increase in circulating and lung infiltrating CD4⁺ T cells. Phenotyping of CD4⁺ T cells showed an increased expression of activation marker CD137.

Our results suggest that ADRB signaling contributes to the establishment and progression of lung metastases, potentially through the modulation of myeloid derived suppressor cells and CD4⁺ T cells. These findings demonstrated that propranolol, an approved drug for clinical use in various non-cancer indications, may offer an opportunity to improve disease outcomes for patients with metastatic disease.

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IMMUNOTHERAPY INDUCED REMODELING OF THE MYELOID CELL COMPARTMENT FACILITATES T-CELL INFILTRATION AND TUMOR REGRESSION

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Although immunotherapies have revolutionized cancer treatments, there is an ongoing research effort to decipher the mechanisms promoting clinical response with these treatments.

Recent data from several groups, including ours, revealed that both lymphoid and myeloid compartments are remodeled by effective immunotherapies. Our team revealed that a positive cooperation between CD8+ T cells and the activated tumor-infiltrating myeloid cells needs to take place in tumors for an optimal tumor regression. However, the dynamic changes in the immune populations and functions, as well as how these cells interact with each other, in regressing tumors after immunotherapy, remains unclear.

To address those questions, we characterized the immune infiltrate of transplanted PyMT mammary tumors regressing after injection of a STING (Stimulator of Interferon Genes) agonist. We performed scRNAseq of sorted myeloid cells and CD8+ T cells to uncover the identity of cell subsets involved in the cooperative rejection of tumor cells.

By following the dynamic changes of these populations in treated and control mice, we noticed that monocyte-like subsets accumulate transiently upon regression while macrophage clusters tend to disappear. In parallel, the CD8+ effector and memory subsets were increased during the regression at the expense of exhausted populations. We validated these data through unsupervised FACS analysis, allowing us to follow up with the fluctuations of these populations across different phases of tumor regression.

Moreover, our scRNAseq data enabled us to generate in silico predictions of the interactions between macrophages / monocytes and CD8+ T cells subsets. We are currently functionally looking into the increased chemotaxis and adhesion binding as predicted interactions between effector / effector memory CD8+ T cells and CXCL9+ Ly6Chi monocytes. Our preliminary data using dynamic imaging on fresh tumor slices show that CD8+ T cells from treated tumors are able to move faster than those in control tumors and infiltrate the tumor core. In addition, we observed that the effector CXCR6+ CD8+ T cells transiently infiltrate tumor islets during tumor regression, before moving back to the stromal areas. Additional experiments are ongoing to characterize further the spatial distribution of myeloid cell subsets with the aim to uncover the mechanisms by which CD8+ T cells and monocyte-derived cells cooperate in immunotherapy-treated tumors.

Altogether, our study emphasizes that immunotherapy reshape the tumor microenvironment beyond T-cells and we hope to decipher which specific cell-cell interactions promote tumor rejection with a protective immunity.

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SPATIAL TRANSCRIPTOMICS IDENTIFIES SUBTYPES OF LYMPHOID AGGREGATES AND THEIR KEY GENE SIGNATURES IN HUMAN GLIOBLASTOMA

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Glioblastoma (GBM) is an aggressive brain cancer that is resistant to T cell-reactivating immunotherapies due to poor T cell infiltration. Tertiary lymphoid structures (TLS) are ectopic lymphoid aggregates that form in inflamed tissues and are associated with positive prognosis in numerous cancers. Recently, we discovered that the occurrence of TLS correlates with increased T cell presence in human GBM, making them attractive targets to improve T cell infiltration. However, the role and diversity of lymphoid aggregates in GBM remain to be elucidated.

In this study, we coupled spatial transcriptomics with immunofluorescence staining techniques to identify, classify and study the composition of lymphoid aggregates in GBM patients. Lymphoid clusters identified in tumor sections were manually annotated based on their appearance, location and T to B cell composition. Aggregates that formed around vessels and extended beyond the perivascular space were classified as TLS, while aggregates that remained confined to the perivascular space were classified as perivascular niches (PN). In addition, PN and TLS were sub-classified as “T cell-dominant” (T), “B cell-dominant” (B) or “mixed” (M) depending on their B and T cell composition. Next, we profiled the annotated lymphoid aggregates using the GeoMX® spatial transcriptomics platform.

Spatial deconvolution and differential gene expression analysis identified key characteristics of each subtype of lymphoid aggregate and their involvement in different biological processes. B-TLS exhibited high expression of genes involved in B cell differentiation/activation, BCR signaling and NFκB regulation, but low proportions of professional antigen-presenting cells. M-TLS displayed the most robust cytotoxic T cell signature, as well as high expression of HLA molecules and genes involved in type-I IFN response. T cell-rich structures harbored the highest proportion of T helper cells. Interestingly, T-TLS displayed the most pronounced fibroblastic signature and high expression of genes involved in both extracellular matrix organization and cell motility, but low expression of HLA molecules. Finally, T-PNs were characterized by gene signatures associated with regulation of TCR signaling/T cell activation and antigen processing/presentation.

In conclusion, this study highlights that lymphoid aggregates in human GBM are diverse and likely to exert different functions depending on their nature and T to B cell content. Unravelling their composition and role will pave the way to elucidate which structures are most beneficial in GBM, and whether they can be boosted through immunotherapy.

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TCR DEXTRAMER® REAGENTS CAN MEASURE ANTIGEN PRESENTATION ON CELLS

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When developing cancer immunotherapies, the detection and quantification of antigen-presenting cells (APC) is important for 1) stratification and selection of patients with demonstrated expression of the target antigen, 2) confirming tissue-specific expression of the target, and 3) monitoring target expression during treatment. In the context of TCR-T cell-based therapy, the specificity and sensitivity of the selected TCR must also be validated before proceeding to clinical development. To support such efforts, we have developed TCR Dextramer reagents to allow detection of peptide presentation by antigen-presenting cells. Here, we demonstrate the use of TCR Dextramer reagents for the sensitive detection of specific peptide epitopes on the surface on peptide-pulsed APCs.

T2 cells or HLA-A*02:01-positive peripheral blood mononuclear cells (PBMC) were pulsed with peptide, stained with peptide-specific TCR Dextramer reagents, and finally analysed using flow cytometry. To address the effect of TCR affinity for its cognate MHC-peptide complex, two TCR Dextramer reagents were prepared based on TCR monomers with high and low affinities for their cognate MHC-peptide complex.

The high and low affinity TCR Dextramer reagents were analysed for recognition of their cognate peptide, negative control peptide, and a mixture of both peptides on T2 cells. For both TCR Dextramer reagents and for single peptides as well as mixtures, the cognate peptide was detected below 20 nM pulsing concentration with only minor loss of sensitivity for the mixtures and the low-affinity TCR Dextramer reagent.

Our novel TCR Dextramer reagents allow detection of specific peptide epitopes on peptide-pulsed PBMCs and T2 cells, both as single peptides and peptide mixtures, and detection of peptides down to 20 nM pulsing concentration is possible in these model systems. This makes TCR Dextramer reagents useful to validate candidate TCRs for therapy as well as studying antigen presentation on cells.

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INVESTIGATING THE SUSCEPTIBILITY OF DRUG-RESISTANT OVARIAN CANCER CELLS TO ONCOLYTIC VIROTHERAPY

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Ovarian cancer (OC) is the deadliest gynaecological malignancy in women. First-line treatments are initially effective, however, up to 75% of OC patient's relapse. Relapsed disease can acquire resistance to standard chemotherapies. In addition, the tumour microenvironment (TME) also contributes to drug resistance. This intrinsic drug resistance exists in patients before the administration of anticancer drugs through tumour cell interactions with the host stroma including immune cells, endothelial cells, fibroblasts, and metabolites. Therefore, novel treatment options are urgently required to combat both acquired and intrinsic drug resistance in OC.

Oncolytic Viruses (OV) are emerging immunotherapy that can directly infect and lyse tumour cells whilst also inducing an anti-tumour immune responses. Currently, OVs are being tested for the treatment of a range of cancers and importantly, can be genetically engineered to deliver additional therapeutic payloads (such as miRNA) to enhance treatment efficacy. miRNAs have been shown to regulate numerous drug resistance genes such as MDR1 in OC, and modulating miRNA expression can alter cancer cell sensitivity to chemotherapies.

- 1- Microculture tetrazolium tests (MTT) assay: Was used to determine OC cells growth/viability against paclitaxel, cisplatin or carboplatin drug treatments.
- 2- Paclitaxel-resistant cell lines: SKOV-3 and 1847 resistant cell lines (SKOV-3R and 1847R, respectively) were generated when cultured with increasing doses of paclitaxel for a long period of time.
- 3- Cell viability using Zombie Aqua™ Fixable Viability Kit: Was used to assess the viability of parental and resistant cell lines when treated with increasing MOI (Multiplicity of infection) of Maraba-1-GFP (MG-1-GFP), Herpes simplex virus-1-GFP (HSV1-GFP), Reoviridae (Reo) and coxsackie (CVA) virus treatment.
- 4- 51 Chromium release assay: Was used to detect the percentage of parental 1847, SKOV-3 (1847P & SKOV-3P, respectively) and resistant 1847, and SKOV-3 cell death (1847R & SKOV-3R, respectively) when treated with effector NK cells. Effector cells were generated by treating healthy PBMC donors with 0.1 MOI of either MG-1-GFP, HSV1 or Reo virus.
- 5- miRNA expression in OC cell lines was analysed by qPCR using SYBR™ green reagents.

To generate paclitaxel-resistant OC cell lines, OC cells were cultured in increasing doses of drug. Increased cell viability of resistant cell lines compared to parental cell lines was observed. Moreover, paclitaxel-resistant cell lines were also resistant to carboplatin, compared to parental cell lines, suggesting the generation of multidrug-resistant phenotype. Importantly, (i) drug-resistant OC cells were susceptible to NK cell killing and NK cell killing was enhanced by OV treatment, and (ii) direct OV oncolysis was not abrogated, and in fact, was sometimes increased in drug resistant cell lines, compared to parental cells. Additionally, overexpressing miR-145 in OC cells was observed following with miR-145 encoded MG-1 virus infection.

We have identified that OV therapies could be used to eradicate drug-resistant OC cells. Detailed characterisation of drug resistant OC cells now aims to identify therapeutic miRNA payloads that could be used to restore chemotherapy sensitivity and boost OV efficacy.

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REWIRING INNATE AND ADAPTIVE IMMUNITY WITH TLR9 AGONIST TO TREAT OSTEOSARCOMA

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Osteosarcoma (OS) is the most common primary bone tumor in children and adolescent. Surgery and multidrug chemotherapy are the standard of treatment achieving 60-70% of event-free survival for localized disease at diagnosis. For metastatic disease, however, the prognosis is dismal, making urgent the identification of new, more efficient and possibly less toxic, strategies.

In immune competent OS mouse models bearing two contralateral lesions, we tested the efficacy of intralesional administration of a TLR9 agonist against the treated and not treated contralateral lesion evaluating abscopal effect. Multiparametric flow cytometry was used to evaluate changes of the tumor immune microenvironment. Experiments in immune-deficient mice allowed the investigation of the role of adaptive T cells in TLR9 agonist effects, while T cell receptor sequencing was used to assess the expansion of specific T cell clones.

TLR9 agonist strongly impaired the growth of locally-treated tumors also showing a robust effect against the contralateral, untreated lesion. Multiparametric flow cytometry analysis showed a reduced fraction of CD206+ M2-like macrophages over the total F4/80 population, paralleled by an increased infiltration of dendritic cells and activated CD8 T cells in both lesions. Notably, CD8 T cells were needed for induction of the abscopal effect, whereas they were not strictly necessary for impairing the growth of the treated lesion. T cell receptor (TCR) sequencing of tumor infiltrating CD8 T cells showed the expansion of specific TCR clones in the treated tumors and, remarkably, their selected representation in the contralateral untreated lesions.

Overall these data indicate that the TLR9 agonist acts as an in situ anti-tumor vaccine, activating an innate immune response sufficient to suppress local tumor growth while inducing a systemic adaptive immunity with selective expansion of CD8 T cell clones, which are needed for the abscopal effect.

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ENHANCING THE ANTITUMOR ACTIVITY OF ANTI-CD47 ANTIBODIES THROUGH FC OPTIMIZATION

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The CD47/SIRPα axis plays a crucial role in cancer immunosurveillance. While anti-CD47 antibodies have shown promise in several preclinical models, results from early phase clinical trials have shown limited clinical benefit and significant on-target toxicity, suggesting that the sole blockade of CD47 by the antibody Fab domain might not be sufficient for effective tumor control. A critical question that remains to be answered is whether interactions between the antibody Fc and Fc gamma receptors (FcγRs) also contribute to the activity of these therapies. Our study aims to investigate the role of the Fc domain in the *in vivo* antitumor activity of anti-CD47 antibodies using immunocompetent species-matched models, overcoming limitations of previous studies conducted in immunocompromised models or with interspecies differences between mouse (m) and human (h) CD47, SIRP-α and FcγRs.

We modified the Fc domain of the anti-mCD47 antibody MIAP301 to generate antibodies with varying affinity to mFcγRs: 1) MIAP301-mIgG2a Fc, binding to preferentially to activating mFcγRs, 2) MIAP301-mIgG1 Fc, binding to the inhibitory mFcγRIIB, and 3) MIAP301-mIgG1-D265A Fc, which lacks binding to any mFcγRs. We evaluated the antitumor activity of these antibodies in MC38 and B16 tumor models using both immunocompetent C57BL/6J mice and mice lacking activating FcγRs. Analysis of the tumor immune microenvironment was performed after treatment with these antibodies using multiparameter flow cytometry. Additionally, we generated a mouse humanized for the expression of hCD47, hSIRP-α and hFcγRs through CRISPR/Cas9-mediated gene-targeting strategy, and by backcrossing to our hFcγR mice. We used this model to compare the antitumor activities of the anti-hCD47 antibody Magrolimab (5F9-hIgG4), and an Fc-optimized variant that enhances binding for all the activating hFcγRs (5F9-GAALIE) generated in the lab.

The MIAP301-mIgG2a Fc variant led to the most significant reduction in tumor burden when compared to the control or other Fc variants in WT mice in both MC38 and B16 models. This therapeutic effect was abrogated in mice lacking activating FcγRs and when macrophages were depleted using clodronate. Treatment with the MIAP301-mIgG2a correlates with significant increase in CD11b⁺F4/80⁺ macrophages, antigen-specific T cells, and decreased number of regulatory T cells (Tregs). CD47/SIRP-α/FcγR humanized mice recapitulate the expression profile of CD47 and SIRPα found in human cells. In contrast to experiments done in C57BL/6 mice, increasing dosing concentrations of both 5F9-hIgG4 and 5F9-GAALIE antibodies led to on-target anemia and thrombocytopenia in hCD47/hSIRP-α/hFcγR mice, recapitulating results obtained from patients in clinical trials. To overcome toxicity, we found that intratumoral administration of the Fc optimized 5F9-GAALIE results in enhanced long-term antitumor immunity, abscopal antitumor effect, and minimal on-target toxicity when compared to 5F9-hIgG4 or control alone or in combination with PD-1 blockade

The antitumor activities of anti-CD47 antibodies require interactions with activating FcγRs. We found that local administration of an Fc-engineered anti-CD47 antibody with enhanced binding to activating FcγRs modulates myeloid and T-cell subsets in the tumor microenvironment, resulting in improved long-term systemic antitumor immunity and minimal on-target off-tumor toxicity. Overall these results highlight the importance of Fc optimization in the development of effective anti-CD47 therapies.

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UNRAVELING IMMUNE CHECKPOINTS IN PDAC: FOCUS ON THE TIGIT AXIS

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Pancreatic ductal adenocarcinoma (PDAC) remains one of the deadliest cancers with a five-year survival rate of less than 10%. Its resistance to therapy is strongly influenced by the unique tumor microenvironment (TME). This TME is characterized by a dense desmoplastic stroma consisting of cancer-associated fibroblasts, immune cells, and extracellular matrix components. This creates not only a physical barrier that hinders drug delivery and immune cell infiltration but also fosters an immune suppressive environment. The emerging field of immune checkpoint inhibitors resulted in sustained anti-tumor response in several cancer types. However, PDAC patients enrolled in these clinical trials demonstrated no clinical benefit. Therefore, new and effective targets for novel immunotherapy combinations need to be sought. In that light, this study investigates the potential of targeting TIGIT, a receptor involved in immune regulation, as a therapeutic approach for PDAC treatment.

In this study, a comprehensive analysis of the TIGIT axis was performed using matched peripheral blood and tumor samples from 25 PDAC patients. Multicolor flow cytometry was employed to examine the expression of various receptors, including TIGIT, TACTILE, and DNAM-1, on immune cells of interest. Co-expression analysis with PD-1 and PD-L1 was also conducted. Additionally, immunohistochemistry was utilized to assess the expression of relevant ligands, such as CD111, CD112, CD113, and CD155, on tumor tissue slides. The data obtained from these analyses will further be correlated with clinicopathological information of the patients.

Preliminary findings from this study revealed a significant decrease in the number of natural killer cells within the tumor tissue compared to peripheral blood samples. Conversely, regulatory T cells (Tregs) were found to be significantly increased in the tumor tissue, with higher expression of TIGIT compared to circulating Tregs. Interestingly, cytotoxic cells in the tumor expressed significantly lower levels of DNAM-1 compared to their peripheral blood counterparts, indicating potential immune suppression in the TME. These results provide insights into the complexity of the TIGIT axis in PDAC and highlight potential targets for future immunotherapeutic strategies.

The study findings suggest that targeting the TIGIT axis could be a promising therapeutic approach for PDAC. The observed alterations in immune cell populations and receptor expression within the TME indicate the potential for immune evasion and suppression mechanisms. Further correlation with clinicopathological data will provide a foundation for the development of rationally designed combination immunotherapy strategies. This research contributes to a better understanding of the TIGIT axis in PDAC and offers valuable insights into potential targets for future immunotherapeutic interventions.

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ENHANCING IMMUNOTHERAPY OUTCOMES IN CERVICAL CANCER: INSIGHTS INTO RANK AND IMMUNE CHECKPOINT CO-TARGETS

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Conventional treatment of cervical cancer (CC) has reached a plateau, resulting in low five-year survival rates, particularly for advanced stages. Immunotherapy shows promise in improving long-term outcomes for oncology patients. However, only a minority of CC patients respond to immunotherapy despite the immunogenic nature of the disease. The RANK-axis has recently emerged as an immune suppressive pathway in cancer, potentially augmenting the effects of immunotherapy. However, robust evidence supporting this concept is still lacking. To address this gap, we conducted a study to examine the impact of RANK on the interaction between tumor and immune cells in CC. We also assessed the immune landscape in CC patient samples by profiling a selection of immune checkpoints on CC cell lines. We are currently exploring the potential of combining the most interesting immunotherapy co-targets with RANK-blocking therapy using high-throughput in vitro co-cultures.

We utilized 2D co-cultures with NK-92 cells and various CC cell lines to evaluate the effect of RANK on tumor cell killing and NK cell activation through flow cytometry. Lentiviral transduction of LacZ (control) and RANK was performed on all CC cells, while NK-92 cells underwent Mock or RANKL mRNA nucleofection. Flow cytometry was employed to screen immune checkpoints on CC cell lines before and after treatment with exogenous rhRANKL in order to identify intriguing co-targets. Our selection of markers was based on TCGA analysis and literature research. Immunohistochemistry was then conducted on 49 formalin-fixed and paraffin-embedded CC patient samples to assess the expression of the most abundant markers correlated with RANK. Lastly, we are conducting high-throughput NK-tumor cell co-culture experiments with live cell imaging to target the identified immune checkpoint axes in combination with RANK-blocking therapy.

Our study revealed that RANK inhibits tumor cell killing by NK-92 cells. We identified noteworthy co-targets for RANK inhibition in CC, such as PD-L1, which were found to be more abundantly expressed than PD-L1 through IHC analysis of 49 patients. The ongoing combination immunotherapy co-culture experiments are still underway.

Interim analysis of our study has identified intriguing (co-)targets for immunotherapy in CC. Our ongoing work aims to identify the optimal combination immunotherapy approach targeting RANK.

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PREVENTION OF TERMINAL T CELL EXHAUSTION FEATURES BY TREATMENT WITH CHROMATIN REMODELING INHIBITORS

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Repeated stimulation during chronic viral infections and within the tumor microenvironment without resolution progressively diminishes a T cell's ability to respond, a process referred to as T cell exhaustion or T cell dysfunction. In vitro assays are needed that both model T cell exhaustion/dysfunction as well as incorporate a therapeutic window to both prevent late-stage terminal T cell exhaustion as well as potentially re-invigorate T cells at the tail end of the exhaustion continuum. Exhausted T cells display dramatically upregulated surface expression of co-inhibitory receptors such as PD-1, have reduced effector cytokine secretion, and have an altered epigenetic landscape, which can alter functionality. mSWI/SNF ATPase-specific inhibitors and degraders have been shown to attenuate T cell exhaustion and increase memory T cell phenotypes. Inhibition of SMARCA4/2 ATPase activity was recently shown to result in a significant lowering of PD-1+/TIM-3+ double-positive exhausted T cell populations, increasing progenitor exhausted cells (PD-1+/TIM-3-), lowering the terminally exhausted CD39+ population and enhancing persistence associated with lowered apoptosis in treated cells. Here, we employed our previously reported in vitro T cell exhaustion model to test the SMARCA4/2 ATPase inhibitor FHT-1015 as well as two Proteolysis Targeting Chimera compounds (PROTACs), ACBI1 and AU-15530, to assess their effect on T cell differentiation and function.

To model chronic T cell stimulation in vitro, we repeatedly stimulated PBMCs from 4 donors with CD3/CD28 Dynabeads. FHT-1015, ACBI1, or AU-15530 were treated with the last two Dynabead treatments. Following stimulation, supernatant was harvested for IL-2, IL-6, TNF-alpha, and IFN-gamma cytokine analysis, and T cells were collected for flow cytometry immunophenotyping analysis (PD-1, TIM-3, LAG-3, CD38, CD4, CD8, CD3, Live/Dead, and terminal exhaustion marker CD39).

Treatment of exhausted T cells with FHT-1015 during repeat stimulation resulted in selective reversal of key co-inhibitory receptor surface expression; TIM-3, LAG-3, and CD39 (66% and 35% CD39 reduction in CD4+ and CD8+ T cells, respectively). However, PD-1 surface expression was unchanged, while that of CD38 was elevated with FHT-1015. Importantly, progenitor-like PD-1+, TIM-3- T cells were increased in FHT-1015-treated T cells (1.7-fold in CD4+, 2.7-fold in CD8+ T cells relative to untreated) but not in PROTAC-treated T cells. Cytokine secretion of IL-2, IL-6, TNF-alpha, and IFN-gamma was all reduced with FHT-1015 treatment (~4.5- and ~3.6-fold for IFN-gamma and TNF-alpha, respectively, for 2X FHT-1015 treated cells). PROTAC treatment produced lesser or no reductions in co-inhibitory receptor expression (CD39 and TIM-3) relative to FHT-1015. IFN-gamma and TNF-alpha were also reduced to a lesser degree with PROTACs. These results illustrate the utility of this assay to screen drug candidates for preventing T cell exhaustion and discriminating the strengths of different therapeutics.

We demonstrate the application of our in vitro T cell exhaustion assay to test for immunomodulatory drug candidates able to alter T cell exhaustion development functionality during and after terminal differentiation.

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IDENTIFICATION OF IMMUNOTHERAPY GENE TARGETS ASSOCIATED WITH LUNG CANCER USING TCGA DATASET IN THE CONTEXT OF MUTATION SIGNATURES

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Lung cancer is the leading cause of cancer-related deaths worldwide, accounting for around one in five of all cancer deaths. The use of bioinformatics may provide insight into the common characteristics and interactions between genes of lung cancer within significant clusters. Lung cancer is the leading cause of cancer-related deaths worldwide, accounting for around one in five of all cancer deaths. This study investigates interaction pairs of lung adenocarcinoma (LUAD) from The Cancer Genome Atlas project. Our research identified significant interactions between genes, non-small cell lung cancer (NSCLC) related mutation signatures, the gene targets of lung cancer-associated miRNA, and possible LUAD immunotherapy gene targets.

We used the DAVID and COSMIC databases in order to determine the genes involved in NSCLC. We then ascertained the gene fusions of the genes of interest. We also created an annotation table, which determines the top three mutation signatures of each of the 96 mutations. We were able to utilize CBioPortal, COSMIC, UCSC Genome Browser, and Transvar in order to identify the common NSCLC DNA mutations of each gene of interest, and using our annotation table, we identified the mutation signatures most associated with NSCLC. Then, we did a CD Batch Search of the genes of interest to possibly determine genes that have a functional association with each other. We also identified two miRNAs in the TCGA dataset that are associated with lung cancer and their target genes. Finally, using TIMER, we found the correlation between immune infiltration of certain immune cells (B cells, T cell CD4+, T cell CD8+, neutrophils) and gene expression of certain genes in the context of our mutation signature results.

Regarding the gene fusions, we found 5 genes of interest: ALK and ROS1 for their numerous (4) gene fusions and NRG1, CD74, NTRK1, and ROS1 for their gene fusions with CD74. Certain mutational signatures appeared in many of the 17 genes studied: SBS39 appeared in 13, SBS86 appeared in 12, SBS17 appeared in 9, and SBS15 in 5. Additionally, there were no common DNA mutations in CD74 between CBioPortal and COSMIC. CD Batch Search found that BRAF, ROS1, ALK, and CDK6 all share one superfamily, cl21453. STAT5B and STAT3 share multiple, but PIK3R1 share cl15255 with them, KIF5B and PPF1BP1 also share cl37069. Also, miRNA hsa-mir-186 and hsa-mir-570 target the same 4 genes, out of the 7 total genes targeted between the miRNA. TIMER results found that neutrophils have a positive correlation with 9 of the 13 genes associated with at least one notable mutation signature (SBS39, SBS86, SBS17, SBS15). T-cell CD8+ immune infiltration has a positive correlation with CDK6 and STAT5B, but it has a negative correlation with SLC3A2. T-cell CD4+ has a positive correlation with 5 of the 13 genes. B cells have a positive correlation with 3 genes and a negative correlation with 3 other genes.

ALK's and ROS1's 4 gene fusions demonstrate how the genes involved in the NSCLC KEGG Pathway interact with NRG1, CD74, and ROS1, which are not part of the pathway even though they have been identified by COSMIC to be associated with NSCLC. Additionally, the fact that CD74 has no common mutations demonstrates that CD74 is associated with NSCLC not because of its mutations, but because of its gene interactions, like gene fusion. The frequent appearance of SBS39 and SBS86 suggests that these two mutation signatures are associated with NSCLC; moreover, the fact that these two signatures often appear in the same genes suggests that the signatures' high-frequency C>G mutation is associated with NSCLC. The two miRNAs largely target the same genes, which suggests that the miRNA may play a role in the genes' association with NSCLC. Finally, the results from TIMER suggest possible immunotherapy gene targets; for example, enhancing the

expression of certain target genes (e.g. KIF5B, CD74) may increase immune infiltration of T cell CD4+.

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CODON-SPECIFIC MRNA TRANSLATION OPTIMIZATION REGULATES TUMOR IMMUNITY THROUGH MHCII IN MELANOMA

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mRNA translation reprogramming supports cancer development, immune evasion and resistance to immunotherapy. Changes in tRNA regulation and codon-specific mRNA translation have been associated with cancer metastasis and resistance to therapy. Wobble tRNA modifications are required for specific codon decoding during translation and for maintenance of protein homeostasis.

We used a combination of mouse models, patient derived lines, single cell RNA-seq, ribosome profiling and TCR sequencing to assess the impact of the loss of the wobble tRNA modifications in reshaping the immune landscape of poorly immunogenic melanoma cells.

Here we show that ablation of enzymes catalyzing wobble uridine (U34) tRNA modification (U34 enzymes) promotes anti-tumoral immune response through remodeling of MHCII antigen presentation. We show that U34-enzymes deficiency in melanoma leads to the specific infiltration of cytotoxic Th1 CD4+ T cells, but not of CD8+ T cells, that fully rely on antigen presentation by MHCII complex in melanoma cells. Mechanistically, U34-enzymes deficiency perturbs protein homeostasis through codon-specific translation regulation, activates autophagy and leads to remodeling of antigen presentation by MHCII in melanoma cells. Accordingly, TCR signaling is engaged in CD4+ T cells, and specific immunodominance is observed in melanoma tumors deficient for U34-TM enzymes. Consistently, U34-signature inversely correlates with Th1 cells infiltration in melanoma patients.

Taken together, our results show that codon-specific mRNA translation, through modulation of wobble uridine tRNA modification, represents a new strategy to regulate tumor immunology and to control tumor growth in melanoma through antigen presentation.

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ESTABLISHING A CLINICALLY RELEVANT MOUSE MODEL OF MESENCHYMAL GLIOBLASTOMA FOR STUDYING IMMUNOTHERAPEUTICS AND TME TARGETING AGENTS

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Historically, pre-clinical GBM models have largely failed to predict response in the clinical setting. Here, we establish a more clinically faithful model using the syngeneic NFpp10a-Luc2 mesenchymal-GBM cell line to study response to resection and temozolomide (TMZ), adjuvant/neo-adjuvant anti-PD1 and Regorafenib (REGO) therapy.

Cells were orthotopically implanted in C57BL/6-mice. Response to surgical and therapeutic interventions was assessed using bioluminescence imaging (BLI). Murine Microenvironment cell-population (mMCP)-counter, GSEA and multiple interactive labelling by antibody neodeposition (MILAN, high-dimensional single-cell multiplex analysis) were employed to characterise treatment-related TME effects.

We observed survival advantage in aged mice undergoing resection (resection:33.5 days vs non-resection:18 days; $p=0.0166$). TMZ/anti-PD1 had no impact on tumour growth (TMZ: $p=0.9001$, anti-PD1: $p=0.7933$) or survival (TMZ: $p=0.3035$, anti-PD1-: $p=0.6328$). Neo-adjuvant anti-PD1 also conferred no survival advantage in young mice (33 vs 35 days; $p=0.9429$). REGO/REGO+TMZ treatment conferred no survival benefit in young mice (REGO: $p=0.0735$ and REGO+TMZ: $p=0.3945$).

mMCP-counter (which estimates the abundance of TME-cell populations from gene-expression data) showed resection upregulated B-cells and mast-cells, whereas TMZ caused a decreased abundance of vessels. Anti-PD1 treatment enriched B-cells, mast-cells, and CXCR3 expression ($p=0.0045$). REGO/TMZ+REGO treatment upregulated cytotoxic-lymphocyte populations.

MILAN analysis showed resection increased cytotoxic-T-cells (20.3% vs pre-resection:7%). In contrast, TMZ/REGO monotherapy increased tumour-cells and decreased cytotoxic-T-cells. Anti-PD1 decreased macrophage abundance (39.8% vs control:14.5%). Likewise, TMZ+REGO decreased macrophages (11.3% vs control:35.3%) but upregulated B-cells and vessels.

Overall, we have characterised response of the NFpp10a mouse model to resection, TMZ, anti-PD1 and REGO. We have shown that the model is insensitive to chemotherapy and TME-targeting agents (including immunotherapeutics), mirroring patient-response patterns. Nevertheless, we observe transcriptomic and proteomic changes following TME-targeting treatments. Further analyses of these TME-associated resistance properties may help guide novel combinatorial treatment regimens. Overall, the NFpp10a model of mesenchymal GBM may be employed in future pre-clinical studies to accurately guide future clinical trials.

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COMBINING PD1 BLOCKADE WITH EPIGENETIC THERAPY THROUGH AN HDAC INHIBITOR ACHIEVES SUPERIOR RESPONSE RATES IN HPV-ASSOCIATED SQUAMOUS CANCERS. THE PEVO TRIAL

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Epigenetic modulation plays a major role in escaping tumor immunosurveillance and confers resistance to immune checkpoint inhibitors. Preclinical evidence suggests that modulating the epigenome might improve the efficacy of immunotherapy. We proposed that squamous cell cancers, which share several molecular features irrespective of their anatomical origin and are often associated with Human Papilloma Virus (HPV) infections, may benefit from the addition of histone deacetylase (HDAC) inhibitors to anti-PD1 treatment. Rare squamous cancers like anal cancer represent an unmet clinical need, in which responses to immunotherapy alone (anti-PD1, with or without anti-CTLA4) therapy remain low, and no standard of care beyond first-line chemotherapy is available

PEVOsq is an open-label, non-randomized, multi-center, basket phase II trial, evaluating the efficacy of pembrolizumab in combination with the HDAC inhibitor vorinostat in patients with recurrent and/or metastatic squamous cell carcinoma of the cervix, head and neck, anus, vulva/vagina, penis, and lung. Patients had to be PD1/PD-L1 antagonist-naïve. There was no selection based on PD-L1 expression nor on HPV status, and no restriction in terms of prior lines of treatments. Pembrolizumab dose was 200 mg Q3W IV, and vorinostat 400 mg QD PO. Sample size was determined for each cohort using an A'Hern design. Primary endpoint was objective response rate (ORR) according to RECISTv1.1. Secondary endpoints included safety, progression-free survival (PFS), overall survival (OS), and duration of response (DOR). All patients underwent systematic biopsy collection at baseline, on-treatment (after cycle 2) and at progression for a vast array of translational analyses, which includes PD-L1 and tumor-infiltrating lymphocyte (TIL) quantification, Whole Exome Sequencing, RNA sequencing, HLA sequencing, HPV integration analysis, mass spectrometry-based analysis of histone modifications, long-read sequencing by Oxford Nanopore for native DNA methylation analysis.

Among 112 included patients, 111 were evaluable for safety and 107 for efficacy. The lung cohort was closed prematurely because of lack of enrollment. Median age was 61 years old [range: 18-85] and the median number of prior lines of therapies was 1 [range: 0-4].

28 patients (26%) had an objective response and median DOR was 9.7 months [95%CI: 3.1-15.2]. Response rates were highest in anal (9/27, 31%, 15.3-50.8 95% CI) and cervical (9/24, 39%, 19.7-61.5 95% CI) cancers, with OS ; biomarkers associated with improved OS included HPV positivity (median OS 6.8 months in HPV- vs 17 in HPV+, p=0.03), WES-quantified Tumor Mutational Burden (10.9 vs not reached, p=0.02), (11.1 vs not reached) and PD-L1 CPS score. Additional translational analyses including correlation of transcriptional signatures and HPV integration features are ongoing

and will be presented at the conference.

Pembrolizumab and vorinostat were stopped for toxicity in 9% and 39% of patients, respectively. 60% of patients had a dose-reduced of vorinostat for toxicity. Pembrolizumab safety was as expected. Main vorinostat toxicities included hematological toxicity, gastrointestinal disorders, and asthenia and creatinine increase

Pembrolizumab combined with vorinostat showed encouraging antitumor activity in squamous cell carcinoma with unselected PD1/PD-L1 status, especially in HPV-associated cancers. Response rates in these tumors are higher than previously reported in trials with immunotherapy alone. Epi-immuno combinations represent a promising strategy in HPV-induced tumors of unmet medical need like anal cancer.

Clinical trial information: NCT04357873.

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ANTIBODY REPERTOIRES OF TUMOR-INFILTRATING B CELLS

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Tumor-infiltrating B cells are correlated with a better prognosis in lung and ovarian cancer. However, their role remains largely unknown.

To assess the potential anti-tumor activity of the antibodies produced by these cells we are analyzing recombinant cognate antibody repertoires from tumor infiltrating B cells from lung and ovarian cancer.

Antibody repertoire libraries were generated using a droplet microfluidic single B cell platform which enables the cloning and expression of cognate antibody repertoire libraries from up to 1 million tumor derived B cells. These libraries can be expressed as full-length IgG and the thus obtained recombinant antibody mixtures can be used in standard screening assays. This platform also retains the capability to identify monoclonal antibodies responsible for a given functionality identified in an antibody mixture.

Antibody repertoire libraries were established from 5 NSCLC donors with a high abundance of tertiary lymphoid structures and from a single donor with HGS ovarian cancer.

An initial assessment of the obtained repertoires by next generation sequencing showed that each of the samples of approximately one cubic centimeter of tumor tissue yielded thousands of antibody clonotypes and that the majority of these antibodies show signs of affinity maturation and clonal expansion.

Tumor-derived antibodies from NSCLC with tertiary lymphoid structures are surprisingly diverse. The observed signs of affinity maturation and clonal expansion may point to an antigen-driven mechanism. We are currently characterizing the recombinantly expressed antibody libraries in a battery of assays combining both binding- and functional readouts.

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SCSTUDIO: A WEB PORTAL FOR INTUITIVE AND FLEXIBLE ANALYSIS OF PUBLIC SCRNA-SEQ DATA

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The increasing availability of single-cell RNA sequencing (scRNA-seq) data has recently allowed the study of a vast variety of biological tissues and systems with unprecedented detail. However, most scRNA-seq analysis tools still require programming knowledge, which hinders their utilization by researchers without computational expertise. In the last decade, efforts towards the development of applications with visual interfaces for scRNA-seq data analysis resulted in programs that still present several shortfalls, namely in the usability by non-computational scientists (no automated access to publicly available data, inflexibility, “black box” algorithms, no joint analysis of multiple datasets, etc.).

R (software environment for statistical computing), using packages from Bioconductor (public repository of R tools for the analysis of high-throughput genomic data, <https://www.bioconductor.org/>).)

We are developing scStudio, a web-based tool for scRNA-seq data analysis by non-computational researchers. One main advantage of scStudio will be enabling users to automatically retrieve datasets from the Gene Expression Omnibus (GEO) data repository, that currently holds the majority of publicly available single-cell data, thereby democratizing their access by laboratories that cannot afford to generate them. In addition, scStudio is based on a modular structure, to allow easy and straightforward integration of constantly emerging new methods for single-cell data analysis and thereby their immediate availability to all users.

Altogether, with scStudio we will not only refine previous efforts to create an interface for single-cell data analysis by non-computational researchers, but also make available novel tools to allow them to take full advantage of the potential of scRNA-seq data exploration. Finally, scStudio will be made available as open-source software. A demo prototype of scStudio is already accessible through the host lab’s web server (<https://compbio.imm.medicina.ulisboa.pt/>).

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HEME CATABOLISM BY TUMOR-ASSOCIATED MACROPHAGES CONTROLS METASTASIS FORMATION

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Tumor-associated macrophages (TAMs) are heterogeneous orchestrators of cancer-related inflammation, whose functional and topographical diversity influences disease progression and response to therapy. Although the pathological significance of TAMs heterogeneity is still poorly understood, TAMs reprogramming is viewed as a promising anticancer therapy.

Flow cytometry analysis and FACS sorting, preclinical fibrosarcoma and melanoma mouse models, mRNA-Seq, confocal microscopy, immunohistochemistry.

Here we show that a distinct subset of TAMs (F4/80hiCD115hiC3aRhiCD88hi) endowed with high rates of heme catabolism by the stress-responsive enzyme heme oxygenase-1 (HO-1) plays a critical role in shaping a pro-metastatic tumour microenvironment, favoring immunosuppression, angiogenesis and epithelial-to-mesenchymal transition (EMT). This population originates from F4/80+HO-1+ bone marrow precursors, accumulates in the blood of tumor bearers and preferentially localizes at the invasive margin through a mechanism dependent on the activation of Nrf2 and coordinated by the NF-kB1/CSF1R/C3aR axis. Inhibition of F4/80+HO-1+ TAM recruitment or myeloid-specific deletion of HO-1 blocks metastasis formation and improves anticancer immunotherapy. Relative expression of HO-1 in peripheral monocyte subsets, as well as in tumor lesions, discriminates survival among metastatic melanoma patients.

Overall, these results identify a distinct cancer-induced HO-1+ myeloid subgroup as a new anti-metastatic target and prognostic blood marker.

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IMMUNE-MEDIATED REGULATION OF ADENOSQUAMOUS CARCINOMA OF PANCREAS (ASCP) TUMOR GROWTH

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Pancreatic cancer is one of the deadliest cancers, with a current 5-year survival rate of 12.5%. This rate drops drastically to 3% when it is diagnosed at a late stage, which accounts for over half of all pancreatic cancer cases. For this reason, early interception of the disease is critical to improving overall survival. Among emerging therapeutic strategies to treat pancreatic cancer are drugs that stimulate the immune system to block disease progression. These immunotherapy drugs act by either inhibiting immunosuppressive signals that support tumor growth, or by recruiting or activating immune cells that can kill the tumor cells.

Our lab has recently developed a novel mouse model (Msi2-Myc) in which the MycT58A allele stabilizes the expression of the MYC protein in Musashi2 (Msi2)-positive progenitor cells and stem cells. This model gives rise to both adenosquamous carcinoma of pancreas (ASCP) and acinar cell carcinoma (ACC). This provides us with an opportunity to explore immune-mediated regulation of pancreatic cancer subtypes, with the goal of advancing immunotherapeutic approaches for this disease. To our knowledge, the Msi2-Myc model is the first mouse model of ASCP, which is the most aggressive subtype of pancreatic cancer, with an average patient survival of just 6 months.

Our preliminary studies indicate that ASCP and ACC tumors have very different tumor immune microenvironments, with autochthonous ASCP tumors having a much higher infiltration of T cells than ACC tumors. Interestingly, when we transplanted ASCP tumor cells into the flanks of normal (littermate) mice, we found that ASCP tumors grew very poorly. In contrast, transplanted ASCP tumor cells readily formed tumors in NSG mice. These data suggest that a healthy immune system is capable of effectively targeting and blocking ASCP tumor growth. To test which immune cells mediate ASCP tumor rejection in recipient mice, we depleted B cells and T cells prior to tumor cell transplantation and monitored tumor formation over time. Importantly, depletion of immune cells led to robust ASCP tumor growth that was comparable to that observed in NSG mice. Using bulk RNA sequencing, we are currently mapping the transcriptomic differences in T cells between autochthonous tumor-bearing Msi2-Myc mice and normal littermate mice and plan to identify both intrinsic and extrinsic factors that lead to immune suppression during ASCP tumor development and growth.

Collectively, these studies will provide critical insight into immune-mediated regulation of pancreatic cancer growth and may pave the way for new approaches to therapy for this deadly disease.

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TET2-MUTANT CLONAL HEMATOPOIESIS DRIVES ALTERATIONS IN MYELOID CELL METABOLISM AND FUNCTION IN SOLID TUMOR

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Clonal hematopoiesis (CH) is detectable in upwards of 30% of patients with solid tumors and has been linked to both increased risk of certain tumor types as well as worsened prognosis. The implications of CH-mutant tumor infiltrating lymphocytes (TILs) on solid tumor immunobiology and response to immune checkpoint therapy remain uncharacterized. Using our novel model of Tet2^{+/-} bone marrow chimerism to mimic the CH phenotype in mice with solid tumors, we previously reported that the Tet2-mutant myeloid cells were highly abundant in the pancreatic tumor microenvironment and were transcriptionally enriched in both type I and II interferon signaling compared to wild-type (WT) controls. Further, CD8⁺ T cells in tumors with Tet2^{+/-} TILs had a more terminally exhausted phenotype after immune checkpoint therapy. This suggests that Tet2^{+/-} TILs drive significant alterations in the immune microenvironment and such mechanisms requires further investigation.

To further dissect the role of CH on solid tumor biology, we have developed bone marrow chimera mice that harbor a CH clone heterozygous for a loss-of-function mutation in the catalytic domain of Tet2 (Tet2^{+/-} CH); the second most commonly mutated gene in human CH. Briefly, lethally irradiated CD45.1 recipient mice were transplanted with 5x10⁶ whole bone marrow cells at a 1:1 ratio of CD45.1 support marrow to either CD45.2 cells heterozygous for the mutation of Tet2 (to establish the Tet2^{+/-} CH mouse) or wild-type CD45.2 cells with no Tet2 mutation (to establish the Tet2^{+/+} WT controls). Following a bone marrow reconstitution period of 8 weeks, mice received 0.35x10⁶ mT4 cells, a Kras^{+/G12DTP53+/R172HPdx1-Cre} (KPC) derived model pancreatic adenocarcinoma (PDAC), orthotopically. CH was confirmed by tracking CD45.1 and CD45.2 cells in the peripheral blood. In the first cohort, single-cell (sc-) RNAseq was performed on TILs from each group (n=3/group) after FACS sorting for CD45.1 and CD45.2. In the second cohort, tumors were fixed for paired spatial proteomic/metabolomic profiling and CyTOF. Unbiased spatial mass spectrometry images were acquired in negative and positive ion mode (for metabolites and glycans, respectively) using a Bruker timsTOF fleX MALDI QTOF mass spectrometer at 20 μm resolution.

Performing scRNA-seq analysis on CD45.2-specific cells allowed for the direct comparison between Tet2^{+/-} and Tet2^{+/+} TILs derived from bone marrow precursors. We identified 8 functional macrophage subsets; 2 of which were skewed in abundance based on Tet2 mutational status. Macrophages harboring Tet2^{+/-} were enriched in the Mrc1⁺ (CD206) cluster that upregulated genes involved in phagocytosis and immunotolerance, while WT macrophages were enriched in the Spp1⁺ cluster involved in fibrosis and hypoxia. It is not surprising that these differential functional phenotypes correlated with evident metabolic reprogramming. At the transcriptional level, WT macrophages were enriched for glycolysis while Tet2^{+/-} macrophages demonstrated significant reliance on OXPHOS. Spatial metabolomic profiling also revealed a significant increase in unsaturated fatty acids; including arachidonic, linoleic and oleic acids, as well as essential amino acids; including taurine and adenine, in regions of tumors with Tet2^{+/-} TILs—all of which could contribute to metabolic control of inflammation in Tet2^{+/-} chimera mice.

Our research suggests that Tet2^{+/-} myeloid cells significantly alter the metabolic microenvironment and drive immunosuppression through differential cell function and nutrient availability. Understanding how these metabolic alterations ultimately affect response to immunotherapies will be critical in the treatment of patients with CH across a variety of solid tumors.

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CGAS/STING-DRIVEN CANCER CELL-DERIVED TYPE I INTERFERONS INSTRUCT TUMOR MONOCYTE FUNCTIONAL POLARIZATION

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Monocytes are highly plastic immune cells that often infiltrate solid tumors and restrain antitumor immunity by being immunosuppressive. Repolarizing suppressive tumor monocytes to become immunostimulatory is a desirable strategy to boost antitumor immunity and overcome resistance to checkpoint blockade therapy. However, little is known about the specific cues that drive tumor monocyte polarization, which has made the characterization of distinct suppressive and stimulatory cancer-associated monocyte populations elusive.

In this study we used single cell transcriptomics to profile mouse and human lung and colon tumors. Additionally, ex vivo studies assessing the function of tumor myeloid cells in various conditions were used. In vivo experiments were used to determine the relationship between tumor growth and treatment with therapeutic antibodies as well as manipulations to the cGAS-STING pathway. Finally, we used bulk RNAseq data obtained from tumor biopsies from a Regeneron-led clinical trial of anti-PD-1 therapy in cutaneous squamous cell carcinomas (CSCC).

Using single cell transcriptomic profiling of human and mouse tumors, we identified discrete tumor monocyte subsets with unique functional properties. Additionally, using high dimensional flow cytometry profiling we define novel surface markers that faithfully distinguish immunostimulatory and immunosuppressive monocyte subsets, which are differentially enriched in syngeneic mouse tumors. Our unique framework enabled the functional characterization of distinct tumor monocyte populations and serves as a strategy to decipher tumor monocytes with robust antitumor qualities. Mechanistically, we show that cancer cell-derived type I interferons drive immunostimulatory monocyte polarization, which is associated with efficacy of anti-PD-1 immunotherapy. Furthermore, we found suppressive monocytes can be converted into immunostimulatory monocytes in vitro and in vivo via induction of cancer cell IFN production by cGAS-STING pathway activation. Clinically, enrichment of our experimentally-defined immunostimulatory monocyte gene signature is common in immunotherapy-responsive MSI-high colorectal adenocarcinomas (COAD) and in CSCC that responded to treatment with our anti-PD-1 antibody cemiplimab.

In conclusion, our work establishes a framework to discriminate and polarize immunostimulatory monocytes that may enhance the antitumor immune response to checkpoint blockade therapy.

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TARGETED DELETION OF CD244 ON MONOCYTES PROMOTES DIFFERENTIATION INTO ANTI-TUMORIGENIC MACROPHAGES AND POTENTIATES PD-L1 BLOCKADE IN MELANOMA

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In the myeloid compartment of the tumor microenvironment, CD244 signaling has been implicated in immunosuppressive phenotype of monocytes. However, the precise molecular mechanism and contribution of CD244 to tumor immunity in monocytes/macrophages remains elusive due to the co-existing lymphoid cells expressing CD244.

To directly assess the role of CD244 in tumor-associated macrophages, monocyte lineage-specific CD244-deficient mice were generated using cre-lox recombination and challenged with B16F10 melanoma. The phenotype and function of tumor-infiltrating macrophages along with antigen-specific CD8 T cells were analyzed by flow cytometry and single cell RNA sequencing data analysis, and the molecular mechanism underlying M1 macrophage differentiation, antigen presentation, phagocytosis, and autophagosome formation was investigated ex-vivo. Finally, the clinical feasibility of CD244-negative monocytes as a therapeutic modality in melanoma was confirmed by adoptive transfer experiments.

CD244^{f/f}-LysMcre mice demonstrated a significant reduction in tumor volume (61% relative to that of the CD244^{fl/fl} control group; 1061 ± 130.5 mm³ vs 1734 ± 145.1 mm³; P<0.005) 14 days after tumor implantation. Within tumor mass, CD244^{f/f}-LysMcre mice also showed higher percentages of Ly6C low macrophages over Ly6C high macrophages, along with elevated gp100+IFN γ +CD8 T cells, providing a direct evidence that monocyte-lineage-expressing CD244 serves as a negative regulator in tumor immunity. Flow cytometry and RNA sequencing data revealed that ER stress upon tumor challenge led to elevated CD244 expression on monocytes and hindered anti-tumorigenic Ly6C^{low} macrophage generation, phagocytosis, and MHC-I antigen presentation by suppressing autophagy pathways. Combining anti-PD-L1 antibodies with CD244-KO bone marrow-derived macrophages markedly improved tumor rejection compared to the anti-PD-L1 antibody alone or in combination with wild-type bone marrow-derived macrophages. Consistent with the murine data, transcriptome analysis of human melanoma tissue single-cell RNA-sequencing dataset (SCP398 from single-cell portal), revealed close association of CD244 with inhibition of maturation and functions of macrophages. Furthermore, cell type deconvolution analysis within melanoma patients bulk RNA-seq datasets from TCGA database showed that the presence of CD244-negative monocytes/macrophages significantly increased patient survival in primary and metastatic tumors.

In conclusion, our study highlights the novel role of CD244 on monocytes/macrophages in restraining macrophage differentiation and promoting T cell exhaustion in melanoma. Our findings suggest that CD244-deficient macrophages could potentially be used as a therapeutic agent to enhance antigen-specific T cell immunity in immunologically "cold" tumors and reinvigorate exhausted T cells into memory cells in combination with checkpoint inhibitors. Furthermore, CD244 expression in monocyte-lineage cells serve as a prognostic marker in cancer patients. Our study sheds light on an unexplored aspect of immune regulation in cancer and has important implications for the development of novel immunotherapeutic strategies, in conjunction with checkpoint blockade to effectively target tumor growth and enhance T cell immunity.

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ROLE OF TRIM28 IN INFLAMMATION-INDUCED CARCINOGENESIS

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An inflammatory microenvironment is a crucial feature of tumors. Tumor-associated macrophages (TAMs) represent a major population of infiltrating leukocytes and have been identified as a key orchestrator of cancer-promoting inflammation. Cancer-related inflammation triggers myelopoietic alterations that support the expansion of myeloid populations endowed with suppressive activities (i.e., TAMs and MDSCs), thereby supporting malignant progression and conferring resistance to therapies. Our project aims to identify new important molecular players driving the pro-tumoral activities during cancer development and progression.

To decipher the molecular events underlying the pro-tumor phenotype of TAMs, a mass spectrometry-based phospho-proteomics analysis was performed comparing TAMs from a murine fibrosarcoma (MN/MCA1) with control naïve, M1 and M2 polarized macrophages. We focused on TRIM28 protein, and we first evaluate its phosphorylation state in control peritoneal macrophages challenged with different inflammatory TLR ligands by Western Blot Analysis. Furthermore, by using conditional knock-out mice bearing selective TRIM28 deletion in either myeloid or intestinal epithelial cells (IECs), we investigated its contribution to colorectal cancer (CRC) development, mimicking both human sporadic CRC (i.e. AOM model) and colitis-associated cancer (CAC) (AOM/DSS model). TRIM28-dependent modifications on the tumor cell phenotype were further validated in vitro, by using the murine colon adenocarcinoma cell line MC38, silenced for TRIM28 gene by CrispCas9. In addition, to decipher the mechanism that the ablation of TRIM28 affect CAC development, we analyzed the trascrptional profile of tumors grown in TRIM28VillinCre mice and TRIM28flox/flox control mice, by scRNASeq analysis.

The phosphor-proteomic results highlighted TRIM28 as the top-ranking phosphorylated protein in TAMs. TRIM28 is a pleiotropic protein and its was described as regulator of DNA repair, pluripotency, proliferation and cell survival. We therefore hypothesized its role as a molecular interface between inflammation and carcinogenesis. Noteworthy, while TRIM28 was phosphorylated to varying degrees by the different TLR ligands, its phosphorylation was abolished in macrophages deficient of the molecular adapter MyD88. Noteworthy, conditional depletion of TRIM28 in IECs (TRIM28VillinCre mice), but not in myeloid cells (TRIM28LysCre mice), conferred resistance against cancerogenesis, resulting in decreased number of tumor lesions in the CAC model. Since, TRIM28VillinCre did not show differences in tumor development when challenged only with AOM (i.e. absence of DSS-induced inflammation), we suggest that TRIM28 plays a specific role in IEC carcinogenesis under inflammatory conditions. In agreement, TRIM28-deficient MC38 cancer cells exhibited decreased migratory capability, proliferation and self-renewal ability. scRNASeq analysis of tumors grown in TRIM28VillinCre mice and TRIM28flox/flox control mice revealed changes in both cell frequencies and their transcriptional profiles in both the immune and the tumor cell compartments.

Our results indicate TRIM28 as possible functional connector between inflammation and cancer, suggesting the possible development of new strategies to prevent inflammation-associated cancers development.

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AUTOMATED MANUFACTURE OF DNPM1 TCR-ENGINEERED T CELLS FOR THERAPY

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Treatment of relapsed or refractory acute myeloid leukemia (AML) still represents a challenge for physicians and is mainly associated with poor prognosis with a 5-year survival rate in Europe of only 25%. A neoantigen encoded by mutated nucleophosmin 1 (dNPM1) is expressed in approximately 30% of patients and presents an attractive target for immunotherapy with T cell receptor (TCR)-engineered T cells. Manufacturing of TCR-modified T cells, however, is still limited by a complex, time-consuming and laborious procedure. Therefore, this study specifically addressed the requirements for a scaled manufacture of dNPM1-specific T cells in an automated, closed and Good Manufacturing Practice (GMP)-compliant process using the CliniMACS Prodigy. Small scale studies demonstrated that the HLA-A*02:01 restricted recombinant TCR is dependent on CD8 co-receptor interaction to bind the peptide-HLA complex with high affinity. We therefore focused on the development of a CD8 T cell-specific process.

In 23 individual runs, 2E8 CD8-positive T cells were enriched from cryopreserved leukapheresis, subsequently activated, lentivirally transduced, expanded and finally formulated.

By adjusting and optimizing culture conditions, we additionally reduced the manufacturing time from twelve to eight days while still achieving a clinically relevant yield of up to 5.5E9 dNPM1 TCR-engineered T cells ($4.2E9 \pm 1.4E9$). The cellular product mainly consisted of highly viable CD8-positive T cells ($97.5\% \pm 1\%$) with an early memory phenotype. The cellular product was stable for up to 48h. Overall, mean vector copy number was 2.9 ± 1.1 . Importantly, TCR-engineered T cells specifically lysed dNPM1-positive target cells in vitro as well as in vivo.

In conclusion, we demonstrated that our CD8 process is robust and reproducibly yielded suitable numbers of potent dNPM1 TCR-engineered T cells, preparing the way for initiating a clinical trial to treat patients with relapsed or refractory AML.

P355

NATURALLY OCCURRING ANTIBODIES AS NEXT-GENERATION IMMUNOTHERAPEUTICS FOR HIGH-RISK NEUROBLASTOMA

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Neuroblastoma (NB), the most common extracranial solid tumor in children, presents a major clinical challenge for pediatric oncology. Despite advances in multimodal therapies, high-risk NB still has a poor prognosis and a high relapse rate. Treatment with the approved monoclonal antibody Dinutuximab has shown promising results in terms of overall survival, but its severe side effects and limited efficacy in recurrent tumors, necessitate the development of novel immunotherapeutic alternatives.

In a recent study involving a cohort of 248 individuals from a healthy population, we made an intriguing discovery. Approximately 6% of the participants possessed antibodies within their serum that induced potent cytotoxicity in NB cells by activating the complement cascade. To identify the target of these naturally occurring antibodies (nAbs), the glycocalyx of NB cells was systematically modified, and CRISPR/Cas9-based knockout cells were generated that lacked crucial enzymes for ganglioside synthesis. Through these approaches, we identified the target (NBGL) of these nAbs specifically induce cytotoxicity only in NB cells expressing this target on their surface.

Next, we aimed to identify B-cell clonotypes specific to NBGL from peripheral blood mononuclear cells (PBMCs) obtained from the nAbs positive donors. For this purpose, we used the new 10X barcode enabled antigen (BEAM) technology. BEAM-Ab combines antigen-specific fluorescence activating cell sorting (FACS) with subsequent single cell sequencing of selected B cells. This allowed us to obtain full-length, paired V(D)J sequences of the target-specific B cell receptors (BCR). These sequences now serve to produce recombinant antibodies, Bi-specific T-cell engagers (BiTEs) as well as chimeric antigen receptor (CAR) T cells and will be characterized for their specificity and efficiency against NB cells expressing NBGL.

Our findings hold great promise for the development of a novel immunotherapeutic option for high-risk neuroblastoma patients. The recombinant antibodies derived from these approaches provide potential advantages, such as reduced side effects compared to the traditional therapy, as they are based on naturally occurring antibodies found in healthy donors. However, further research and testing are needed to determine their effectiveness in preclinical and clinical settings.

P356

DEVELOPMENT OF A CAR-T CELL THERAPY FOR SOLID TUMORS AGAINST A CARBOHYDRATE TARGET

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Ca-10 is a carbohydrate tumor antigen expressed in the murine Ehrlich tumor and recognized by the monoclonal antibody A10, which has reactivity against different human solid tumors. In this work we describe the antigen Ca10 as a feasible target for an antitumor immunotherapy for solid tumors based on the use of CAR-T cells.

CAR was designed from the A10 antibody sequence. The ScFv chain was produced in a soluble form in transfected HEK 293T cells in order to test for its correct expression and antigen recognition capability which were assessed by ELISA and flow cytometry. Jurkat cells and human primary CD3+ cells from healthy blood donors were modified with a second generation Ca10ScFvCAR construct (Ca10-CAR) by lentiviral transduction and subsequently used to evaluate their capacity to recognize Ca10+ cells "in vitro".

The ScFv fragment was able to specifically recognize the target antigen both in a soluble form and when it was expressed on the surface of Ca10+ tumor cells. Jurkat were efficiently transduced with the Ca10-CAR and showed specific activation when cultured with Ca10+ cells as assessed by PLC γ phosphorylation and CD69 expression. In human primary T cells, we have achieved a CAR transduction efficiency of up to 50%. When cultured with Ca10+ cells, Ca10-CAR expressing T cells specifically produce IFN- γ and were able to efficiently kill the target cells.

Our results suggest that the antigen Ca10 may be a specific and effective target for the treatment of solid tumors by CAR-T cells.

P357

PREDICTIVE VALUE OF COMPLETE BLOOD COUNT AND MPHHS SCORE IN ADVANCED NON-SMALL CELL LUNG CANCER PATIENTS TREATED WITH IMMUNE CHECKPOINT INHIBITORS

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Background: Lung cancer is responsible for 1.8 million deaths per year, with the highest cancer mortality rate, in which 85% of patients fall into the non-small cell lung cancer (NSCLC) group. Immunotherapy targeted at immune checkpoint inhibitors (ICIs) has shown significant advances, being composed of monoclonal antibodies capable of modulating the homeostasis of co-stimulatory and co-inhibitory signals, which are critical in maintaining immunological tolerance. ICIs shows better outcome compared to standard second-line chemotherapy, but reliable prognostic markers are lacking.

Aim: This study aimed to investigate the prognostic role of the complete blood count (CBC) and to develop a valuable prognostic model for patients with NSCLC undergoing ICIs therapy.

Methods: A retrospective medical records review was performed in advanced NSCLC patients treated with ICIs at Barretos Cancer Hospital (Brazil). The values of baseline CBC were analyzed, with a median value used as a cutoff. Demographic characteristics, such as age, sex, smoking status, histology, staging, PD-L1 status, and treatment, were collected from medical records upon admission. The primary outcomes evaluated included overall survival (OS) and progression-free survival (PFS), assessed through Kaplan-Meier analysis using the log-rank test. Additionally, Cox regression analysis was employed to evaluate the prognostic factors and receiver operating characteristic analysis (ROC) was employed for predictive value. All analyzes were performed using the SPSS software.

Results: The study cohort comprises 55 patients, with an average age of 62 years old (range: 48.2 - 79.8 years old), consisting of 58.2% males and 41.8% females. The smoking history of the patients revealed that only 10.9% had never smoked. Adenocarcinoma was the most predominant histological type, comprising 60.0% of the cases, followed by 34.5% of SCC. All patients have advanced staging, with 23.6% showing no mediastinal involvement (stage IIIA), 16.4% demonstrating mediastinal involvement (stages IIIB and IIIC), and 60.0% exhibiting distant metastasis (stages IVA and IVB). In the cohort, 65.4% showed PD-L1 positive status, with 23.6% having low expression (1 - 49%) and 41.8% high expression (>50%). The most frequently recommended treatment was anti-PD-1, prescribed to 78.2% of the patients, followed by anti-PD-L1 for 21.8% of cases. The study also showed significant association among CBC components, assessed from the baseline results of ICIs treatment, and clinical outcome of NSCLC patients. Lower levels of hemoglobin (<12.0g/dL; p= 0.027) and hematocrit (<37.3g/dL; p= 0.022) were associated with poorer OS. Patients with lower monocyte counts (<712.0/mm³) and platelet counts (<251.0K/mm³) exhibited significantly higher OS (p= 0.006 and p= 0.040, respectively) as well as improved PFS (p= 0.050 and p= 0.040). Additionally, the ROC curve analysis of baseline monocyte and platelet counts yielded significant results. The area under the curve (AUC) for OS based on monocyte count was 0.868 (p<0.000), and for predicting PFS it was 0.754 (p=0.002). Among platelets, the AUC predicting OS was 0.700 (p=0.018), and for PFS it was 0.713 (p=0.008). Furthermore, patients with an MPHHS score (monocytes x platelets per hemoglobin x hematocrit) of <393.5 displayed higher rates of OS (p=0.002) and PFS (p=0.017). The MPHHS score demonstrated

a high predictive value for OS, with an AUC of 0.827 ($p=0.000$). Similarly, the AUC predicting PFS was 0.749 ($p=0.002$), and it effectively predicted treatment response with 0.771 ($p=0.004$).

Conclusion: In advanced NSCLC patients treated with ICIs, baseline hemoglobin, hematocrit, monocyte, and platelet counts (MPHH score) may serve as a useful predictive marker of clinical outcomes. Moreover, lower MPHH scores have shown the ability to predict enhanced OS and PFS, as well as identify patients who are more likely to respond favorably to ICI treatment.

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TUMOR-REDIRECTED iNKT CELLS INDUCE ROBUST ANTITUMOR RESPONSE BY DUAL TARGETING CANCER AND SUPPRESSIVE MYELOID CELLS

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Adoptive immunotherapy with T cells engineered with tumor-specific T cell receptors (TCRs) holds promise for cancer treatment. However, suppressive cues generated in the tumor microenvironment (TME) can hinder the efficacy of these therapies, prompting the search for strategies to overcome these detrimental conditions and improve cellular therapeutic approaches. Invariant natural killer T (iNKT) cells are a T lymphocyte subset expressing a conserved semi-invariant TCR that (iTTCR) that reacts to lipid antigens, such as alpha-galactosyl ceramide (α -GalCer), presented by the monomorphic molecule CD1d. iNKT cells actively participate in tumor immunosurveillance by restricting suppressive myeloid populations in the TME. This intrinsic anti-tumor function, and their restriction to a monomorphic molecule identical in all individuals, suggest that iNKT cells may support the generation of a universal platform for adoptive cell therapy in cancer patients.

As a proof-of-concept study, mouse iNKT cells were transduced with a TCR specific for major histocompatibility complex MHC-restricted tumor-associated peptide (TCR-iNKT) and assessed in vitro and upon transfer in vivo against tumors expressing the nominal antigen. The adoptive iNKT cell transfer was also combined with restimulation by the strong iNKT cells agonist α -GalCer administered using the multi-stage vector (MSV) nanotechnology that ensures controlled delivery at tumor site.

Harnessing iNKT cells with a second TCR specific for a tumor-associated peptide, generated bi-specific effectors for CD1d- and MHC-restricted antigens in vitro. Upon in vivo transfer, TCR-iNKT cells showed the highest efficacy in restraining the progression of multiple tumors that expressed the cognate antigen compared with non-transduced iNKT cells or CD8+ T cells engineered with the same TCR. TCR-iNKT cells, in which both the endogenous invariant TCR and the exogenous tumor-specific TCR exert anti-tumor effects, achieved robust cancer control by simultaneously modulating intra tumoral suppressive myeloid populations and killing malignant cells. This dual antitumor function was further enhanced when the TCR-iNKT cell transfer was combined with MSV therapeutic booster, resulting in an increased survival of the treated mice.

These preclinical results support the combination of tumor-redirected TCR-iNKT cells and local α -GalCer boosting as a potential therapy for patients with cancer and point to iNKT cells as an attractive adoptive cell therapy platform for engineering with anti-tumor TCRs or CAR providing an appealing alternative to conventional T cells for the treatment of solid and hematological malignancies.

P359

PERITUMORAL MACROPHAGES ADOPT AN ALTERNATIVELY ACTIVATED STATE TO RECRUIT EOSINOPHILS AND PROMOTE IMMUNE RESPONSE TO BREAST CANCER

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As breast tumors grow, they accumulate macrophages that are heterogenous in terms of localization and function. In both mice and humans these macrophages can be broken down into two main subtypes: tumor-associated macrophages (TAMs) and mammary tissue macrophages (MTMs). TAMs are thought to be immunosuppressive and act by inhibiting cytotoxic T lymphocytes. However, less is known about the function of MTMs, including what cells they interact with over disease progression and what their role is during tumor regression.

To interrogate this, we used multiplexed immunofluorescent imaging technology (CODEX), a system that allows us to stain tumor tissues with 30+ markers and thereby visualize most major immune cell populations simultaneously. We combined this technology with single-cell RNA sequencing (scRNA-seq), macrophage-specific depletion mouse models, flow cytometry, and cytokine profiling to understand how TAMs and MTMs respond during anti-tumor immunity.

Using macrophage-specific depletion, we found that MTMs prevent tumor growth, suggesting that these cells are functionally distinct from TAMs. We then investigated the spatial niches occupied by these cells using CODEX to identify key cell-cell interactions. Across early and late-stage tumors, TAMs were found to be intra-tumoral while MTMs were found to be peritumoral. Additionally, MTMs specifically localized with eosinophils, immune cells that are typically associated with allergic disease but have also recently been shown to mediate anti-tumor immunity.

Single-cell RNA sequencing and immunophenotyping showed that MTMs displayed the signature of alternative activation, a state critical to allergic inflammation and associated with cell types such as T helper 2 cells (TH2) and eosinophils. To further explore the MTM-eosinophil interaction, we induced anti-tumor immunity by depleting regulatory T cells (Tregs). Upon Treg depletion, MTMs increased their alternatively activated state and this was accompanied by an influx of eosinophils. Tumors had canonical increases in inflammatory Type 1 cytokines (i.e. IFN γ), while the only increases in Type 2 cytokines were eosinophil-specific proliferation and chemotactic factors (e.g. IL-5 and eotaxins). These eosinophils were found to home specifically to the peritumoral MTM region and not the TAM region. Mechanistically, the MTM-eosinophil interaction was initiated by the alarmin IL-33, which promoted the alternatively activated phenotype shift of MTMs and the release of eosinophil-attracting factors such as eotaxins. Recruited eosinophils can be then polarized by IFN γ in the breast cancer microenvironment and synergize with CD8 T cells.

This work implicates the MTM-eosinophil axis in promoting anti-tumor immunity and provides a rationale for the development of therapies that engage this specific macrophage subset.

P360

TARGETED LOCAL DISRUPTION OF PRO-INFLAMMATORY OR PRO-FIBROTIC STIMULI IN CANCER-ASSOCIATED FIBROBLASTS SKEWS THEIR IDENTITY AND SLOWS TUMOR GROWTH

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Functional understanding of cells residing in the tumor microenvironment (TME) requires controlled experiments ideally in native tissue contexts. Experimental manipulations using systemic or local drug applications are challenging to direct to the cell- or target-of-interest depending on location of cells and distribution of targets. Experimental manipulations using genetic tools to conditionally knockout (KO) a gene-of-interest or deplete a cell-of-interest can be complicated by systemic KO effects and secondary effects caused by cell death of target cell.

To specifically investigate stromal cell manipulations restricted to the tumor region and adjacent tissue, we developed a viral delivery method to target any gene-of-interest for KO in fibroblasts (FBs) at high efficiency requiring only 2 weeks of lead time instead of months of laborious transgenic mouse generation and breeding. We use this technology to disrupt previously identified fibroblast (FB)-macrophage (MAC) communication axes in mouse models of pancreatic ductal adenocarcinoma (PDAC) or melanoma and investigate how manipulation of the FBs affects their activation states and subsequent polarization of tumor-associated macrophages (TAMs). The model is based on using a transgenic mouse with FB-specific Cas9-expression (Pdgfra-Cre;Rosa26-LSL-Cas9-EGFP), subcutaneous (s.c.) injection of self-complementary AAV expressing a validated guide RNA (gRNA) targeting a gene-of-interest and subsequent s.c. injection of tumor cells (KPC-derived PDAC cell line or YUMM5.2 melanoma) at the site of prior AAV injection.

We routinely achieve 70-90% KO efficiency in cancer-associated fibroblasts (CAFs) and decided to target two different receptors on fibroblasts for KO: the oncostatin M receptor (Osmr) – a proliferative and pro-inflammatory mediator when triggered by its ligand OSM – and transforming growth factor beta 2 (Tgfbr2) – a pro-fibrotic mediator when triggered by its ligand TGF- β 1.

Flow cytometric analysis showed that KO of Osmr in CAFs led to a more steady-state double-positive (DP) SCA-I+ Ly6C+ phenotype and less to a pro-fibrotic double-negative (DN) SCA-I- Ly6C- NCAM1hi phenotype. Concomitant with a skewed CAF population, tumors with Osmr-KO FBs also harbored more immunestimulatory MHC-IIhi TAMs compared to controls. KO of Tgfbr2 phenocopied the effects of Osmr-KO resulting in less pro-fibrotic DN SCA-I- Ly6C- NCAM1hi FBs and more MHC-IIhi TREM2lo TAMs. Additionally, to benchmark our KO approach to conventional conditional gene KO, we compared s.c. PDAC tumor growth kinetics in FB-restricted Tgfbr2 KO mice (Col1a2-CreER;Tgfbr2fl/fl) to our ad hoc viral KO model (Pdgfra-CreERT2;R26-LSL-Cas9-EGFP plus AAV-gTgfbr2) and noticed delayed PDAC growth in both models compared to controls.

These findings validate our approach in manipulating CAFs locally growing in the TME and highlight the importance of pro-inflammatory (via OSMR) and pro-fibrotic (via TGFBR2) cues received in CAFs for polarizing TAMs and supporting tumor growth. We anticipate that this approach holds great potential for further exploration of gene targeting in stromal cells residing in tumors and beyond.

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A VEIN-RICH TUMOR MICROENVIRONMENT OF INTRAHEPATIC CHOLANGIOCARCINOMA CORRELATES WITH INCREASED TERTIARY LYMPHOID STRUCTURES AND FAVORABLE PATIENT OUTCOME

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Intrahepatic cholangiocarcinoma (iCCA) is the second most frequent primary liver cancer, which is an aggressive cancer that is currently classified largely into large-duct and small-duct types. These two types of cancers have different carcinogenic pathways and clinicopathological characteristics. Understanding the tumor immune microenvironment and its related vascular system is important for developing novel and efficient therapies. We focused on tertiary lymphoid structure (TLS) as a hallmark of antitumor immunity and investigated the clinicopathological significance of TLSs and the influence of vascular microenvironment on TLS formation in iCCAs.

We examined 261 iCCA cases surgically resected in our hospital between 2000 and 2019 to evaluate the clinicopathological impacts of TLS formation. TLSs were classified into two groups according to their position: intratumoral TLSs (iTLSs) existing in the regions occupied by cancer and peritumoral TLSs (pTLSs) existing outside the cancer regions but within 500 μ m from the invasion margins. We also analyzed tumor-infiltrating lymphocytes and the vascular system using immunohistochemistry. Single-cell and bulk RNA sequencing analyses were performed using datasets downloaded from public databases.

This study demonstrated new findings concerning TLS formation, its clinicopathological significance in different iCCA types, and the vascular microenvironment of abandoned TLSs. First, TLS was formed more frequently in large-duct type (34.3% iTLS and 40.6% pTLS) than that in small-duct type (25.5% iTLS and 16.0% pTLS), and was prognostic only in large-duct type by Kaplan-Meier analyses. High iTLS and pTLS grades were significantly associated with prolonged disease-free survival (DFS) and overall survival (OS) period in large-duct type. Multivariate survival Cox regression analyses revealed that the presence of pTLS was a significant factor for both DFS ($P < 0.0001$) and OS ($P < 0.0001$) in large-duct type. Second, the vascular microenvironment of TLS-rich iCCA showed a significantly high vein density with a tendency for low immature vessels than that of TLS-poor iCCA, even though the total microvessel density was not altered. In addition, T cell infiltration was significantly high in TLS-rich iCCA, and both the presence of TLS and high vein EC were significantly associated with molecular networks representing an active immune response in iCCA transcriptomics analyzed using the Weighted Gene Co-expression Network (WGCNA). Third, vein density was a prognostic factor for long DFS ($P = 0.030$) in patients with large-duct type iCCA and DFS ($P = 0.012$) and OS ($P = 0.045$) in patients with small-duct type iCCA in the multivariate analysis.

We demonstrated, for the first time, that a TLS-rich microenvironment has high vein density, which represents the antitumor direction of the tumor microenvironment, with a favorable patient outcome.

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LOW-DOSE ACETYLSALICYLIC ACID REDUCES LOCAL INFLAMMATION AND TISSUE PERFUSION IN DENSE BREAST TISSUE IN POSTMENOPAUSAL WOMEN

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One major risk factor for breast cancer is high mammographic density, but no preventive therapy for women with dense breast tissue is currently available. As high mammographic density is associated with increased inflammation, we aimed to determine whether low-dose acetylsalicylic acid (ASA) affects local inflammation or induces structural and dynamic changes in dense breast tissue.

A total of 53 healthy postmenopausal women categorized as having dense breast tissue on their regular mammography screen were randomized to receive ASA 160 mg/day or no treatment for 6 months. Magnetic resonance imaging (MRI) for structural and dynamic quantifications and microdialysis for sampling in vivo proteins from breast tissue and abdominal subcutaneous fat as a measure of systemic effects before and after 6 months were performed. A panel of 92 inflammatory proteins were quantified in the microdialysates.

Results: Out of the 92 proteins, 20 were significantly altered in breast tissue after ASA treatment, whereas no changes were detected in abdominal subcutaneous fat. In the no-treatment group, no changes in protein levels were observed in any tissue. No changes in breast density, measured by MRI lean tissue fraction, were found in either group. In the ASA group, clearance of contrast agent was significantly decreased and was correlated with the local concentration of VEGF.

ASA may shape the local breast tissue into an anti-tumorigenic microenvironment. Thus, low-dose ASA may be a nontoxic alternative strategy for preventing breast cancer among postmenopausal women with high mammographic density. Trials investigating the effects of low-dose ASA and risk of primary breast cancer are warranted.

P363

UNDERSTANDING CASTRATION RESISTANCE PROSTATE CANCER TUMOR IMMUNE MICROENVIRONMENT FOR DEVELOPING BETTER THERAPEUTIC STRATEGIES

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Castration resistant prostate cancer (CRPC) patients with primary refractory disease or who relapse after androgen receptor signaling inhibitors have very poor prognosis. Very few therapeutic options remain for these patients. In the last two decades, immunotherapy has brought better outcomes for many patients who suffer from cancer types resistant to long, previously established therapies. However, clinical trials with immune checkpoint inhibitors (CPIs) have not brought any real improvement for CRPC patients. Part of the problem is the fact that the complexity of the tumor microenvironment of prostate cancer remains poorly studied and hormone signaling pathways are involved in the plasticity of tumors and their resistance to CPIs. Here, we show the first results of a complex and ambitious study focused in understanding the immune landscape of PCa using genetically engineered mouse models (GEMMs) and the capacity of drug combinations for restoring tumor immunogenicity with the objective of identifying treatment combinations that could synergize with CPIs.

Conditional and inducible genetically engineered mouse models (GEMMs) generated in the Aytes' Lab recapitulate the spectrum of PCa genotypes, including metastasis and CRPC. We have performed single cell RNA sequencing of the tumor immune fraction of these mice to understand how distinct genetic determinants of prostate cancer progression impinge on the composition of the tumor immune microenvironment. We also performed histological and phenotypical characterization by flow cytometry to validate sequencing results.

In parallel, we have generated human PCa cell lines that successfully integrate a fluorescent and luminescent gene reporter of the interferon (IFN) type I pathway activation. Using IFN type I activation as a surrogate for enhanced antigenicity, we performed high-throughput including repurposing and compound library screenings in IFN type I responsive and unresponsive cells to identify candidate drugs that would synergize with CPIs.

Here we demonstrate that the immune landscape of GEMMs differs among mice with distinct and specific PCa driver alterations. Tp53 deletion appears to be major driver of cancer immune infiltration. Importantly, castration contributes to modify and shape this immune landscape towards a less regulatory and more cytotoxic phenotype. Neutrophils, macrophages and CD8 T cells are the populations more highly regulated by these changes.

We also have identified several compounds and drugs that have the capacity of restoring the immunogenicity of tumor cells by regulating the IFN type I pathway. Interestingly, different PCa cell types have different rate responses to these stimuli, indicating that different genetic features also contribute to the immunogenic capacity of these cells. Some of the tested drug candidates can stimulate the pathway at higher levels than positive controls, indicating the potential of these drugs to move to hit expansion and validation for synergistic combinations with CPI.

The composition of the immune microenvironment in PCa remains poorly defined, hampering the design of better strategies to deploy immunotherapies for PCa patients. Recurrent loss of function in PTEN, p53, and/or BRCA2 are hallmarks of CRPC patients. Here we provide evidence that the repertoire of genetic alteration in the cancer cells shapes the immune infiltrating phenotype of these tumors. We also have identified drugs able to restore PCa cell immunogenicity. Our preliminary data suggest that drug-induced enhancing of antigenicity is a plausible avenue to expand CPIs for PCa treatment and that these synergies may need to be tailored for different cancer drivers.

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INVESTIGATION OF LOCAL IMMUNOMODULATORY COMPOUND ACTIVITY IN AN EX VIVO TUMOR TISSUE SLICE ASSAY

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Immunologically cold tumors represent a major challenge to immunotherapy. Such tumor microenvironments are characterized by a lack of T cell infiltration and strong infiltration of immunosuppressive myeloid cells, such as tumor-associated macrophages and immature granulocytic or myeloid leukocytes. Therapeutic targeting and re-programming of suppressive myeloid cells is hence a promising treatment modality. In vitro systems for drug testing cannot reflect the complex tumor-mediated education of myeloid cells. To reduce in vivo testing, predictive test systems are needed for testing of innate immunomodulatory compounds.

In this study, an ex vivo test system was established, in which subcutaneous untreated tumors left over from other studies, were cut into tumor tissue slices (TTS). To this end, TC-1, 4T1, and CT-26 tumor samples were cut into 300 µm thick slices. The TTS were cultivated on an air-liquid interface for up to 7 days.

First, TTS viability and cellular composition over time was analyzed via flow cytometry. Stable tissue composition was observed over the 7 days. Next, responsiveness to different classes of pattern recognition receptor (PRR) agonists, which represent immunomodulatory compounds and stimulate mainly myeloid cells, was evaluated. Cytokine secretion was detected upon stimulation as well as re-programming of tumor-associated macrophages, indicated by upregulation of inducible nitric oxide synthase (iNOS).

Taken together, the data confirm the TTS assay as a promising ex vivo model for the initial screening of immunomodulatory compounds. In conclusion, evaluation of local drug effects in the TTS assay that uses leftover tumor samples from other studies may strongly reduce the number of mice needed for in vivo testing.

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INTERLEUKIN 17A ROLE IN PANCREATIC ADENOCARCINOMA IMMUNE MICROENVIRONMENT AND PRE METASTATIC NICHE ORCHESTRATION

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Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive and lethal cancer with an average of 5-years survival rate in less than 10% patients. High lethality is related to low surgical resection availability, absence of symptoms in early stages, treatment resistance and distal metastases. Notably, PDAC displays massive immunosuppressive desmoplastic reaction which hampers immune infiltrating cells recruitment reducing drug efficacy and promoting tumor progression. Activated cancer associated fibroblasts (CAFs) are a heterotypic predominant population in the PDAC extra cellular matrix (ECM), tumoral stroma, indeed, is composed of proteins derived from CAFs, including collagens, laminin and fibronectin. Aside their role in matrix remodeling, CAFs are involved in the signaling between cancer cells and immune suppressive cells. Levels of T helper 17 (Th17) cells and interleukin-17 (IL17) were also found to be elevated in pancreatic fibrotic reaction. Existing data suggest that different members of the IL17 family play a key role in the PDA progression by affecting both tumor and stromal cells. Notably, in our last work we showed that IL17A depletion strongly modified the PDAC microenvironment and the cancer associated fibroblasts (CAF) transcriptome.

Exploiting our IL17A^{-/-} KPC model we demonstrate that IL17A promote recruitments of immunosuppressive (pro-tumoral) cells while IL17A ko leads to matrix remodeling and boosts immune infiltrate with an improved T cell-mediated anti-tumor response.

CAFs and PDAC cells isolation from mice spontaneously developing tumor (KPC models)

Orthotopic and tail vein PDAC cells injection in mice models

Mass spec analyses

3D invasion assay

Immunostaining analyses

ELISA and ELISpot assay

Single cell spatial transcriptomic and RT-PCR analyses

We observed phenotypic alterations in fibrotic desmoplasia and transcriptomic in IL17A^{-/-} KPC mice model, suggesting IL17A impact on PDAC immune microenvironment (TIME) and tumor progression. By decellularization of primary tumor from our KPC models (wt and IL17A^{-/-}) we isolated the tumoral matrix and performed mass spec analysis. We found alterations in collagen, metalloproteases and vesicle trafficking pathways.

Exploiting 3D hydrogel plate (inert support) we could monitor matrix raising induced by CAFs-PDAC cells co-culture. We analyzed phenotypic alteration and collagens composition of the 3D co-cultures in the presence or in the absence of anti-IL17A.

From PDAC cells isolated from wt and IL17A^{-/-} KPC models we obtained spheroids which faithfully recapitulate the PDAC tissue traits. After characterization we challenged the spheroids in a 3D invasion assay alone or in co-culture with CAFs (wt and IL17A^{-/-}); tumoral sprouting and invasive potential was altered by the presence or absence of CAFs of both genotypes or by the administration of anti-IL17A antibody.

Transcriptomic analyses by RT-PCR showed pronounced epithelial to mesenchymal (EMT) transition in spheroids derived from PDAC wt cells compared to IL17A^{-/-}.

To corroborate the in-vitro results we inoculated PDAC cells in wt and IL17A^{-/-} mice models and studied metastatic dissemination in both genotypes.

We were able to:

- isolate metastatic PDAC cells together with metastatic associated fibroblasts (MAFs), and propagate them in 3D culture obtaining “metapneumospheres”;
- monitor the metastatic dissemination and TIME rearrangement at early stages in the presence or absence of IL17A.

Deep analysis of IL17A related to PDAC microenvironment will significantly impact our understanding of its role in PDAC tumor progression and metastatic dissemination and will give hints on potential combined targeted therapies which can improve immune response in patients.

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DEVELOPING MULTITARGETING CHIMERIC ANTIGEN RECEPTOR (CAR) T CELLS FOR TREATING GLIOBLASTOMA (GBM)

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GBM is the most common and aggressive primary brain tumour, with an ~12-month survival prognosis. Chimeric antigen receptor (CAR) T cell therapy is an immunotherapy currently being evaluated in GBM. Despite encouraging clinical results, this treatment still fails partly due to GBM heterogeneity. Heterogeneity in GBM includes the variability between cancer cells within the same tumour (intra-tumoural heterogeneity) and between different patients (inter-tumoural heterogeneity). Thus, heterogenous CAR target expression can lead to antigen-loss relapse after CAR T therapy.

Multitargeting CAR T cells in preclinical studies can overcome antigen-loss relapse by simultaneously targeting a more comprehensive set of GBM antigens. However, currently pursued antigens are not expressed in all patients, providing an additional challenge to increase the universality of multitargeting anti-GBM CARs. As such, we hypothesize that multitargeting CAR T cell strategies, combined with more inclusive CAR targets, may overcome intra- and inter-tumoural heterogeneity in GBM.

We initiated a comprehensive GBM target discovery program using 9 RNA and protein datasets consisting of 715 samples. Upregulated genes were identified in primary/recurrent GBM patients compared to healthy brain controls, and then an algorithm was applied to predict cell surface proteins that are not expressed in other vital tissues. We identified a 19-CAR target panel called OmniCART that can be used in a specific combination to target the intratumoural heterogeneity of one GBM, while an alternative combination can address the intertumoural heterogeneity of a different GBM. To evaluate OmniCART-specific multitargeting CAR T cell killing, we use human brain tumour stem cells (BTSCs) that recapitulate GBM antigen heterogeneity. These BTSCs were resected from spatially distinct regions of GBMs (tumour core (X) and leading edge (Z)) and are orthotopically co-implanted into mice (XZ model).

OmniCART-specific target-binding domains are engineered into a 2nd generation 4-1BB/CD3 ζ CAR backbone derived from a Canadian CAR T phase I/II clinical trial (NCT03765177). The CAR vector has unique cloning sites to rapidly exchange the target-binding sequence and includes specific epitopes (e.g., MYC, FLAG) within the CAR structure to measure CAR surface expression. Further, truncated cell surface receptors (e.g., CD19t, EGFRt) are engineered into the construct as a marker of lentiviral transduction and as a potential safety switch.

Two multitargeting approaches are used: CAR pool and multivalent CAR. CAR pool uses multiple separate CAR T products that are co-administered into mice, whereas multivalent CAR T cells express multiple different CARs on the same T cell. Two prototypic OmniCART constructs against CD276 and EphA2, which are established GBM antigens and were identified in our OmniCART panel, are utilized in our preliminary studies.

When implanted orthotopically into mice, we found XZ models recapitulate the spatial heterogeneity observed in original patients. In two XZ models, heterogenous antigen expression was detected between X and Z lines for 8 OmniCART antigens. Together, our XZ models exhibit phenotypic and genotypic GBM heterogeneity. Further, we developed four CAR constructs against CD276 and EphA2 that can kill 100% of antigen-positive cells at a 10:1 effector:target ratio in vitro. However, in vivo data demonstrates initial anti-tumour responses after single-targeting α CD276 CAR T cell treatment eventually leads to relapse with decreased CD276 expression. Ongoing studies are examining our two multitargeting CAR approaches.

We characterized XZ models displaying GBM CAR target heterogeneity that may contribute to antigen-loss relapse observed with α CD276 CAR T cells. Multitargeting against more inclusive CAR targets may reduce antigen-loss relapse induced by CAR T therapy for GBM. This project aims to develop a personalized multitargeting CAR T cell therapy for all GBM patients.

P367

PREDICTIVE POTENTIAL OF METABOLIC-RELATED BIOMARKERS IN ANTI-PD-1 TREATMENT OUTCOMES FOR ADVANCED MELANOMA

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The immunosuppressive tumor microenvironment (TME) poses a significant challenge to the effectiveness of anti-tumor immune responses. While immune checkpoint inhibitors have shown promising response rates in multiple cancers, including melanoma, only a limited subset of patients derive significant benefits from these treatments. This emphasizes the need to unravel the underlying mechanisms of metabolic adaptation. This study aimed to identify metabolic-related biomarkers capable of predicting the therapeutic outcomes of anti-PD-1 treatment in patients diagnosed with advanced melanoma.

The study encompassed a cohort of 54 melanoma patients who underwent anti-PD-1 immunotherapy at Barretos Cancer Hospital (Barretos-SP, Brazil). Using primary FFPE tumor samples, we investigated the expression of 748 genes associated with metabolic processes and immunometabolism, utilizing the NanoString nCounter® Metabolic Pathways Panel. Furthermore, we assessed the release of immune mediators into plasma through Hu Th1/Th2/Th17 Cytokine kit by Cytometric Bead Array (CBA). Patients who experienced tumor progression or stable disease for less than 6 months were categorized as non-responders, while responders included patients who exhibited partial and complete response or had stable disease for over six months. Differential gene expression (fold change > 1.5, p<0.05) and cytokine concentrations were associated with patient response and survival following immunotherapy. Statistical analyses were performed using nSolver Advanced Analysis software, IBM SPSS Statistics 23, and R version 4.2.0.

The patient cohort had a median age of 57 years, with the majority having superficial spreading (33.3%) and nodular (29.4%) melanoma subtypes. Among the participants, 63.0% were treated with immune checkpoint blockade (ICB) as their initial treatment. Twenty-eight (51.9%) patients experienced disease progression, and 16 (29.6%) achieved partial or complete responses. The median survival post-ICB was 22.7 months. Age at diagnosis played a role in treatment response, where 71.4% of younger patients (<57 years old) were categorized as non-responders, whereas 65.4% of older patients exhibited positive responses to the anti-PD-1 blockade. Thirty-four genes exhibited differential expression (DE) between the response groups, with 22 genes found to be overexpressed in non-responder patients. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis identified arginine and proline metabolism, as well as arginine biosynthesis pathways enriched in this group. Using a multivariable Cox regression model, Cyclin D1 (CCND1; HR = 0.440; 95% CI: 0.202 – 0.960; p=0.039) and SOS Ras/Rac guanine nucleotide exchange factor 1 (SOS1; HR = 2.554; 95% CI: 1.144 – 5.703; p=0.022) were independently associated with overall survival following immunotherapy. Moreover, higher expression of Family with Sequence Similarity 30 Member A (FAM30A) was associated with younger patients (p=0.014). These findings align with previously published studies, underscoring the pivotal roles of arginine and proline metabolism in carcinogenesis and tumor growth. Additionally, CCND1 has been reported to influence T-cell activation and proliferation by influencing metabolic reprogramming. Similarly, SOS1 contributes to T-cell activation and metabolic alterations through the activation of Ras signaling pathway.

Our findings suggest that metabolic-related biomarkers hold promise for predicting the therapeutic outcomes of anti-PD-1 treatment in patients with advanced melanoma. Further investigations into

the roles of these biomarkers may enhance treatment strategies and ultimately improve patient outcomes. Overall, this study contributes significantly to the expanding body of evidence regarding the metabolic and immunological nature of tumors, advancing our understanding in this field.

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TARGETING GLIOBLASTOMA WITH INTRANASAL VIRAL IMMUNOTHERAPY

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Glioblastoma (GBM) is a devastating and currently incurable form of primary brain tumor. Poor immunogenicity and the presence of an immunosuppressive tumor microenvironment represent significant challenges for effective GBM immunotherapy. On the other hand, oncolytic viruses, which induce immunogenic tumor destruction and inflammation, have shown promise as potential therapy for "cold" tumors like GBM. In this study, we investigated the feasibility of using intranasal route as a novel non-invasive way of delivering immunotherapeutic virus in a mouse GBM model.

Mice were implanted with orthotopic syngeneic glioma (CT2A-VLDLR/FLuc/GFP) and treated with single (1×10^7 PFU) dose of fully replicative Semliki Forest virus (SFV) A774-nLuc either intranasally or intravenously. Tumor growth was measured with IVIS imaging and mice were sacrificed upon tumor-related symptoms. Both survival and tumor growth were compared to PBS-treated control mice. Presence of virus in the tumor region was analyzed by plaque titration and nLuc expression. In addition, expression of cytokines in the tumor microenvironment after intranasal virus delivery was measured with Proteome Profiler Mouse XL Cytokine Array (R&D Systems) and compared to PBS-treated control.

Intranasal injection of SFV prolonged median survival of glioma-bearing mice 10 days compared to the intravenous delivery or PBS control, indicating enhanced therapeutic potency. Ex vivo imaging of intranasally treated mice showed nLuc-expression both in the olfactory bulb indicating that virus could reach brain from the intranasal cavity. Interestingly, nLuc-signal could be also seen in the proximity of the tumor giving evidence that SFV can reach the tumor site located deeper in the brain. Replicative virus was detected in tumor-bearing brain region also with plaque titration, giving further evidence of virus reaching the tumor. Cytokine profiling revealed elevated IFN-beta concentration in the treated tumors. In addition, intranasal SFV therapy promoted IL-17 signature in the tumor microenvironment, potentially contributing to the therapeutic effect.

Here we show for the first time, effective intranasal delivery of oncolytic virus into intracranial tumor in mice. Surprisingly, intranasal delivery showed improved therapeutic potency over intravenous virus injection. This indicates that the unique connection between intranasal cavity and the brain can be exploited to facilitate effective delivery of SFV-based therapy in orthotopic mouse glioma model. Our results also indicate that intranasal SFV therapy can be used to modulate the glioma cytokine microenvironment with potential therapeutic benefit. These results can be translated into the development of effective oncolytic immunotherapy against GBM.

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MULTISCALE CHARACTERIZATION OF IMMUNE-RELATED COLITIS IN IPILIMUMAB-TREATED CANCER PATIENTS

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The use of CTLA-4-targeting immune checkpoint inhibition (ICI) in combination with PD-(L)1 blockade can improve the efficacy of various tumor immunotherapies, but also increase severe adverse events, in particular immune-related colitis (irColitis). A better understanding of the immunological mechanisms underlying irColitis at the site of inflammation, along with systemic inflammation, is needed to effectively reduce these side effects. Though irColitis inflammatory events have been associated with CD8+ T cells and CD4+ T helpers as drivers of pathological changes and inflammatory response, no study has systematically integrated multiscale data, from blood, gut, and stool. Here, using gut biopsies collected at the time of irColitis and resolution, and peripheral blood throughout treatment, we investigate the density and spatial distribution of immune-infiltrating cells in the mucosa and lamina propria with integrated multiplex immunohistochemistry (MICSSS) and machine learning, and soluble factors involved during irColitis.

We prospectively enrolled 57 cancer patients receiving standard-of-care CTLA-4-containing combination immunotherapies, and 15 of them developed irColitis during treatment. Colitis was diagnosed by clinical and endoscopic evaluation. Tissue biopsies were obtained from 10 irColitis patients (28 biopsies, ~3 per patient) during flexible sigmoidoscopy or colonoscopy at colitis onset. Blood samples were collected at pre-treatment, at 6 weeks on ICI, irColitis onset, and post-symptom resolution. Serum proteins were assessed using Olink's proximity extension assay, and anti-GM-CSF auAb were measured by ELISA. Tissue cell phenotypes, states, and spatial distribution in inflammatory lesions were quantified by MICSSS. MICSSS allows the quantification of 10 markers on a single FFPE slide using consecutive staining cycles. Digital images of each stain were analyzed with QuPath, R, and MARQO. MARQO analytical pipeline was developed to perform machine learning semi-supervised quality control, tissue masking, registration, segmentation, classification, and quantification of cells in tissues. This provided single cell outputs that were visually reviewed by a pathologist. Mixed linear models and unsupervised clustering were used to evaluate changes in protein expression and tissue cell composition associated with irColitis. Significance was defined as $\text{Log}_2\text{FC} > 1$ and $\text{FDR} < 0.05$ (false discovery rate adjusted p-values).

Our study revealed that 69 proteins were dysregulated during ICI treatment regardless of colitis status. Patients during ICI treatment after irColitis onset exhibited increased IFN-g, MMP-10, IL17A-C and FGF-21. In contrast, patients without irColitis showed similar levels of these proteins to baseline. Also, patients who experienced irColitis showed an increase in anti-GM-CSF IgG and IgA antibodies compared with baseline. Interestingly, inactive (quiescent) tissues areas of irColitis exhibited heterogeneous lower expression FOXP3, CD68, IgG, and CD20, with immune aggregates in some samples; alternatively in active irColitis showed a heightened immune response with clusters of effector (CD3+CD8+) and regulatory T cells (CD3+FOXP3+) along with IgG expressing B/plasma cells near macrophages (CD68+). Ongoing analyses include MICSSS single-cell sequencing and spatial transcriptomics on additional samples.

These findings suggest a multifactorial dysregulation of gut homeostasis, tissue healing, and lymphoid immune modulation in irColitis patients, highlighted by Th1/Th17 dual activation coupled with stromal disrupting factors circulating in blood, GM-CSF autoantibody likely contributing to Treg modulation (PMID:35623454), and T cell and plasma cell-enriched tissue cellular composition. We also present a novel integrated technology to measure and quantify tissue architecture. They may aid in the identification and stratification of patients with irColitis and in the implementation of targeted mitigation strategies

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MULTIMODAL PIPELINE USING SINGLE-CELL RNA SEQUENCING AND MASS SPECTROMETRY REVEALS OXIDATIVE PHOSPHORYLATION AS AN ENRICHED PATHWAY IN REGULATORY T CELLS

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Regulatory T cells (Tregs) in the tumor microenvironment (TME) can disrupt effector T cell infiltration and contribute to the reduced clinical efficacy of immunotherapies. Thus, targeting Tregs is of interest to promote tumor clearance and the anti-cancer immune response. We are specifically interested in targeting Treg metabolism and to that end, we describe a novel multimodal pipeline that can be utilized to identify and probe metabolic pathways that are enriched in Tregs over other immune cells.

Our workflow was developed in normal Tregs as a proof-of-concept, consisting of sorting Tregs via fluorescence-activated cell sorting (FACS), culturing Tregs, using single cell RNA-sequencing to identify differentially expressed genes (DEGs) in Tregs related to metabolism, and probing the metabolic profile of Tregs with mass spectrometry techniques.

In the first step of our multimodal pipeline, CD4+CD25+CD127low Tregs are sorted via FACS from peripheral blood mononuclear cells (PBMCs) that are isolated from human buffy coats. Since Tregs represent a minor population of PBMCs, the sorted Tregs are activated and expanded in culture followed by subsequent rounds of FACS as needed to obtain a highly pure sample of Tregs of sufficient cell numbers. Single-cell RNA sequencing is then performed on a mixed sample containing both PBMCs and Tregs, in efforts to compare the enriched Treg population to other immune cell populations represented by the PBMC dataset. Using the Seurat pipeline for single-cell RNA-sequencing analysis, Treg DEGs are then identified and gene set enrichment analysis (GSEA) is applied to determine Treg DEGs involved in pathways related to metabolism. Liquid chromatography-mass spectrometry (LCMS) of both Tregs and PBMC samples is then performed to probe their metabolic profiles as well as desorption electrospray ionization-mass spectrometry imaging (DESI-MSI) on the Tregs. In leveraging our multi-technology platform in normal Tregs, oxidative phosphorylation (OXPHOS) was identified as a top pathway enriched in Tregs over PBMCs. Additional analyses with LCMS revealed differences in the metabolic profiles of both cell types, and DESI-MSI on the Tregs further identified abundant species such as phosphatidylcholines and phosphatidylglycerols. Manipulation of the species identified by mass spectrometry, which could play a role in the mitochondrial membrane of Tregs and warrants further investigation to that end, may impact the activity of transmembrane proteins involved in OXPHOS in Tregs.

Taken together, these findings suggest the targeting of the OXPHOS pathway as a potential therapeutic strategy. Efforts to investigate this are underway, including treating Tregs and PBMCs with inhibitors of OXPHOS to elucidate the hypothesized preferential sensitivity of Tregs to inhibition of this pathway, and probing this further in the context of Tregs from the TME. Ultimately, our multimodal pipeline underscores the utility of integrating single-cell RNA sequencing and mass spectrometry techniques in identifying novel opportunities to target pro-tumorigenic immune cells in the TME.

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CONTROL OF TREG FUNCTION BY CROSS-REACTIVITY BETWEEN INTESTINAL AND SELF ANTIGENS

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Improper control of inflammation drives the pathological loss of tissue function in common conditions including cardiac arrest, fatty liver disease and cancer, where proinflammatory cytokines act as growth factors for tumor initiation and progression. Proinflammatory cytokine production following injury is important for tissue regeneration. However, the magnitude and duration of inflammatory cytokines must be kept in check by a subset of regulatory T cells (Tregs) that are marked by expression of the protein ROR γ and trained in the gut. Without ROR γ + Tregs, the skeletal muscle (SkM) fails to recover following injury resulting in persistent inflammation and tissue fibrosis. A similar ROR γ + Treg axis is found in liver inflammation and ROR γ + Treg populations are also found in the heart after cardiac arrest. However, the drivers that facilitate the ROR γ + phenotype in Tregs, and target tissue accumulation, are unknown but antigen specificity of ROR γ expressing Treg T cell receptors (TCR) are undoubtedly a strong candidate. Interestingly, one TCR, mTreg24, has been consistently observed in Tregs in injured SkM, on days 2-4 after injury, across 17 mice analyzed over 10 years, suggesting the recognition of a conserved antigen. In the present study, we sought to identify the antigen(s) that the mTreg24 TCR recognizes and determine how antigen specificity controls Treg phenotype and function.

T cells from TCR transgenic mice expressing the Treg TCR of interest (mTreg24) were co-cultured with antigen presenting cells (APC) loaded with protein lysates to identify the sources of antigens capable of activating mTreg24. mTreg24-APC co-culture was then performed with synthesized candidate peptides to identify exact peptide targets of the mTreg24 TCR.

Peptide-MHC-II tetramers were designed from the identified peptide targets in the co-culture screening and generated by the NIH Tetramer Core. We injured the SkM using cardiotoxin (Ctx) and performed tetramer staining to track and characterize the phenotype of CD4+ T cells reactive to this specificity in the SkM and secondary lymphoid organs of mice. We performed similar tetramer tracking experiments in mice where the identified non-self-dietary antigen was eliminated by placing mice on diets composed of amino acids rather than whole proteins.

The mTreg24 TCR recognizes antigens from gut contents, specifically those derived grain products found in standard mouse chow. We generated candidate lists of peptides from published mass spectrometry data of proteins extracted from grains. This peptide screen revealed the diet-derived cognate specificity of the mTreg24 TCR. We screened self-peptides, sharing a similar amino acid sequence to the identified dietary antigen, and identified a cross-reactive self-antigen target for the mTreg24 TCR that is expressed in the SkM.

We generated peptide-MHC-II tetramers to track this dietary antigen specificity in vivo. We found a large population of tetramer binding Tregs in SkM 3 days following injury. These tetramer binding Tregs in the injured SkM are largely ROR γ +, indicative of their provenance in the gut. Further, tetramer binding Tregs are enriched in the mesenteric lymph nodes and biased towards the ROR γ phenotype.

Finally, when the cognate dietary antigen was removed, by placing mice on amino acid diet, recruitment of tetramer specific cells is dramatically reduced in the injured SkM. Compared to mice on standard chow diet, where tetramer binding Tregs in the injured SkM are largely ROR γ +, the majority of tetramer binding Tregs in the injured SkM of mice on amino acid diet are ROR γ -.

Our data supports a model whereby non-self-antigens drive the adoption of the ROR γ + Treg phenotype and expand these specificities. During an inflammatory event, ROR γ + Tregs that cross-

react to a self-antigen in the inflamed tissue are quickly recruited to control the inflammatory tone and facilitate repair to return the tissue to homeostasis.

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EFFECT OF GLUCOCORTICOIDS ON ONCOLYTIC VIRUS MEDIATED KILLING IN GLIOBLASTOMA

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Glioblastoma (GBM) is the most aggressive brain tumour in adults, with average survival of only 18 months post diagnosis. Oncolytic viruses (OVs), are emerging as valuable tools to supplement standard therapies. GBM patients receive a synthetic glucocorticoid, Dexamethasone (Dex) as part of standard therapy, which is a potent anti-inflammatory. Dex may interfere with the efficacy of immunotherapies, however, it remains unclear how Dex might affect OV action in GBM

GBM cell line (MO59K) and derived-patient cell lines (GBM13, GBM20) were treated with Dex in the presence or absence of oncolytic Reovirus. GBM cells were also co-cultured with PBMC treated with Reovirus. The impact of the combining treatment of Dex with Reovirus on direct oncolysis of GBM cells and indirect killing of GBM cells by Reovirus-activated PBMC was examined by flow cytometry

Our data showed that Dex (100nM) significantly impaired direct killing of GBM cells by Reovirus. This effect was not observed on normal progenitor cells. Flow cytometry analysis showed no significant difference in expression of the Reovirus entry receptor (JAM-1) or in the levels of reovirus infection post Dex treatment. This indicates that Dex does not affect the ability of Reovirus to infect or replicate within GBM cells. Pre-treatment with Dex also significantly impaired indirect killing of GBM cells by PBMC treated with Reovirus. Dex treatment also reduced the activation state of NK cells infected with Reovirus. The inhibitory effect of Dex on PBMC mediated killing was lost when NK cells were depleted using CD56 microbeads, confirming the role for NK cells.

Dex impairs direct oncolysis of GBM cells by Reovirus and abrogates NK-mediated killing of GBM cells. Next, RNA-sequencing will be used to determine the pathways regulated by Dex that effect the response of GBM cells to Reovirus.

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ALPHA-ENOLASE AND FAR UPSTREAM ELEMENT-BINDING PROTEIN 1 CIRCULATING AUTOANTIBODIES AS NEGATIVE PROGNOSTIC FACTORS FOR PANCREATIC CANCER PATIENT SURVIVAL

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Pancreatic ductal adenocarcinoma (PDA) has a dismal prognosis with a 5-year overall survival (OS) rate of only 12%. With increasing incidence, it is projected to become the second leading cause of cancer-related deaths by 2030. There are different reasons for poor PDA prognosis, such as the absence of early diagnostic markers and the lack of effective therapies to increase patient survival. Serum marker Carbohydrate Antigen (CA) 19.9 is used for the diagnosis of PDA, but it is poorly sensitive and specific.

Autoantibodies (aAb) to several oncogenic proteins in tumor patients have been reported. An antibody-dependent response against tumor proteins could thus be used to detect low antigen concentrations and indicate imminent tumor progression.

Through a serological proteome approach, we identified alpha-enolase (ENO1) as a key glycolytic enzyme upregulated in PDA patients. We showed that aAb to phosphorylated ENO1 discriminated healthy subjects from PDA patients, and usefully complemented the diagnostic performance of serum CA 19.9, achieving approximately 95% diagnostic accuracy in both advanced and resectable PDA. In addition, we observed that in PDA patients, circulating anti-Far upstream element-binding protein 1 (FUBP1) aAb were higher than in healthy subjects.

In this study, the prognostic role of FUBP1 and ENO1 tissue expression and the aAb response to them was analyzed in PDA patients.

Circulating ENO1 and FUBP1 aAb were analyzed by ELISA (n=470), while tissue expression was observed by immunohistochemistry (n=45). OS was estimated using the Kaplan-Meier method, while the Cox model was used to estimate the hazard ratios (HR) adjusted for the main prognostic factors. Logistic models were applied to assess associations between death and its risk indicators. All statistical analyses were performed with Stata version 15.

The analysis of circulating aAb showed that the levels of ENO1 and FUBP1 aAb were upregulated in PDA patients.

The expression of FUBP1 and ENO1 in the selected tumoral and peri-tumoral tissues from PDA resected patients, revealed an increased expression of both antigens in tumoral tissues. In addition, a significant correlation between circulating FUBP1 aAb and its expression in tumors was observed. As circulating aAb to FUBP1 and ENO1 were increased in PDA patients, their prognostic role was evaluated by assessing their levels with the risk of death. A Cox regression model was employed to analyze other variables that can affect OS of PDA patients. Levels of aAb to FUBP1 and ENO1 were subdivided into tertiles (low, intermediate and high). Performance status, Ca19.9, high anti-ENO1 and intermediate anti-FUBP1 aAb levels were negative prognostic factors.

A significant p-value interaction between values and disease site indicates that anti-FUBP1 aAb level is a good prognostic marker when the tumor is in the tail-body of the pancreas.

Using a spline tool to test the prognostic values of anti-ENO1 and FUBP1 aAb, we observed that the relationship between anti-FUBP1 aAb levels and prognosis is evident for non-resected PDA patients with elevated aAb values. Anti-ENO1 aAb levels showed no relationship with mortality risk when

PDA patients were resected, but there was a relationship for high titer of ENO1 aAb when patients did not undergo surgery.

Low and intermediate levels of anti-ENO1 and anti-FUBP1 aAb, respectively, correlate with a decreased OS in PDA patients and can provide useful medical information, not only for the prognosis of the disease, but also for the site of onset of PDA, allowing a better outcome of currently available therapies.

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A TRANSCRIPTION FACTOR SCREENING PLATFORM FOR IMMUNE CELL REPROGRAMMING

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Rewiring cell identity using direct cellular reprogramming holds great promise for regenerative medicine. Once identified, the expression of selected combinations of Transcription Factors (TFs) in somatic cells lead to induction of a desired cell fate. Reprogramming immune cells for immunotherapy was recently established from fibroblasts to conventional type 1 dendritic cells (cDC1) with the TFs PU.1, IRF8 and BATF3. We hypothesize that combinatorial action of different TFs results in the specification of immune cell identities controlling discrete modules of the immune response.

We generated a library of immune TFs by identifying ~400 TFs restricted to immune cell populations based on single cell gene expression profiles using an aggregated ranking approach. Next, we have established an unbiased platform to identify combination of reprogramming factors. By implementing a modified single-cell mRNA-sequencing protocol, we were able to simultaneously measure the transcriptome of cells and track instructive TF combinations using TF-specific barcodes.

We validated our screening platform by overexpressing 9 individually barcoded TFs with enhancing or repressing effects on cDC1 reprogramming. On average, induced cDC1s contained 2-5 barcodes, which is ideal for direct reprogramming. We reconstructed the reprogramming trajectory and mapped induced cells to natural cDC1s. Reassuringly, successfully reprogrammed cells harbored the highest frequency of PU.1, IRF8 and BATF3. Measuring barcode expression in single cells uncovered the TF stoichiometry required for successful reprogramming. Additionally, we identified and experimentally validated one additional TF that improved efficiency and fidelity of cDC1 reprogramming. Finally, we have further demonstrated that cDC1 reprogramming factors were identified when pools of ~20 and ~40 TFs were used to elicit reprogramming.

Collectively, this work provides evidence for a multiplexed approach to unravel lineage-determining TFs by direct cell reprogramming. This platform will allow to map the transcriptional networks underlying immune cell specification and reprogramming, with significance for the development of novel immunotherapies.

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EXPLORING THE PHENOTYPE AND ANTIGEN SPECIFICITY OF ENGRAFTING CD8 T CELL CLONES IN PATIENTS UNDERGOING ADOPTIVE CELL THERAPY

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Adoptive cell transfer of tumor-infiltrating lymphocytes (TIL) shows great promise in treating various cancers. The clonal dynamics of T cells after infusion and the persistence of tumor-reactive clones can affect treatment efficacy, however, the impact of phenotype and specificity on clone engraftment remains unclear.

To address this we generated single-cell analyses of infusion products, TILs themselves and peripheral blood pre and post-adoptive TIL transfer from five metastatic melanoma patients. We sorted antigen specific T cells based on patient-specific pMHC multimer panels consisting of 100 different epitopes comprising patient-unique neoantigens, tumor-associated antigens and virus-derived antigens.

We have profiled these with respect to gene expression, surface markers, TCRs and pMHC recognition, and assigned antigen specificity to the T cell clones. Hence, we have identified TCRs responsive to neoantigens, tumor-associated antigens and viral-derived antigens. Amongst these we have identified both TCRs cross-reacting with several pMHCs and multiple TCRs specific to only one epitopes. We have subsequently validated a large proportion of the identified TCR-pMHC pairs by re-expressing the individual receptors, and are currently exploring their functional properties. We integrated surface protein and gene expression into a single embedding, revealing both the CD8 T-cell states and trends in phenotypical dynamics from infusion product and consecutive time points in circulation. We tracked T cell clones in each follow-up and quantified engraftment as a mean deviation of clone frequency of each time point from the infusion product. Subsequently, we found several phenotypical characteristics of T cell clones that correlate with engraftment.

Overall, our study provides novel insights into the characteristics of persistent TCR clones in patients receiving adoptive cell therapy and demonstrates the capacity to link transcriptomic, surface expression, TCR, and pMHC specificity of clinically relevant T cells, hence describing features of relevance for the development of personalized cancer immunotherapies.

P376**THE ROLE OF TUMOR-SPECIFIC T CELL FREQUENCY AND PHENOTYPE IN PREDICTING RESPONSE AND RECURRENCE TO A DENDRITIC CELL-BASED VACCINE**

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Immunotherapy has been shown to be a powerful treatment option for different types of cancer. A proportion of high-grade glioma patients can benefit from dendritic cell-based vaccination. However, we still lack the understanding of which patients can benefit the most from these treatments. This study aims to assess the alterations in tumor-specific T cells triggered by a dendritic cell-based vaccine, with the intention of identifying predictive markers for treatment response and recurrence following vaccination.

In our prospective, non-controlled phase I/II clinical trial, we enrolled a total of 37 patients diagnosed with glioblastoma or grade 4 astrocytoma. Following the initial recurrence post-surgery, these patients commenced a monthly regimen of intradermal injections, while still receiving bevacizumab. These injections consisted of hybridoma cells generated through the fusion of allogenic dendritic cells with autologous tumor cells. To assess the immune response elicited by vaccination, we analyzed the blood samples of the participants. The frequency of tumor-specific CD8 and CD4 T cells was determined using autologous tumor lysate as the antigen source, and proliferation as a readout. The phenotype of CD4 T cells was determined by intracellular cytokine staining followed by UMAP analysis to identify the different CD4 T cell subpopulations.

Vaccination significantly prolonged overall survival in high-grade gliomas compared with patients from the Genomics Data Commons (GDC) database. In several patients, functional scores improved and the tumor shrank after the first doses of vaccine. Yet, in many of them, this benefit was transient, as the cancer later progressed and led to the patient's death. To understand the factors that determine response to the vaccine, we examined the frequency and phenotype of tumor-specific T cells in the blood of a patient who initially showed a partial response to the vaccine but evolved to progressive disease 10 months later. Before vaccination, the frequency of circulating tumor-specific CD4 and CD8 T cells was below the detection limits of the assay. In addition, tumor-specific CD4 T cells had a higher proportion of IL-10 and a lower proportion of IFN- γ -producing cells relative to polyclonally activated cells. The vaccination increased the frequency of circulating tumor-specific CD4 and CD8 T cells to levels above the assay detection limit after 2 and 3 doses, respectively. Concomitantly, the MRI showed tumor shrinkage. We also observed an increase in Th1 cells, and a reduction in all other CD4 T cell subpopulations. The patient continued to improve, with clinical deterioration occurring only after the 10th dose. The recurrence was preceded by a drop in the frequency of circulating tumor-specific CD4 T cells, which began after the 7th dose and intensified on reanalysis two doses later. We also observed a drastic change in the cytokines produced by tumor-specific CD4 T cells at dose 9, with the majority of the cells producing IL-17. Tumor-reactive Tregs also increased. The final MRI was performed at dose 12, and showed a large tumor mass occupying both frontal lobes. Treatment was discontinued at the request of the parents, and the patient died 15 months after the start of the study as a result of tumor growth. Upon expanding our analysis to a larger patient population, we observed a notable trend. Vaccination increased the frequency of CD8 tumor-specific T cells in 5 out of 7 patients tested. Remarkably, only individuals with an induced CD8 tumor response survived beyond one year from the time of study enrollment, indicating a potential association between CD8 tumor response and extended survival.

Our study suggests that tumor-specific T cell frequency and phenotype play an important role in cancer control in the context of vaccination. Monitoring the blood tumor-specific T cells proved to be a powerful tool for predicting response to immunotherapy and disease recurrence.

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IL-1R8 DEFICIENCY ENHANCES THE ANTI-TUMOUR ACTIVITY OF DOXORUBUCIN WITHOUT AFFECTING CARDIAC FUNCTION

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In recent years, the combination of chemo and immunotherapy achieved significant benefits for oncological patients. The treatment with anthracycline, such as Doxorubicin (Doxo), combined with immune checkpoint inhibitors was shown to elicit immunogenic cell death, activating neoantigen T cell and tumor-specific immune responses against different types of cancer, including sarcoma. Increased severity of side effects and risk of adverse events have been described during chemo-immunotherapy, such as exacerbated inflammation, hepatotoxicity and dose-dependent cardiotoxicity. Interleukin-1 (IL-1) plays a key role both in cardiac and cancer-related inflammation. IL-1 receptor 8 (IL-1R8), a member of the IL-1 receptor (ILR) family, acts as a negative regulator of ILR and Toll-like receptor downstream signaling pathways, and is involved in inflammation regulation. IL-1R8 acts as an immune checkpoint for both NK and CD8+ cells (Molgora et al., 2017 and not shown), and its genetic blockade is associated with immune-mediated anti-tumor activity in murine models. With the aim to develop IL-1R8 as a new immune checkpoint, we investigated its activity in combination with classical chemotherapy.

We evaluated the combination of IL-1R8 deficiency with Doxo treatment to assess the effect on tumor growth and cardiac toxicity. In particular, wild-type (wt) and Il1r8^{-/-} mice were subcutaneously transplanted with a fibrosarcoma cell line (FS6) and the effect of Doxo treatment was assessed at a high (20 mg/kg) or low (10 mg/kg) dose. Tumor growth was assessed every two days until 30 days after transplantation. Echocardiographic analysis was performed in order to evaluate cardiac function upon treatment with high and low dose of Doxo. Cardiac fibrosis, assessed by Sirius red staining, and IL-1R8 expression in cardiac tissue were analyzed in order to investigate mechanism/s involved in the phenotype observed.

We observed that the high dose regimen allowed the complete eradication of the tumor in both wt and Il1r8^{-/-} mice, while the low dose treatment led to a significant smaller tumor size in Il1r8^{-/-} vs wt mice, indicating that IL-1R8 deficiency increased the efficacy of low dose Doxo treatment, allowing tumor regression. Echocardiographic investigation showed decreased cardiac function only upon high dose Doxo treatment, while no functional defects were observed in low dose regimen. No differences were observed in the cardiac function when comparing wt and Il1r8^{-/-} mice after high dose Doxo treatment, indicating that blockade of this immune check point does not exacerbate Doxo-induced cardiotoxicity. Expression analysis in cardiac tissue revealed that IL-1R8 is expressed only in endothelial cells and leukocytes but not in cardiomyocytes. The histological investigation showed a reduced cardiac fibrosis in Il1r8^{-/-} animals compared to their wt counterparts, due to still uncharacterized mechanisms.

Collectively, our results suggest that targeting IL-1R8 combined with Doxo treatment improves the anti-tumor activity of this anthracycline without increasing its cardiotoxic effect.

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MITOCHONDRIAL DYNAMICS REGULATE INTERFERON SIGNALING AND AGE-RELATED CHANGES IN HSPCS

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Blood cells are predominantly short-lived and hematopoietic stem cells (HSCs) are required throughout life to replenish multilineage and more committed precursors, ultimately giving rise to mature blood cells. Despite major advances in our understanding of the biology of the hematopoietic system, numerous critical issues remain to be addressed. Some of those are the mechanisms underlying the aging of hematopoietic stem and progenitor cells (HSPCs). With age, HSPCs expand and become more myeloid biased, leading to immune impairment and increased risk of clonal hematopoiesis (CH) and myeloid malignancies, such as myelodysplastic syndromes (MDS).

We analyzed mice with conditional deletion of Mfn2 in the hematopoietic system (Mfn2 fl/fl Vav-Cre+, called Mfn2KO) and wild type littermates (WT) as germline deletion is embryonic lethal. We performed RNA-seq and sc-RNAseq on sorted HSCs or on whole BM and spleen cells from WT and Mfn2KO mice with RT-qPCR validation. We also crossed Mfn2KO mice with global Tet2^{-/-} (Tet2KO) to analyze peripheral blood for cell blood counts, HSC frequency and cellular (myeloid versus lymphoid lineage) composition in the BM and in the spleen. We generated other double KO (DKO) mouse model crossing Mfn2KO with Mavs^{-/-}, Sting^{-/-}, Stat1^{-/-} or the type I interferon receptor^{-/-}. To study the effect of the microbiome we sterilized the mice with a cocktail of antibiotics for one week.

This work is based on the finding that Mitofusin 2 (MFN2), a protein involved in mitochondrial fusion, is required for the maintenance of HSCs with extensive lymphoid potential and for overall HSC quiescence. Here we show that deletion of Mfn2 causes an 'aged' HSPC phenotype in young mice, with relative expansion of the stem and progenitor cell compartment in both medullary and extramedullary compartments, with exacerbated loss of lymphoid potential during aging. Crossing Mfn2 knock-out (KO) mice with Tet2KO, the most frequently mutated gene in CH and MDS, we also noticed a more rapid expansion of myeloid cells with impaired lymphoid lineage differentiation as well as signs of extramedullary hematopoiesis and enlarged spleen in Mfn2/Tet2 double KO (DKO) mice vs single Tet2KO and Mfn2KO ones. RNAseq combined with single cell-RNAseq experiments revealed striking up-regulation of Interferon stimulated genes (ISGs) in HSCs as well as whole bone marrow (BM) and spleen cells from Mfn2KO mice compared to wild-type (WT). Detectable Interferon (IFN α) was furthermore found in the serum of Mfn2KO mice. Moreover, we found that Mfn2 does not functionally interact with Mitochondrial activator of viral signaling (Mavs), a central mediator of innate immunity signaling associated with mitochondria. Instead, double deletion of Mfn2 with the DNA sensor Sting or key IFN mediators such as Stat1 or the type I interferon receptor fully rescued the effect of Mfn2 deletion on HSPC frequency, cycling and interferon induction. The mechanism behind this phenotype is mediated by Calcium, because altering intracellular calcium levels rescued ISGs induction in Mfn2KO macrophages (MDM) as well as a significantly decreased tonic interferon signalling in WT MDM. Interestingly, aged Mfn2-deleted BM showed decreased hematopoietic regenerative function compared to WT with a full rescue deleting Stat1 together with Mfn2. Finally, eliminating the intestinal and lung microbiota abrogated ISGs induction as well as HSPCs expansion in the BM of Mfn2-deleted mice.

Our data indicate that MFN2 is required to shield hematopoiesis and, in particular, HSCs from tonic IFN-I signaling induced by the microbiome, inhibiting IFN α production in a STING dependent fashion. Taken together, these findings suggest that manipulating mitochondrial dynamics constitutes an approach to alleviate some aspects of aging, myeloproliferative diseases as well as genetic interferonopathies.

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NPX887, A FULLY HUMAN MONOCLONAL ANTIBODY TARGETING HHLA2, BLOCKS KIR3DL3-MEDIATED IMMUNOSUPPRESSION AND INITIATES ANTIBODY-DEPENDENT CELLULAR CYTOTOXICITY OF HHLA2+ TUMORS

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Human endogenous retrovirus H long terminal repeat associating protein 2 (HHLA2) is a member of the B7 family that has both immune suppressive and stimulatory functions on T and NK cells through interaction with either the co-inhibitory receptor killer cell immunoglobulin-like receptor, three immunoglobulin domains and long cytoplasmic tail 3 (KIR3DL3) or the co-stimulatory receptor transmembrane and immunoglobulin domain containing 2 (TMIGD2). HHLA2 binding to KIR3DL3 blunts downstream immune activation while the interaction of HHLA2 with TMIGD2 enhances T and NK cell activity. The HHLA2-KIR3DL3 axis is emerging as an important immune checkpoint in cancer. While HHLA2 expression in normal tissues is limited, it is highly expressed in many cancers where it is often associated with more severe pathology, fewer tumor infiltrating lymphocytes, and poor patient outcome. Utilizing HHLA2 antibodies that block the co-inhibitory interaction with KIR3DL3 but spare the co-stimulatory interaction with TMIGD2 may be a promising strategy to promote the anti-tumor activity of T and NK cells in the tumor microenvironment. NextPoint Therapeutics has developed NPX887, a fully human, antagonistic immunoglobulin G1 (IgG1) monoclonal antibody targeting HHLA2 that blocks binding to KIR3DL3 but spares binding to TMIGD2. NPX887 was engineered to induce antibody-dependent cellular cytotoxicity (ADCC) activity.

NPX887 binding to HHLA2 expressed on the cell surface was determined by flow cytometry using cell lines engineered to express HHLA2, tumor cell lines, and dissociated primary tumors. On-cell blocking experiments using recombinant proteins were used to measure the ability of NPX887 to block the interaction of HHLA2 with its receptors, KIR3DL3 and TMIGD2. The ability of NPX887 to block the inhibitory interaction of HHLA2 with KIR3DL3 and enhance immune cell activity was determined using Jurkat reporter assays and an NK-92 MI cell line killing assay. Finally, the ability of NPX887 to induce ADCC of HHLA2+ tumor cells was assessed using both a CD16 ADCC reporter signaling assay and a primary human PBMC killing assay.

NPX887 bound to HHLA2 on 300.19 cells stably expressing both human and cynomolgus HHLA2 as well as tumor cell lines endogenously expressing HHLA2. In addition, NPX887 bound to tumor cells from dissociated tumor samples collected from patients with renal cell carcinoma and lung adenocarcinoma. NPX887 blocked the binding of recombinant HHLA2 to 300.19 cells engineered to express KIR3DL3, but spared the binding of recombinant TMIGD2 to 300.19 cells overexpressing HHLA2. NPX887 treatment inhibited KIR3DL3-induced suppression of T cell activation, but spared TMIGD2 co-stimulation in Jurkat co-culture assays with HHLA2+ cell lines. Furthermore, NPX887 treatment enhanced the killing of HHLA2+ tumor cells by the KIR3DL3+ NK-92 MI cell line. NPX887 induced CD16 reporter cell signaling against HHLA2+ tumor cells in a dose dependent manner. In addition, NPX887 induced ADCC killing of HHLA2+ tumor cells by primary human immune cells.

The HHLA2 axis represents a novel immune checkpoint that mediates tumor immune evasion by suppressing both NK and T cell activity. Furthermore, HHLA2 is an attractive tumor antigen given its limited expression in normal tissue and enhanced expression in many tumors. NPX887, a fully human, monoclonal antibody targeting HHLA2 represents an attractive approach to treat certain HHLA2+ cancers by both inducing ADCC and by potentiating anti-tumor immune responses.

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EARLY FINDINGS FOR THE ROLE OF CASPASE 8 IN ANTI-CANCER IMMUNITY

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Caspase-8 (Casp8) is an initiator of extrinsic apoptosis and is frequently downregulated in various cancer types. Despite this downregulation and its widespread functions in apoptotic and non-apoptotic signaling pathways, its role in altering anticancer immunity has not been explored. Utilizing murine tumor models (MC38 colon carcinoma and B16 melanoma), we sought to establish an in vivo role for Casp8 in the tumor microenvironment to alter anti-cancer immunity.

Because Casp8 deletion is embryonic lethal, mlkl was additionally ablated in these mice to generate Casp8^{-/-}-mlkl^{-/-} (DKO) mice. These DKO mice develop a lymphoproliferative disorder (LPR or ALPS in humans). Because this disease can not be reconstituted from hematopoietic stem cells, bone marrow (BM) transplants are utilized in these studies to address the role of Casp8 in the tumor microenvironment without complications from LPR.

Tumor Injection

Bone Marrow Isolation/Transplantation

Tissue Digestion

Flow Cytometry

Figure 1: Tumor growth and survival of Casp8^{-/-}-mlkl^{-/-} mice.

Three DKO and four WT/Het mice were injected with B16 melanoma tumors and monitored for growth over time. Two out of the three DKO mice grew larger B16 tumors sooner, while the third mouse appeared to have a large delay in tumor growth. Survival data shown.

Figure 2: Tumor growth and T-cell analysis in RAG mice reconstituted with Casp8^{-/-}-mlkl^{-/-} bone marrow.

RAG mice were irradiated and reconstituted with BM from either DKO or WT/Het mice for one month before being injected with MC38 colon carcinoma cells subcutaneously. Although non-irradiated RAG mice grew larger tumors than reconstituted mice, tumor size was not significantly different between mice reconstituted with DKO vs WT/Het bone marrow. However, PD-1 expression on CD8 T cells was increased in both the spleen and the tumor. Further, both the splenic and intratumoral CD8 T-cells showed increased levels of short-lived effector cells (SLECs) and decreased levels of memory like progenitor cells (MPECs).

Figure 3: Tumor growth and immune cell analysis in WT/Het and Casp8^{-/-}-mlkl^{-/-} mice reconstituted with WT/Het Casp8^{-/-}-mlkl^{-/-}.

WT/Het (labeled WT for simplicity) and DKO mice were irradiated and reconstituted with either WT or DKO bone marrow. After a two-month recovery, these mice were injected with MC38 tumors subcutaneously and tumor size was assessed. After 26 days there was a significant increase in tumor size between the DKO->DKO compared to the WT->WT. Although no difference was observed in the percentage of PD1⁺ CD8 T cells in the spleen between WT->WT and WT->DKO, there was an increase in both groups of mice which received DKO bone marrow (DKO->WT and DKO->DKO) with an additional increase seen in the DKO->DKO group. Despite this, no significant difference in PD-1⁺ CD8 T cells was seen in the tumor. Finally, there were increased percentages of myeloid cells (CD11b⁺) in the tumors of the two groups of mice which received DKO bone marrow (DKO->WT and DKO->DKO).

Though many of these experiments need repeated, these data provide a strong foundation elucidating the role of Caspase-8 in anti-tumor immunity. Despite the recipient (WT, RAG, DKO), CD8 T cells derived from BM from DKO mice show increased PD-1 levels. Further, tumors in mice

which lack Caspase-8 (and MLKL) in the TME grew larger than those with functional Caspase-8. Finally, an increase in myeloid cells was also seen in tumors of DKO-reconstituted mice, suggesting Caspase-8 may decrease the percentage of myeloid cells in the tumor. Future studies measuring T cell exhaustion and exploring Caspase-8 knock-out in specific cell types should help elucidate these early findings exploring the novel role of Caspase-8 in anti-tumor immunity.

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TRANSCRIPTION FACTOR GENE NETWORKS REGULATING DIVERGENT HUMAN B CELL FATES

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Activated B cells bifurcate into antibody-secreting plasmablasts (PBs) or germinal center B cells (GCBCs), the balance of which determines the quality and magnitude of humoral immune responses. While short-lived PBs provide a rapid burst of low-affinity antibodies, GCBCs delay differentiation in secondary lymphoid organs where their immunoglobulin genes undergo somatic hypermutation and affinity-based selection to generate precursors of long-lived plasma cells that secrete high-affinity antibodies. We aim to comprehensively elucidate the dynamic TF gene networks that regulate the generation and function human PBs and GCBC precursors. We and others have previously demonstrated that reciprocal and sequentially acting negative feedback loops between pairs of signaling-induced TFs, IRF4 and IRF8 and Blimp1 and Bcl-6, regulate the bifurcation of murine activated B cells into PBs and GCBCs. While elevated IRF4 and Blimp1 expression promotes PB differentiation, elevated IRF8 and Bcl-6 expression restrain PB differentiation and enable GCBC formation instead.

To study the dynamic TF gene networks that regulate human B cell fates, we utilize an in vitro B cell differentiation approach which captures the transition of naïve B cells into activated B cells and their bifurcation into PBs or pre-GCBCs. We have coupled bulk and single cell RNA-seq and ATAC-seq with joint regulatory modeling to predict TF regulation of differentially expressed genes and human B cell fates. We then test these predictions with CRISPR TF perturbations in primary human B cells undergoing activation and differentiation.

In congruence with murine B cells, we show that in vitro activated human B cells bifurcate into distinct IRF4^{hi}/IRF8^{lo}/BLIMP1^{hi}/BCL6^{lo} (PB) or IRF4^{lo}/IRF8^{hi}/BLIMP1^{lo}/BCL6^{hi} (pre-GCBC) regulatory states upon activation and several days of rapid cell division. Through bulk RNA-seq and ATAC-seq analysis, we identified differentially expressed genes and differentially accessible regions that distinguish activated B cells, PBs, and pre-GCBCs. Paired single cell RNA-seq/BCR-seq analysis revealed that individual B cell clones can indeed bifurcate to generate both PB and pre-GCBC fates. While PBs underwent extensive clonal expansion, GCBC cells exhibited less clonal expansion resulting in greater BCR clonal diversity. Topic modeling of single cell multimodal (RNA-seq/ATAC-seq) analysis revealed PB- and GCBC-specific chromatin and gene expression topics which emerge during B cell activation. Through joint regulatory modeling of chromatin accessibility and gene expression dynamics and CRISPR perturbations, we provide evidence for key TF regulators of human B cell differentiation and function.

By temporally profiling the gene expression and chromatin accessibility in human B cells undergoing activation and differentiation, we have assembled a dynamic TF gene network model that underlies the generation of heterogeneous human B cell states. This work will provide new molecular insights into the dynamic regulatory apparatus that controls the quality and durability of humoral immune responses in humans and facilitate vaccine design.

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BTLA PLAYS A CRITICAL ROLE IN HEMATOLOGIC MALIGNANCIES AND SERVES AS PROMISING TARGET FOR CANCER IMMUNOTHERAPY

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Checkpoint molecules regulate the activation of immune cells upon antigen presentation. Two classes of immune checkpoint receptors are recognized – stimulatory, e.g. LIGHT receptor, promoting immune cell proliferation and effector functions and inhibitory, e.g. BTLA and PD-1, suppressing immune cell activation by inhibiting TCR/BCR and CD28 signaling. Tumor cells often overexpress ligands of inhibitory receptors, such as HVEM and PD-L1, as a mechanism to escape anti-tumor responses. Interestingly, increased expression of inhibitory receptors, such as BTLA, PD-1 and CTLA-4, has been described in several tumors as well. However, the role of these receptors in tumor setting remains unknown.

Here, we analyzed the expression and mutations of BTLA in large set of hematologic tumors from publicly available RNA-seq and Whole exome sequencing (WES) data using tools available in UseGalaxy server. FPKM values were called using Seqmonk. To evaluate the expression of BTLA in various cell lines, flow cytometry was used and data were analyzed using BD FACSDiva software. Proteins HVEM and UL144, which are natural ligands of BTLA, were expressed using the BV-Sf9 expression system. To stimulate BTLA, JVM-2 cells were treated with HVEM or UL144 at concentration 5, 10, 20, 60 and 100µg/ml. After 72 hours, the viability of cells was analyzed using MTT assay. To identify, which signaling pathways are deregulated after UL144 treatment, JVM-2 cells were lysed in RIPA and the level of selected proteins and phosphorylated proteins was analyzed using Western blot.

We found, that BTLA is highly expressed in Acute myeloid leukemia and Chronic lymphocytic leukemia (CLL) at mRNA level. Neither BTLA nor downstream signaling proteins were mutated in analyzed CLL tumors, suggesting on active BTLA signaling in these tumors. Next, we analyzed protein level of BTLA in cell lines derived from hematologic malignancies using flow cytometry. We observed that BTLA is highly expressed in cell line MEC-1 derived from chronic lymphocytic leukemia (rate of expression 90 ± 2) and JVM-2 derived from mantle cell lymphoma (83 ± 4). Medium expression was in cell lines SUDHL-6, MOLT-16 and CA46 (ranging from 16 ± 7 up to 36 ± 3) and very weak expression in cell lines K562 and RAJI (2.1 ± 1.7 and 1.5 ± 1.15 , respectively). To further evaluate the role of BTLA in tumor growth and survival, we targeted this protein by its natural ligand HVEM as well as UL144. UL144 is an ortholog of HVEM encoded by human cytomegalovirus. In opposite to HVEM, UL144 specifically binds to BTLA and not to the stimulatory receptor LIGHT. HVEM did not inhibit proliferation of JVM-2 cells. Interestingly, UL144 reduced proliferation of JVM-2 cells at concentration 100µg/ml (fold change > 2) as analyzed by MTT assay 72 hours post treatment and this occurred through events involved in BCR/TCR signaling, including downregulation of SHP-1, an inhibitor of BCR/TCR signaling, which we detected already 24 hours post treatment.

Altogether, our data suggest that BTLA is highly expressed in various hematologic malignancies and targeting BTLA can inhibit tumor cell growth by controlling events involved in BCR and TCR signaling.

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P383**INTERFERON-GAMMA RESPONSIVENESS OF HUMAN SINONASAL MUCOSAL MELANOMAS**

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Mucosal melanoma (MMs) is a highly aggressive and rare form of melanoma. Head and neck MMs, in particular those arising in the sinonasal (SN) cavity, represent the most common MMs. MM outcome is very poor and there is still no definitive consensus on the optimal management of MMs, particularly on the role of adjuvant treatments for loco-regional control. BRAF V600 mutations are found in a negligible percentage of MMs and systemic chemotherapies have demonstrated little or no survival benefit, in contrast with cutaneous melanoma (CM). Compared to CM, the analysis of the IC in MM is fragmentary and indicate that MMs are poorly infiltrated by immune cells. Complex cellular and molecular interactions within the tumour microenvironment might explain this immune desert phenotype. Escape from the recognition and elimination by the immune system encompasses a set T cells exclusion (TCE) mechanisms. Cancer cell-intrinsic molecular abnormalities play a key role in driving the TCE, as previously shown in CMs, including gain of function (WNT- β catenin, MYC) and loss of function (LKB1, PTEN, p53) mutations. These abnormalities eventually result in the modulation of T cells attracting chemokines (CKs) or in the recruitment of immunosuppressive cells promoting TCE. TCE escape is also mediated by cancer cells being unresponsive or hyporesponsive to interferon gamma (IFN- γ). The role of interferons in cancer immune editing has been well established by pre-clinical mouse models. Specifically, IFN- γ is a pleiotropic molecule and represents a master regulator of tumor cell immunogenicity by controlling the expression of proteins involved in antigen processing and presentation. Moreover, IFN- γ signaling modulates the expression of the inhibitory receptors programmed cell death 1 ligand 1 (CD274/PDL1) and cytotoxic T-lymphocyte associated protein 4 (CTLA4) responsible for adaptive immune resistance to ICB. Finally, IFN- γ exposure of tumor cells exerts direct tumor suppressor functions by unleashing anti-proliferative and pro-apoptotic signals. Genetic and epigenetic abnormalities in members of the IFN- γ pathways are responsible for primary or acquired hypo- or unresponsiveness to IFN- γ responses, as well documented for CMs. Moreover, recent studies indicate that cancer stem cells are insensitive to interferons including IFN- γ and outgrowth after ICI treatment.

Taking advantage on unique SN-MM patient-derived cellular systems we explored TCE mechanisms in MMs, specifically the IFN- γ responsiveness.

All patients included in this study received surgery with curative or palliative intent for a sinonasal mucosal melanoma (SN-MM) between 2003 and 2016. Five human SN-MM cell lines, designated as SN-MM -1 to -5, previously generated from tumor biopsies and characterized by our group were used in this study. Formalin fixed and paraffin embedded (FFPE) tumor biopsies and cell-block sections were used for histology and immunohistochemistry (IHC). Image analysis of CD45 and CD8 stained sections was performed by using a Digital Microscopy approach.

Our findings on a cohort of forty-eight Sinonasal MM (SN-MM) patients indicate that MM are immune desert and lack of PD-L1 expression on tumor cells. We performed RNA-sequencing and in vitro functional assays to evaluate the cytotoxic and anti-proliferative effect of IFN- γ as well as the up-regulation of MHC-I and PD-L1. Although IFN- γ pathway is preserved in all SN-MM cell lines, SN-MM cell lines show a heterogeneous functional response to IFN- γ . Moreover, the production of T-cell attracting chemokines is maintained. RNA-sequencing analysis, flow cytometry, western blotting and ELISA assay were performed on our five SN-MM cell lines.

These findings suggest that administration of therapies promoting an appropriate T cell infiltration combined with immune checkpoint blockade therapy (e.g. anti-PD-L1) could result in a clinical benefit for poorly immunogenic SN-MM.

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Efficient and minimally perturbative production of CAR-T cells via peptide-mediated delivery of CRISPR RNPs

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Introduction

Precise genome editing of primary human T cells is enabling potent immunotherapies, as demonstrated by the recent first clinical use of *TRAC*-CAR-T cells for treating cancer. In this strategy, a chimeric antigen receptor (CAR) transgene is targeted to the T-cell receptor (TCR) alpha constant (*TRAC*) locus, thereby replacing the surface expression of the endogenous TCR with a CAR that is under the homogeneous and optimal physiological regulation of the TCR. However, the use of electroporation to deliver genome-editing reagents contributes to cell toxicity and requires expensive and, in some settings, unavailable hardware. These burdens limit the development of precisely edited immunotherapies. We have developed a technique called PERC – peptide-enabled ribonucleoprotein (RNP) delivery for CRISPR engineering – for carrying out efficient and high-yield T cell editing without the need for electroporation.

Materials and methods

We screened an array of amphiphilic peptides for the ability to deliver an RNP into primary T cells and lead to editing at the *TRAC* or *B2M* loci. The most effective peptide was A5K, which is derived from and more effective than an HA2-TAT fusion scaffold. In the editing procedure, an enzyme such as Cas9/gRNA or Cas12a/gRNA is formed, then simply mixed with an amphiphilic peptide (in molar excess), and applied to cells. The resulting gene knockouts were up to 90%.

Results

In comparison to electroporation, PERC produced higher yields of edited cells, preserved the naïve and memory phenotype, and was less perturbative to the transcriptome. We also found that PERC could be paired with an adeno-associated virus (AAV) homology-directed repair template (HDRT) to make knock-ins. PERC with AAV achieved up to 75% *TRAC*-CAR knock-in efficiency. Because PERC is minimally perturbative to cell

state, we were able to edit multiple loci in a one-at-a-time manner over time, and thereby mitigate chromosomal translocations, which did occur when RNPs targeting multiple loci were delivered at the same time. We observed 25% dual knock-in of therapeutically relevant genes at *TRAC* and *B2M*, and chromosomal translocations were not detected. *TRAC*-CAR-T cells produced using PERC had in common with electroporated cells a cytotoxic benefit over gammaretrovirally engineered CAR-T cells in targeting leukemia cells in vitro. Additionally, multiplex-edited PERC CAR-T cells demonstrated potency matching that of electroporated cells in a leukemia model in vivo.

Conclusions

The combination of PERC with an AAV HDRT enables editing that is efficient, convenient, minimally perturbative to cell state, and that can be used at multiple loci. We envision that this method could be used to reduce the cell handling steps and infrastructure required to produce precisely engineered T cells, and it could decrease barriers to establishing point-of-care manufacturing.

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A NOVEL ONCOLYTIC VIRUS IMMUNOTHERAPY OVERCOMES IMMUNE RESISTANCE AND PROLONGS SURVIVAL IN ORTHOTOPIC PANCREATIC CANCER IN MICE

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Oncolytic viruses (OVs) have recently emerged as clinically relevant therapeutic options in cancer therapy. Our engineered hybrid vector (rVSV-NDV), comprising elements of oncolytic vesicular stomatitis virus (VSV) and Newcastle disease virus (NDV), and has been shown to stimulate a systemic anti-tumor immune response via immunogenic cell death. We have previously demonstrated that rVSV-NDV treatment leads to prolonged survival and delayed tumor growth in an immune-competent B16 melanoma model in vivo with evidence of abscopal effects. We now investigate whether similar oncolytic and immune-stimulatory effects could be achieved in a more challenging orthotopic model. Pancreatic ductal adenocarcinoma (PDAC) is the most common type of pancreatic cancer and is considered one of the most aggressive forms of cancer with a relatively low five-year survival rate of 12%.

In order to establish a predictive orthotopic PDAC model in mice, a primary cell line isolated from the genetic KPC mouse model was implanted into the pancreas of eight-week-old male C57BL6/J mice by surgical intervention. After tumor engraftment was verified by MRI, rVSV-NDV or buffer control was administered intravenously. For survival analysis, mice were monitored and euthanized at humane endpoints. For kinetic analysis, tumor, liver, and spleen were analyzed via flow cytometry at various time-points post-treatment.

Treatment of PDAC mice with rVSV-NDV leads to a significantly prolonged survival in vivo. We then aimed to investigate the intratumoral and systemic immune cell signature in terms of altered frequencies and activation status in response to OV treatment. As evidence of an early immune response, on day 4 after treatment, we observed higher expression of CD69, Granzyme A, Interferon-gamma and NKG2D on NK cells and of CD69 and PD-1 on CD4+ T cells within the tumor in response to rVSV-NDV treatment, although total cell numbers remained the same. As a late immune response, on day 8 after treatment, we observed a strong increase in CD8+ T cells in the tumor which occurred concomitantly with upregulation of CD69 and PD-1. Additionally, evidence of a systemic immune response was observed. OV treatment led to upregulation of activation markers on T cells and NK cells in the spleen and liver. NK cell activation in the liver and spleen could be observed by one day one after rVSV-NDV treatment, as evidenced by a higher expression of CD69, Granzyme A, Interferon-gamma and NKG2D, as well as increased maturation. NK cells were enriched in the liver and decreased in the spleen in virus-treated mice. An early CD4+ T cell response was observed in the spleen, revealing upregulation of CD69 and PD-1, which was also present on day 8, when we additionally observed the same effect in the liver. A systemic CD8+ T cell response was detected in the spleen and liver by increased CD69 and PD-1 positivity, both on day 4 and day 8 after OV treatment, while increased cell numbers could only be observed on day 8.

In summary, these results demonstrate that oncolytic rVSV-NDV treatment leads to an immune-stimulatory effect on NK cells and T cells in a tumor-specific and systemic manner, likely contributing to the prolonged survival outcome observed in mice. As PDAC is well-characterized as an immune-suppressive malignancy that is generally resistant to immunotherapies, the ability of intravenously-applied rVSV-NDV to mediate therapeutic effects and drive a shift towards immune stimulation within the tumor microenvironment, represents an exciting finding which supports its potential development as a promising new therapeutic candidate for PDAC.

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CD28 AND ICOS DIFFERENTIALLY REGULATE PD-1+ CD8 T CELL RESPONSES

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During persistent antigen exposure, such as in chronic infection and cancer, CD8 T cells that recognize pathogens/tumors differentiate into a hypofunctional exhausted state. Exhausted CD8 T cells are a heterogeneous population: T cell factor (TCF)-1+PD-1+ progenitor exhausted cells (T_{pex}) maintain the antigen-specific T cell pool by self-renewal and differentiation into effector-like or terminally differentiated TCF-1^{neg}PD-1+ CD8 T cells (T_{ex}). T_{pex} are responsible for the proliferative burst following immunotherapy with PD-1 blockade, and data from cancer patients show that the presence of TCF-1+ CD8 T cells in tumors correlates with response to immunotherapy. Furthermore, our data in cancer patients show that T_{pex} are in close proximity to dendritic cells expressing high levels of costimulatory ligands. These data, together with our findings that CD28 costimulation was required for successful PD-1 targeted therapy, support that T_{pex} interactions with dendritic cells are critical to determine the fate of T_{pex} and reinvigoration of T cell responses. T_{pex} have high expression of CD28 and inducible T cell co-stimulator (ICOS), and here we focus on how these different costimulatory pathways shape T_{pex} self-renewal and differentiation.

In mice chronically infected with lymphocytic choriomeningitis virus (LCMV), while abrogation of CD28 signaling by blockade of B7 ligands resulted in a sharp reduction of virus-specific CD8 T cells, ICOSL blockade enhanced virus-specific CD8 T cells responses. Hence, CD28 and ICOS signaling have distinct effects on PD-1+ CD8 T cells. Using inducible genetic deletion of Cd28 or CRISPR/Cas9-mediated knockout of Icos, we interrogated the intrinsic role of these costimulatory receptors on chronically stimulated CD8 T cells.

Loss of Cd28 on virus-specific CD8 T cells in established chronic infection, resulted in reduction of both T_{pex} and T_{ex}. In contrast, Icos deletion significantly increased the pool of virus-specific CD8 T cells, inducing greater expansion of effector-like T_{ex} with improved effector function and significant changes in T_{pex} phenotype (increase in memory-associated markers). Mechanistically, we uncovered that sustained CD28 signaling during persistent antigen stimulation is required to maintain mitochondrial fitness of T_{pex}, and enhanced CD28 signaling increases glycolysis and T_{pex} differentiation into more effector-like CD8 T cells. In contrast, ICOS signaling may modulate PD-1+ CD8 T cells by altering Foxo1 activity.

Our data show that sustained CD28 signaling is required for long-term maintenance of antigen-specific PD-1+ CD8 T cells during chronic stimulation, and enhanced CD28 signaling, beyond priming, may determine effectiveness of T cell responses. Furthermore, our results demonstrate that different costimulatory pathways can have drastically opposite effects on T cells with ICOS signaling seemingly being detrimental to effector-like PD-1+ CD8 T cell differentiation during chronic antigenic

stimulation. These findings provide key insights into events that affect T_H1 biology to improve rational design of combination therapy with PD-1 blockade.

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“GET THIS CELL A GUN”: ARMING NK CELLS FOR EFFICIENT ANTI-CANCER CELL THERAPY.

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The use of NK cells as cell therapy product is a safer alternative than CAR-T cells, either unmodified or as CAR-NK cells NK cells can be used in allogeneic settings without inducing side effects. They mediate antibody-dependent cell cytotoxicity (ADCC) through CD16a/IgG interaction.

To generate a non-genetically modified NK cell therapy product, we developed a manufacturing protocol to produce highly activated expanded NK (eNK) which represent a versatile alternative to CAR engineering products.

Our technology consists of modifying the Fc part of mAbs with a so-called PinTM mutation to improve recognition by the CD16a and hence, long-term stabilization of the mAb on the NK surface. Our results show that it is possible to stably increase the selectivity of eNK by arming them with Pin-modified mAbs. Moreover, eNK multi-arming with several Pin-mAbs allows the targeting of several antigens (Ag) on tumor cell to overcome resistance and heterogeneity in tumor.

eNK are expanded from umbilical cord blood through a 3-week expansion protocol. eNK batches purity is above 95% and 70%-90% of eNK are CD16a+.

Armed-eNK are generated by 1h incubation with Pin-mAbs, followed by washing steps to remove excess of soluble mAbs.

Pin-mAbs eNK arming kinetic and function was assessed by flow cytometry.

We focused on hematological cancer and thus designed a Pin-modified mAb targeting CD20, so-called Pin-CD20.

We first showed that 70% of CD16a+ eNK armed with Pin-CD20 are still armed 72h after the arming procedure. When cocultured with CD20+ hematological cancer cell lines, Pin-CD20 eNK were able to induce higher cell death than eNK exclusively against CD20+ targets.

We next assessed eNK activation through CD16a receptor. The cell arming procedure itself wasn't able to induce CD107a degranulation, however, when co-cultured with tumor cells, Pin-CD20 eNK expressed more CD107a than eNK at cell surface, illustrating higher activation level of armed eNK cells.

Since CD16a F158V polymorphism has been described to impact the affinity of CD16a to IgG and ADCC efficiency in vitro and in the clinic, we investigated whether this could affect eNK arming. We found that CD16a V/V eNK arming is more stable after 48h than CD16a F/F eNK. To test whether eNK efficiency is impacted, we performed ADCC assays 24h or 48h after cell arming. We did not observe a genotype-dependent effect on ADCC efficiency, showing that Pin-technology could be used regardless of the CD16a polymorphism.

Next, we challenged Pin-CD20 eNK or unarmed eNK with two cancer cell lines in vivo, one CD20+ and one CD20-, in the peritoneal cavity of NSG mice. We found that Pin-CD20 eNK preferentially targeted CD20+ cells, killing fewer CD20- cells, whereas unarmed eNK similarly killed both target cell types. These results demonstrated the selectivity of armed eNK toward the mAb's Ag.

Finally, we designed another Pin-mAb targeting CD19, Pin-CD19. We armed eNK with both Pin-mAbs and showed that eNK can be armed evenly with two Pin-mAbs with a similar kinetic as mono-armed eNK. Then, we challenged mono- or double-armed eNK with lymphoma patient cells. We found that both Pin-CD20 and Pin-CD19 armed eNK exert better activity than unarmed eNK, however, double-armed eNK did not improve cytotoxicity in this setting, probably because both protein targets were expressed on lymphoma cells.

In conclusion, we propose to use tumor-specific armed eNK to fight cancer. This new Pin technology produces non-GMO NK resulting in easier regulatory and manufacturing transfer than CAR NK. Through this easy-to-use Pin technology, it is possible to modify the specificity of armed-eNK to target different cancer types. This technology has been patented “Martin Villalba, Pierre Martineau, Bruno Robert, Javier Hernandez, Jessy Presumey and Christian Jorgensen. WO2022023581A1 “Armed NK cells for universal cell therapy” and licensed to CYTEA BIO on August 04, 2020 (<https://cytea.bio/>).

P388**NECTIN-4 EXPRESSION IS ASSOCIATED WITH REDUCED SURVIVAL AND IMPAIRED IMMUNE INFILTRATION IN PANCREATIC CANCER**

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Pancreatic ductal adenocarcinoma (PDAC) has a dismal prognosis with a 5-year survival rate of 11%. Therapy advances in the last decades have been meager. Immunotherapies have revolutionized the treatment of various cancers. However, their efficacy in PDAC is extremely limited, primarily due to a highly immunosuppressive tumor microenvironment and poor immunogenicity. Nevertheless, high tumor infiltration by effector T cells and a proinflammatory, antitumorigenic immune infiltrate are associated with improved survival, suggesting an immunotherapeutic potential. The co-inhibitory immune checkpoint receptor (ICR) TIGIT has been identified as a marker of exhausted T cells, and first studies suggested the interaction with its ligand CD155 on tumor cells as a mechanism to suppress antitumor immunity. This study aimed to deepen the understanding of the TIGIT axis as a mechanism of immune evasion in PDAC. Thus, the expression of TIGIT and its related ICRs CD226 and CD96 by T cells and their ligands Nectin-1,-2,-3,-4 and CD155 by cancer cells were investigated.

CD8⁺ T cells, conventional CD4⁺ T cells (Tconv) and regulatory T cells (Treg) from blood and matched PDAC samples from 87 patients were isolated and characterized using multicolor flow cytometry. In addition, the ligand expression by tumor cells was assessed by immunohistochemistry (IHC) in 69 PDAC specimens from the same cohort, and findings were further validated with TCGA data. Additionally, soluble CD155 (sCD155) and soluble Nectin-4 (sNectin-4) serum levels were assessed in 170 PDAC patients and 40 healthy individuals.

TIGIT expression by intratumoral Tconv and Treg was significantly increased compared to peripheral blood. The expression of CD226 by CD8⁺ T cells and Treg, and CD96 by Tconv was significantly decreased. TIGIT and CD226 expression by intratumoral CD8⁺ T cells were negatively correlated ($r = -0.625$, $P = 0.003$). The TCGA and GTEx bulk transcriptome dataset revealed higher expression of all ligands in PDAC compared to normal pancreatic tissue. Interestingly, high PVR (CD155) expression was associated with good survival, while high PVRL4 (Nectin-4) expression was associated with reduced survival and reduced immune infiltration. These observations from the TCGA data were consistent with our IHC. Here, in a multivariate analysis high CD155 expression by cancer cells in PDAC was also associated with significantly increased survival (Odd's ratio (OR) = 0.41, $P = 0.019$), while high Nectin-4 expression was a risk for reduced survival (OR = 3.07, $P = 0.004$). Furthermore, CD155 expression was associated with reduced TIGIT, but increased CD226 expression by tumor-infiltrating T cells. Conversely, high Nectin-4 was associated with reduced T cell infiltration, increased TIGIT expression by intratumoral T cells and a trend toward decreased CD226 expression. Moreover, sNectin-4 was significantly increased in the serum of PDAC patients compared to age-matched healthy individuals. High sNectin-4 levels characterized patients with significantly reduced survival. While the diagnostic potential was limited (AUC = 0.704 ± 0.092), it improved the prognostic potential of the clinically used biomarker CA 19-9 upon combination (AUC(CA 19-9) = 0.885 ± 0.048), AUC(CA 19-9 + sNectin-4) = 0.939 ± 0.036).

In conclusion, while we and others characterized TIGIT as a marker of dysfunctional T cells and a potential target for immunotherapeutic approaches, this study further contributes to our understanding of TIGIT ligands in PDAC. In contrast to previous studies that only considered CD155 expression, it highlights Nectin-4 expression, but not CD155, as a marker of immunosuppressive

tumors and a potential mechanism of immune evasion. PDAC patients may benefit from targeting TIGIT and Nectin-4, and sNectin-4 may serve as an additional diagnostic biomarker.

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IN-SITU REPROGRAMMING OF CANCER CELLS TO cDC1s ELICITS COMPLETE TUMOR REGRESSION AND IMMUNE MEMORY AS MONOTHERAPY

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Dysfunctional antigen presentation mediated by MHC downregulation and low infiltration of functional professional antigen presenting cells (APCs) are critical immune evasion mechanisms underlying intrinsic resistance to immunotherapies. Among APCs, conventional type 1 dendritic cells (cDC1s) in tumors are key drivers of efficient anti-tumor immunity and associate with better prognosis. We have recently demonstrated that overexpression of transcription factors PU.1, IRF8 and BATF3 (PIB) drives cell fate reprogramming of tumor cells into induced cDC1s able to present endogenous tumor antigens and mount efficient anti-tumor immunity in immune-checkpoint inhibitor (ICI)-resistant models upon in-vivo transfer. To circumvent ex-vivo cell manipulation challenges, Asgard Therapeutics is developing AT-108, a novel immunotherapy based on de-novo recreation of cDC1s' functional properties in tumor cells by in-vivo direct reprogramming, forcing presentation of endogenous tumor neoantigens and inducing personalized anti-tumor immunity.

We validated in-vivo cDC1 reprogramming and anti-tumor efficacy upon subcutaneous implantation of ex-vivo transduced cancer cells in xenografts and ICI-resistant cDC1-dependent mouse syngeneic models: non - T-cell inflamed and low MHC B16, YUMM1.7 and BRAFV600E COX2-/- (established in cDC1-deficient host) models. We used tumor spheroids generated with cancer-associated fibroblasts and anti-inflammatory cytokines to investigate the impact of immunosuppressive microenvironment in cDC1 reprogramming. To select an optimal delivery platform to express PIB within tumors, we compared transduction and reprogramming of replication-deficient PIB-encoding lentiviral (LV), adenoviral (AdV) and adeno-associated viral vectors. Finally, we investigated in vivo efficacy of AT-108 adenoviral gene therapy product after intra-tumoral injection in combination with anti-PD-1 and anti-CTLA-4 treatment, profiled induced systemic T-cell immunity by in vitro restimulation with endogenous tumor antigens, and explored immune memory after re-challenge with parental cancer cells.

In-vivo induced cDC1s drove tumor regression in YUMM1.7 (100% complete response, CR) and BRAF (50% CR) models, suggesting that cDC1 reprogramming drives anti-tumor immunity independently on endogenous cDC1s. We estimated that <1% of iDC1s in tumors allowed CR in YUMM1.7 model, indicating that in vivo efficacy requires a low number of iDC1s. In B16, in-vivo reprogramming delayed tumor growth and synergized with anti-PD-1 allowing 40% CR, expansion of tumor-reactive CD8+ and CD4+ T-cells and abscopal effect, indicating that reprogramming reverses MHC downregulation and enhances tumor antigen presentation leading to expansion of cancer-specific T-cells. PIB-transduced human cancer cells acquired cDC1 (CLEC9A, XCR1) and antigen-presentation markers (MHC-I/II, CD40/80) in-vivo with similar efficiency and kinetics as in-vitro, and the cDC1 reprogramming process was not hindered by immunosuppressive environment. Comparison of delivery platforms revealed that Ad-PIB induced cDC1 phenotype with similar efficiency as LVs but showed superior infiltration of spheroids and in-vivo transduction of tumors, supporting the selection of AdVs for in-vivo PIB delivery. Intra-tumoral injection of AT-108 allowed in-situ phenotypic reprogramming and lead to expansion of tumor-reactive T-cells, resulting in 50% CR in B16 model. Remarkably, CR mice re-challenged with B16 cells at day 100 remained tumour-free for 60 days, suggesting that AT-108 induces long-term memory.

Our study demonstrates that cDC1 reprogramming enhances presentation of endogenous tumor antigens and overcomes immune evasion, inducing personalized anti-tumor immunity in ICI-resistant

tumors as monotherapy or in combination. We provide proof-of-concept for developing an AdV-based, off-the-shelf and personalized cancer immunotherapy based on in-situ cDC1 reprogramming.

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CHECKPOINT INHIBITORS IN A SYNGENEIC MOUSE CANCER MODEL – HOW TO MODEL ACQUIRED RESISTANCE?

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Immune checkpoint inhibitors (ICIs) have revolutionized the treatment of patients with cancer providing long-lasting tumor control. Patients initially responding to ICIs can eventually succumb to the disease due to acquired resistance. Acquired resistance differs from primary resistance, as it refers to cancer cells that at the beginning are successfully attacked by the T cells but gradually adapt to the pressure of the attack leading to resistance. Up to 60% of patients with metastatic melanoma initially benefitting from ICIs acquire resistance to the treatment. Little is known about the mechanisms involved in this type of resistance. Identifying mechanisms involved in acquired resistance is a key challenge to lowering the number of patients that acquire resistance to ICIs.

We propose a definition and model of AR using the syngeneic MC38 mouse model. Acquired resistance is defined as an initial delay in tumor growth followed by tumor progression, leading to a survival >50% longer than the median survival time for control-treated mice. A minimum of three treatments is administered with the last treatment ≤ 7 days before the terminal tumor volume. Immunocompromised and immunocompetent mice were subcutaneously inoculated in the right flank with the MC38 colon cancer cell line and treated with a combination of anti-CTLA-4 and anti-PD-1 antibodies three times the first week followed by once weekly, when tumors reached >50mm³. Tumor dimensions were measured three times weekly. Growth patterns were used to annotate response phenotype as complete regression, acquired- or primary resistance. To verify acquired resistance, tumors at maximal size >1200mm³ were excised. Tumor fragments or sorted cancer cells were transplanted into naïve immunocompetent mice. These mice were subjected to the same treatment with the ICI combination to validate the transformation of the tumor into an acquired resistance phenotype observed as primary resistance to the treatment.

Different response patterns of MC38 tumor-bearing mice were observed to the treatment across multiple experiments despite the identical genetic background of the mice and homogeneity of the cancer cell line. A total of 53% of the tumors acquired resistance to the ICIs, whereas 20% had a complete response and 27% presented with primary resistance. Tumor growth in mice transplanted with either control- or acquired resistance tumor fragments was not found to be different between ICI- and control-treated mice, in contrast to mice transplanted with tumor fragments from immunocompromised mice, where ICI-treated tumors grew slower than control-treated. In mice transplanted with acquired resistance sorted cancer cells, no difference was observed between control- and ICI-treated mice. These results indicate that the tumors had obtained acquired resistance to the ICIs. It was possible to transfer this resistance phenotype with both tumor fragments and sorted cancer cells to naïve mice, however, simply culturing the cancer cells under immunological pressure from the host allowed them to generate resistance mechanisms.

A syngeneic mouse cancer model for investigation of acquired resistance to ICIs was developed and confirmed by in vivo passaging the tumor once. Investigation of tumors with acquired resistance could reveal mechanisms involved in acquired resistance and potentially describe new targets for combinational strategies in the field of cancer immunotherapy.

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EXPLOIT NEUTROPHIL HETEROGENEITY TO INDUCE SYNTHETIC LETHALITY IN BREAST CANCER

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About 80% of breast cancers are hormone dependent. Although, anti-hormonal therapy remains a treatment mainstay, a considerable fraction of these patients does not respond to the therapy and ultimately progress with advanced cancer. Tumor evolution is one of the major mechanisms responsible for acquiring therapy-resistant and more aggressive cancer clones. Whether the tumor microenvironment through immune-mediated mechanisms might promote the development of more aggressive cancer types is crucial for identifying additional therapeutical opportunities. It has been shown that neutrophils highly infiltrate the breast cancer microenvironment, and we have recently demonstrated that these neutrophils support the growth of hormone-driven cancers. Whether tumor-infiltrating neutrophils contribute to genomic instability and may play as mediators of synthetic lethality in the tumor microenvironment of hormone-dependent breast cancer patients is unexplored and a hot topic in the field.

To achieve these goals, my team has set up innovative state of the art methodologies, used different in vivo mouse models of breast cancer and performed validation in the patients.

Recent results from my lab identified a subset of immature neutrophils named Neutrophil Progenitors (NePs) that are enriched and can proliferate in the tumors of highly proliferative hormone-dependent breast cancer patients, an aggressive tumor type characterized by poor prognosis. We demonstrated that these cells directly promote cancer genomic instability favoring the generation of therapy-resistant cancer clones. Mechanistically, we found that the oncometabolite succinate, secreted by tumor-associated NePs, impairs homologous DNA repair, promoting error-prone DNA repair through non-homologous end-joining regulated by PARP-1. Consequently, breast cancer cells acquire genomic instability, resulting in tumour progression and resistance to endocrine therapies. Selective inhibition of these pathways induces increased tumor cell kill in vitro and in vivo. Intra-tumour NePs score correlates with copy number alterations in highly proliferative hormone-dependent tumors from breast cancer patients. Having found that the NePs induce dependence of the tumor cells on alternative DNA repair mechanisms mediated by PARP, we tested and demonstrated that the presence of NePs in the tumor sensitizes the tumors to PARP inhibitors. Treatment with PARP inhibitors counteracts the pro-tumorigenic effect of these neutrophils and reverses endocrine resistance. Furthermore, upon treatment with PARP inhibitor, we found a reprogramming of the tumor microenvironment that empowers the tumor immunogenicity by reverting immune-desert breast cancers in tumors that respond to immune-checkpoint inhibitors.

Our data add novel insights on the neutrophil heterogeneity in breast cancer, describing an unexpected new function for this immune subset as cellular mediator of synthetic lethality in tumors. Our results could explain why a higher proportion of breast cancer patients may benefit from treatment with PARP inhibitors beyond those found mutated in DNA homologous recombination or associated genes. Additionally, our preclinical work provides the basis for testing novel therapeutic drug combination to improve response to endocrine therapy and checkpoint inhibitors in hormone-dependent breast cancer patients.

P392**PD-L1 AND IFN-GAMMA AS REGULATORS OF NON-SMALL CELL LUNG CANCER (NSCLC) CELL PLASTICITY AND IMPLICATIONS FOR IMMUNOTHERAPY RESISTANCE**

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NSCLC is the leading cause of cancer-related death worldwide. Although immune checkpoint inhibitors (ICIs) have demonstrated significantly improved outcomes in the treatment of NSCLC patients, some tumors show a rapid acceleration of growth during immunotherapy defined as hyperprogressive disease (HPD). We aimed to identify distinct plasticity traits in a model of HPD-NSCLC, shedding light on the mechanisms contributing to ICI resistance that involve IFN-g and PD-L1.

Primary cell cultures were established from two stage IV NSCLC samples obtained from the same patient: prior to ICI initiation (NSCLC-B, baseline) and at the time of radiological evidence of hyperprogression under ICI treatment (NSCLC-H).

The cell lines were phenotypically and molecularly characterized through immunofluorescence, Western Blotting and RNA-Seq analysis. To assess plasticity, cellular growth patterns were evaluated in vitro and in vivo. In vitro investigations, including PD-L1 knockout using CRISPR-Cas9 technology, were conducted to explore the influence of PD-L1 and IFN-g on cell plasticity. Statistical analysis was performed by Student's t-test.

Compared to NSCLC-B cells, NSCLC-H cells exhibited higher 2D-clonogenicity ($30 \pm 1\%$ vs $1 \pm 1\%$, $p < 0.001$), 3D-soft agar clonogenicity ($18.15 \pm 0.5\%$ vs $0.15 \pm 0.05\%$, $p < 0.001$), sphere formation ability (82 ± 5 vs 51 ± 2 spheres, $p < 0.05$) and ~ a 20% reduction in the proportion of cells in the G1 phase ($p < 0.05$). NSCLC-H cells also showed the ability to generate organoids. Moreover, tumor growth rate of NSCLC-H–Patient Derived Xenograft (PDX) was significantly higher than that of NSCLC-B–PDX (doubling time: 0.562 ± 0.104 vs 1.430 ± 0.154 wks., $p < 0.05$).

Phenotypical analysis of the cell lines revealed ~10-fold increase in CD44 expression and ~3-fold reduction in PD-L1 expression in NSCLC-H cells compared to NSCLC-B cells. Comparative whole transcriptomic analysis identified differential gene expression: genes known to be involved in processes such as EMT, tissue morphogenesis, response to purine-containing compounds and negative immune system regulation were upregulated in NSCLC-H cells, while genes known to be associated to epithelial cell differentiation and maintenance of cell polarity were downregulated.

The IFN-g circuit was altered in both NSCLC-B and NSCLC-H cells, as IFN-g failed to exert its antiproliferative effect on these cells in 2D-growth assays and to effectively induce PD-L1 expression. NSCLC-B cells displayed lower levels of IFNGR1 compared to NSCLC-H cells, and IFN-g treatment did not increase its expression. NSCLC-H cells exhibited greater activation of JAK1, STAT1, STAT3 (Ser727), MAPK, and IRF3 compared to NSCLC-B cells. Treatment with IFN-g resulted in activation of STAT1, STAT2 and STAT3 (Tyr705 and Ser727). Instead, treatment with anti-PD-L1 mAb atezolizumab enhanced pSTAT3 levels and reduced STAT2 activation.

Atezolizumab increased sphere production by 40% in NSCLC-B cells ($p < 0.01$), while low-dose–IFN-g treatment (1.5 U/ml) resulted in an 18% increase ($p < 0.01$). Moreover, CRISPR-Cas9-mediated inhibition of PD-L1 expression in NSCLC-B cells increased CD44 expression inducing a change in cell morphology towards NSCLC-H–like cells. Interestingly, treatment with both low and high doses of IFN-g induced a 43-61% increase in the number of NSCLC-B colonies in 3D-soft agar assays

(IFN-g 1.5 U/ml, $p < 0.01$; IFN-g 1500 U/ml, $p < 0.001$).

This study highlights the association between NSCLC cell plasticity and immunotherapy resistance. We report a modulation of plasticity by PD-L1 and IFN-g in a primary cell culture established from a treatment-naïve NSCLC patient who developed HPD. Further investigations will focus on understanding the signaling interplay between IFN-g, PD-L1 and CD44 and how the use of ICI influences these signaling pathways. This kind of investigations lays the foundation for exploring the possible detrimental role of ICIs in tumor growth, shedding light on mechanisms underlying tumor hyperprogression.

P393

TARGETING MITOCHONDRIAL VULNERABILITIES TO DRIVE INTRINSIC MELANOMA IMMUNOGENICITY

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Until recently distant metastatic melanoma was considered refractory to systemic therapy and patients with metastatic melanoma. The breakthroughs of immunotherapy have significantly improved clinical outcomes; however, more than half of patients with metastatic melanoma have poor responses when receive current immunotherapies (primary resistance). Furthermore, almost one third of patients with metastatic melanoma who have response at first eventually developed drug resistance within 3 years (acquired resistance). Hence, new strategies that enhance the efficacy of current immunotherapeutic regimens are urgently needed to improve the overall therapeutic outcome. On the other hand, mitochondrial complex I deficiency is the most common mitochondrial enzyme deficiency. Mutations can occur in either the mitochondrial or nuclear DNA. Those mutations, including Ndufs4 deficiency, lead to Leigh syndrome, a neurodegeneration disease due to neuroinflammation. While targeting mitochondrial complex I is emerging as a potent antiproliferative strategy in tumor cells, whether selective mitochondrial complex I deficiency induced inflammation could be exploited for cancer immunotherapy is largely unknown. Therefore, we determined if selective mitochondrial complex I inhibition in tumor cells causes anti-tumor immunity in mouse melanoma models.

Melanoma models were established in immunocompetent C57BL/6 mice and in T cell deficient nude mice and mitochondrial complex I was inhibited by deletion of Ndufs4 in tumor cells or treatment with mitochondrial complex I inhibitor. The tumor samples at the final time points were then subjected to analyses by proteomics, metabolomics, lipidomics, cytokine profiling and the profiling of tumor infiltrated immune cells.

Mitochondrial complex I inhibition restrained the growth of melanoma tumors in C57BL/6 mice but not in nude mice. Proteomic analyses of tumors with Ndufs4 deletion showed upregulation of MHC-I components. Assessment of tumor infiltrating lymphocytes reveals the recruitment of NKT cells and activation of CD8+ T cells. Metabolite profiling in melanomas shows a metabolic shift from choline-betaine to choline-phosphatidylcholine changes in the choline-betaine pathway. At the same time, a profound induction of cytokines was observed in the tumors with mitochondrial complex I inhibition.

Mitochondrial complex I inhibition causes a strong anti-tumor immunity in mouse melanoma models by enhancing MHC-I dependent antigen presentation and metabolomic shift based NKT/CD8+ T cells recruitment and activation. The tumor microenvironment changes caused by MHC-I mediated antigen presentation and cytokine production by NKT cell recruitment and activation might be exploited to improve the efficacy of currently used immunotherapy settings.

P394

COMPREHENSIVE SINGLE CELL SEQUENCING ANALYSIS OF PAIRED TISSUE AND BLOOD SAMPLES FROM HCC PATIENTS TREATED WITH NEOADJUVANT ANTI-PD-1 THERAPY REVEALS TREATMENT-INDUCED CLONAL T CELL DYNAMICS

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While immune checkpoint blockade (ICB) therapy has provided benefit to many cancer patients, the characteristics of anti-tumor T cell responses in non-tumor tissues and in circulation are not fully understood. Neoadjuvant ICB therapy provides a unique opportunity to deeply characterize pre- and post-therapy biomarker samples and identify features of drug activity across matched patient tissues. In a single-arm, open label phase 2 study, 21 patients with resectable (stage Ib, II, and IIIb) hepatocellular carcinoma (HCC) were treated with anti-PD-1 antibody, cemiplimab, in the neoadjuvant setting¹. Patients were treated with two cycles of cemiplimab (350 mg Q3W) prior to resection (median time to resection = 29 days), and 6 of 20 patients who successfully underwent surgical resection had evidence of pathological response to therapy (Responders).

Single cell RNA (scRNA) and T cell receptor (TCR) sequencing were performed on tumor, normal adjacent tissues (NAT), tumor draining lymph node (tdLN), and blood from 20 patients at the time of resection. Additionally, blood samples from many patients were analyzed by scRNA- and TCR-sequencing at baseline, during neoadjuvant therapy, at resection, and during adjuvant therapy. The characteristics of all tumor-expanded T cells at resection were compared across tissues and longitudinally in the blood.

We stratified patient tumors based on degree of T cell infiltration at surgery and identified several populations of PD-1high CD8+ T cells that were enriched in patient tumors compared to other tissues. Responder tumors were enriched with PD-1high Effector CD8+ T cells that expressed high levels of CXCL13 and markers of cytotoxic activity. In contrast, Non-responder tumors contained larger fractions of PD-1high terminally differentiated T cells that expressed multiple T cell dysfunction markers including KLRB1. Using TCR sequence as a fingerprint, we tracked tumor TCR clones across tissues, including longitudinal blood samples. Many of the most clonally expanded TCRs identified in Responders' resected tumors were present in baseline tumor biopsies. However, we also identified expansions of T cell clones that were not detected in baseline biopsies, suggesting that both pre-existing and T cell clones not detected in baseline biopsy expanded in tumors following anti-PD-1 therapy. We also observed significant tumor-expanded TCR sharing across the tdLN, NAT, and blood at the time of resection in patients who responded to therapy. Finally, tumor expanded TCRs were identified in the circulation across treatment timepoints and were more expanded in the circulation of Responders.

In conclusion, we identified several populations of CD8+ PD-1high T cells that were expanded in the tumors of patients who responded to cemiplimab. Both pre-existing and T cell clones not detected in baseline biopsy were expanded in the tumor at the time of resection, and many of these clones were identified in matched NAT and tdLN, particularly in Responders. Our study also suggests that tumor-expanded T cells are frequently found in the periphery, and peripheral expansion might correlate with response to neoadjuvant anti-PD-1 therapy.

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EXPLORING ASSOCIATION OF MELANOMA-SPECIFIC BCL-XL WITH TUMOR IMMUNE MICROENVIRONMENT

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Macrophages take center stage in the tumor microenvironment, a niche composed of extracellular matrix and a heterogeneous group of cells, including immune ones. They can evolve during tumor progression and acquire Tumor-Associated Macrophage (TAMs) phenotype. The release of cytokines by tumor and stromal cells, influence the secretion of cytokines by TAMs, which can guarantee tumor progression and influence the response to therapy. Among all factors able to recruit and polarize macrophages, we focused our attention on Bcl-xL, a multifaceted member of the Bcl-2 family, whose expression is deregulated in melanoma. It acts not only as a canonical pro-survival and anti-apoptotic protein, but also as a promoter of tumor progression.

Human melanoma cells silencing or overexpressing Bcl-xL protein, THP-1 monocytic cells and monocyte-derived macrophages were used in this study. Protein array and specific neutralizing antibodies were used to analyze cytokines and chemokines secreted by melanoma cells. qRT-PCR, ELISA and Western Blot analyses were used to evaluate macrophage polarization markers and protein expression levels. Transwell chambers were used to evaluate migration of THP-1 and monocyte-derived macrophages. Mouse and zebrafish models were used to evaluate the ability of melanoma cells to recruit and polarize macrophages in vivo.

We demonstrated that melanoma cells overexpressing Bcl-xL recruit macrophages at the tumor site and induce a M2-like phenotype. In addition, we identified that interleukin-8 and interleukin-1 β cytokines are involved in macrophage polarization, and the chemokine CCL5/RANTES in the macrophages recruitment at the tumor site. We also found that all these Bcl-xL-induced factors are regulated in a NF- κ B dependent manner in human and zebrafish melanoma models.

Our findings confirmed the pro-tumoral function of Bcl-xL in melanoma through its effects on macrophage phenotype.

P396**FAS-POTENTIATION IMPROVES CAR-T CELL BYSTANDER KILLING IN ANTIGEN-HETEROGENEOUS TUMORS TO PREVENT ANTIGEN ESCAPE**

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Subpopulations of tumor cells that lack targetable antigen (Ag) expression can evade T cell-mediated therapies like CAR-T and Bispecific T cell Engagers (BiTEs), frequently causing relapse in patients (Ag escape), which is an increasing problem as these therapies improve. Rather than treating Ag escape, we propose to prevent it, using geographically localized “Bystander Killing” of Ag(-) cells. We show that CAR-T cells engage Ag(+) cells, upregulate death receptor ligands such as Fas ligand (FasL), and kill nearby tumor cells, Fas-dependently and Ag-independently. Here, we demonstrate that enhanced Fas signaling, by disruption of downstream signal regulation (e.g. cIAP) or by reducing FasL shedding from CAR-T cells, can significantly improve bystander killing. Finally, we begin to describe the spatiotemporal limitations of bystander killing to inform which patients could benefit most with in vitro and intravital live imaging.

To assess bystander killing in vitro, tumor mixtures containing 2:1:1 ratios of CD19(+)Fas(+):CD19KOFas(+):CD19KOFasKO were co-cultured with aCD19 CAR-T cells +/- inhibitors of Fas/FasL regulation (e.g. IAPi, ADAM10i) and analyzed by flow cytometry. Live imaging analysis utilized segmentation and propidium iodide influx as markers of cell type and cell death. In vivo, mice were injected on either flank with mixed tumors containing ratios of Ag(+)/Ag(-)luciferase(+) cells, given intravenous adoptively-transferred CAR-T, and measured by IVIS. To test Fas-dependency, mice were given right-flank Ag(+)/Ag(-)Fas(+) and left-flank Ag(+)/Ag(-)FasKO mixed tumors. Intravital 2-photon microscopy was conducted for 2-4hr; analysis identified caspase-3 cleaved cells following CAR-T engagement.

Using α CD19 CAR-T killing assays, we observed that cIAP1/2 inhibition, potentiates Fas-mediated bystander killing dose-dependently. Notably, IAP antagonism increased CAR-T bystander killing by 50% in Fas(+) but not FasKO cells, correlating with increased FasL expression on T cells. We also observed that ADAM10 inhibition reduces FasL cleavage, leading to a 25% increase in bystander killing. In vivo, we injected mice with mixed Ag(+)/Ag(-) tumors on either flank: right-sided tumors contained Ag(+)/Ag(-) luciferase-expressing (Ag(-)Luc(+)) cells, while left-sided tumors contained Ag(-)Luc(+)FasKO cells. Remarkably, dual flank tumor-bearing mice showed early regressions of both tumors after CAR-T therapy, but later relapsing tumors were mostly FasKO. Inhibition of cIAP1/2 did not improve the early Fas-dependent bystander killing. Nevertheless, dual-flank experiments recapitulated the geographic localization of bystander killing: mice bearing Ag(+)/Ag(-)Luc(+) tumors and contralateral Ag(-)/Ag(-)Luc(-) tumors showed clearance of Ag(-) tumor cells only in the spatial context of Ag(+) cells. We utilized live microscopy imaging to capture bystander killing events over 48hr after co-culture. Utilizing a three-cell system, we were able to track: 1) on-target killing events, 2) bystander killing events, 3) non-specific death, and T cell motility. We observe that, on average, bystander killing takes place 280 minutes after on-target killing. Intravital 2-photon microscopy also showed caspase-3 mediated death in a mixed Ag(+)/Ag(-) tumor but not in a Ag(-) only tumor.

Inhibition of cIAP1/2 improved CAR-T bystander killing by increasing Fas signaling and FasL expression; reduced FasL cleavage by ADAM10 inhibition enhanced bystander killing. In vivo studies remarkably show Fas-independent early bystander killing, but prolonged Fas-dependent tumor control in a geographically localized fashion. Further studies with live microscopy demonstrate a robust model to study the spatiotemporal limitations of bystander killing. Overall, we demonstrate that Fas-potentiating therapies, which increase signaling in tumor cells or reduce FasL cleavage on

T cells, can be combined with CAR-T therapy to mitigate Ag escape in heterogeneous tumor populations.

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IN VIVO GENOME-WIDE CRISPR SCREENS IDENTIFY TARGETS PROTECTING ALLOGENEIC T-CELLS FROM NK AND T-CELL MEDIATED REJECTION

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Allogeneic CAR T-cells derived from healthy donors have many potential benefits over autologous therapies, allowing the immediate access to standardized batches of T cells, improving their efficacy and decreasing the time for manufacture. However, resistance to allogeneic rejection is mandatory for the wide-spread translation of efficient and affordable T-cell therapies.

In the present study, we interrogated in a systematic and unbiased manner the genes enhancing allogeneic T cell survival in vivo using a genome-scale CRISPR knockout screen.

Among 18400 tested genes, we identified the MHC-class I component $\beta 2$ microglobulin ($\beta 2m$) and the cell death receptor Fas (Tnfrsf6) as major targets allowing transferred T cells to survive in fully immunocompetent MHC-mismatched mice. Unlike $\beta 2m$, we demonstrated that Fas-inactivation is preventing both NK-cell and T-cell mediated host immune rejection. We further assessed the translational relevance of the discovery in human CD19 CAR T-cells, which exhibit a markedly improved antitumor functionality, while limiting host recognition and clearance of infused allogeneic CAR T-cells in vivo.

Finally, using base editing technology, we simultaneously silenced the endogenous TCR, FAS, and the suppressor of cytokine signaling 1 (SOCS1) and demonstrated that multiplex modified CAR T-cells show a better killing activity in vitro, suggesting the potential for an off-the-shelf, persistent and polyfunctional allogeneic T-cell product.

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CHOOSING A HUMANIZED MOUSE MODEL FOR PRECLINICAL ASSESSMENT OF ADOPTIVE T CELL TRANSFER WHILE AVOIDING GRAFT-VERSUS-HOST DISEASE

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Humanized mouse models are important tools for preclinical testing of efficacy and toxicity of adoptive T cell transfer (ACT) strategies in cancer treatment. However, it remains a major challenge to identify models that efficiently support human T cell engraftment while limiting graft-versus-host disease (GVHD). The immunodeficient non-obese diabetic (NOD)/severe combined immune deficient (SCID)/common gamma chain knock-out (IL-2R γ null) (NOG) mouse strain has revolutionized human cell engraftment. To further improve engraftment and delay the onset of GVHD, several genetic alterations have been introduced on the NOG background. Among these is transgenic overexpression of the human interleukin-2 (IL-2) gene (hIL-2-NOG), which supports in vivo T cell survival and expansion, and a beta-2-microglobulin (B2M) knock-out (B2M-NOG), which delays the onset of GVHD and extends the window for therapeutic response.

In the present study, we investigated T cell engraftment and GVHD development in NOG, hIL-2-NOG and B2M-NOG mice following ACT with fresh and cryopreserved ex vivo-expanded T cells.

hIL-2-NOG mice developed GVHD following ACT with fresh ex vivo-expanded T cells in a dose-dependent manner, with symptom onset as early as day 17 following infusion with the highest dose of T cells (1.5×10^6). The onset of GVHD was markedly delayed in hIL-2-NOG mice by cryopreserving the T cell product prior to infusion. Cryopreservation increases expression of a selection of exhaustion markers in ex vivo-expanded T cells, when comparing to the fresh product. NOG and B2M-NOG mice did not develop GVHD within the duration of the experiment (Day 0 - 102) and did not seem to support T cell engraftment in the absence of inoculated tumor target cells.

Here, we found that hIL-2-NOG, unlike NOG and B2M-NOG, mice support engraftment of ex vivo-expanded T cells, although this was associated with development of GVHD in a dose-dependent manner following ACT. Cryopreservation of ex vivo-expanded T cells could markedly delay the onset of GVHD, possibly due to its association with T cell exhaustion.

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TUMOUR MITOCHONDRIAL DNA MUTATIONS ENHANCE AEROBIC GLYCOLYSIS TO DRIVE CHECKPOINT BLOCKADE

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Mitochondria are essential metabolic organelles, containing a source of genetic information required for their function, mitochondrial DNA (mtDNA). Mutations in mtDNA are highly prevalent in tumours, with sequencing studies reporting >50% of tumours bear somatic mtDNA mutations (Kim and Mahmood, 2022). However, functional interrogation of mtDNA mutant tumour biology is lacking due to historically limited capacity to manipulate the genome.

To address this, we adapted novel mitochondrial base editing tools (Mok et al., 2020) to model highly recurrent truncating mutations in mitochondrial complex I within murine models of melanoma.

In vitro, mutation-dependent loss of capacity to oxidize NADH by complex I enabled a Warburg-like metabolic shift where cells exhibited increased glycolytic flux with high lactate output, confirming that mtDNA mutation exerts a metabolic phenotype in cancer cells. We next studied their tumour biology in vivo, discovering that metabolic changes seen in mutant tumours altered the composition of the tumour immune microenvironment. Mutant tumours exhibited up-regulation of pro-inflammatory pathways, with no impacts on tumour growth. Importantly, mutant tumours demonstrated a significant decrease of tumour-resident neutrophils, a key suppressor of the host immune response to tumours. This diminished immunosuppression in mutant tumours sensitised them to immune checkpoint blockade (ICB) in a mutation dose-dependent manner. Human clinical trial data reinforced these observations where patients with >50% mutant heteroplasmy cancers also demonstrated a >2.5 fold enhanced response to ICB (Riaz et al., 2017). Curiously, tumours expressing cytoLbNOX (Titov et al., 2016), a cytoplasmic NADH oxidase, showed the strongest response to treatment. These cells exhibit an altered redox state, but with increased NAD⁺:NADH rather than decreased, as seen in complex I mutant cells, suggesting that alterations in redox state are sufficient to sensitise tumours to ICB (Mahmood et al., 2023). Furthermore, we found this sensitisation could be manipulated where increasing tumour-resident neutrophils in mutant or cytoLbNOX tumours decreased sensitisation to ICB, suggesting a role for neutrophils in mediating response.

Taken together, these data provide mechanistic understanding of the effects highly recurrent mtDNA mutations exert in tumour biology, demonstrating potential to stratify ICB response and generate novel therapeutic approaches in oncology.

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PROFILING THE IMMUNE LANDSCAPE AND THE IMPACT OF PRE-SURGICAL THERAPY IN CRC-LIVER METASTASES BY HIGH DIMENSIONAL FLOW CYTOMETRY

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Colorectal cancer (CRC) is the third most common cancer worldwide and up to 50% of patients develop metastatic disease to the liver (CRC-LM). Pre-surgical administration of cytotoxic chemotherapy with either anti-EGFR mAbs or VEGF-targeting agents enables the reduction of tumor load and the risk of local relapse. Chemotherapies can stimulate anti-tumor immunity, either by initiating the release of immunostimulatory molecules from dying cancer cells or by mediating off-target effects on immune cell populations. Moreover, anti-angiogenic therapies have been shown to reinstate intratumor T cell transmigration and effector differentiation, while reprogramming immunostimulatory phenotype in tumor-associated macrophages (TAMs). Whether and how these different pre-surgical regimes specifically reshape the immune tumor microenvironment (iTME) of CRC-LM, resulting in modifications that may have also prognostic impact, remain poorly understood.

Distal normal liver, peritumor and tumor were prospectively collected from CRC-LM patients undergoing surgery with or without pre-surgical therapy. Single cell suspensions from each tissue section were stained with two high dimensional mAb panels to dissect the CRC-LM iTME by flow cytometry. A 26 color-based mAb panel detects functional state of both conventional and unconventional T cells, while a second 21 color-based mAb panel detects myeloid derived suppressor cells, dendritic cells and monocytic-macrophagic populations. Therapy-induced modifications of the immune landscape were assessed by comparing patients that were not pre-treated before surgery (No CT), with patients that were pre-treated within 4 months (T<4 months) before surgery with only chemotherapy (CT) or with chemotherapy in combination with treatments targeting either angiogenesis (CT + a-VEGF) or EGFR (CT + a-EGFR).

At steady state (No CT), unsupervised clustering reveals a preferential tumoral location of CD4+ Tregs and CD4+ $\gamma\delta$ T and CD4+ MAIT cells with reg-like phenotypes, as well as CD8+ T, $\gamma\delta$ T and MAIT cells sharing a tissue resident and highly exhausted phenotype (CD103+CD69+PD-1+CD39highLAG-3-). By contrast, less exhausted CD4+ TFH, CD69+ effector memory and CXCR5+ CD8+ T and $\gamma\delta$ T cells preferentially localize in the peritumoral area. Pre-surgical CT+a-VEGF selectively reshapes the intra tumor T cell landscape. Specifically, total intra-tumor T cell number diminishes with a significant drop of conventional CD4+ Treg cells. Unsupervised clustering shows that CT + a-VEGF modifies the phenotype of intratumor CD8+ T cells, resulting in T cell rejuvenation consisting in a reduction of tissue resident highly exhausted CD103+CD69+PD-1+CD39highLAG-3- population, and increase of non-tissue resident memory precursor CD69+ and less exhausted LAG3high CD39low PD-1- cells, the latter particularly expanded in a subgroup of patients. These CT + a-VEGF modifications are shared between conventional and unconventional cells. Finally, preliminary results regarding the myeloid compartment define at steady state (No CT) an intratumor enrichment of M2-like CD163+CD206+HLA-DRIlow/neg TAMs, which are significantly reduced by pre-surgical CT + a-VEGF.

Our results show that the CRC-LM microenvironment is dominated by a state of immunosuppression and functional exhaustion shared by both conventional and unconventional T cells, together with

M2-like TAM population. Pre-surgical CT + a-VEGF substantially reshapes the metastatic immune landscape, relieving T cell and myeloid immunosuppression and T cell exhaustion, while promoting the increase of more functional subsets in both conventional and unconventional compartments. These flow cytometry data are currently being integrated with gene expression and TCR gene profiling, spatially multiplexing tissue analysis and clinical data of the patients to define the pathways active in the hepatic metastatic microenvironment that may be implicated in the local immune surveillance and clinical outcome.

P401

ROLE OF DIACYLGLYCEROL KINASES IN ACUTE MYELOID LEUKEMIA

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Acute myeloid leukemia (AML) is an aggressive hematological disease accounting for approximately 1% of all cancers, being one of the most common types of leukemia in adults, especially in older people (>75 years). Several possible approaches have nowadays been adopted to overcome this pathology, but the emergence of chemoresistance and high relapse rates highlight the urgency of identifying additional drug targets. Diacylglycerol kinases (DGKs) are a family of enzymes which catalyze the phosphorylation of diacylglycerol in phosphatidic acid, regulating several cellular pathways. Ten mammalian DGK isozymes have been identified in the human genome, where multiple isoforms are co-expressed simultaneously. Their involvement in both cell transformation and immunosurveillance against tumors is reported in the literature, making them an intriguing target for AML treatment. In this study in particular, this aim was pursued by conducting a detailed analysis of expression databases and examining the in vitro effect of DGK inhibitors on leukemia cell lines.

A deep bioinformatic analysis was performed in order to assess DGK expression in AML patients and subsequently to explore the possible correlation between their expression and the survival of AML patients in different datasets, i.e. TCGA, BeatAML, and TARGET. Then, to identify genes positively and negatively correlated with DGK isoform expression, a query on the BeatAML database from CBioPortal for DGK co-expressed genes was carried out. Furthermore, preliminary tests were performed on AML models, namely HL-60 promyelocytic leukemia cells and HEL erythroleukemia cells comparing the results with those obtained with peripheral blood lymphocytes (PBLs) isolated from healthy human buffy coats of anonymous donors: i) the expression of the five principal isoforms of DGK was assessed by quantitative real-time PCR; ii) cell viability upon the treatment with different doses of different DGK inhibitors was evaluated with different assays, finally analyzing their effect on the cell cycle by flow cytometry.

Several DGKs were found to be overexpressed in AML patients, in particular DGKA, DGKD and DGKG, even if slight differences were noted between different datasets, pointing out the challenge to identify a suitable “healthy reference tissue” for a disease like AML. In addition, the correlation between DGK expression and overall survival of AML patients in the TCGA database showed a decreased survival in patients with high DGKA levels, while both BeatAML and TARGET databases indicated that an high DGKH expression is associated with a shorter survival. Moreover, the analysis of the co-expression of genes associated with each isoform highlighted isoform-specific functionalities for DGKs, even if there were shared functions related to the control of cell signaling pathways involved in vesicular trafficking and possibly differentiation. Furthermore, the therapeutic efficacy of DGK targeting was verified, observing an increased mortality after the administration of broad spectrum DGK inhibitors (R59022 and R59949) compared to the DGKA specific ones (CU-3 and AMB639752), as expected, with an arrest in the S phase, pointing out the importance of DGKs in AML cell proliferation and survival. Additional tests with Ritanserin (a serotonin antagonist as R59022 and R59949 specifically inhibiting DGKA) and specific serotonin receptor inhibitors allowed to refine the mechanism under the observed cytotoxic effect of the broad spectrum inhibitors.

These results highlighted DGKs potentiality as therapeutic target for AML, paving the way for further investigations on this direction. On going experiments are exploring cell responses after the transient

silencing of different DGK isoforms to better understand the molecular mechanism and define new molecular targets for AML therapy.

P402

IDENTIFICATION OF MULTICELLULAR IMMUNE HUBS IN TUMOR SPATIAL GENOMIC DATA

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During cancer progression, tumor cells interact with immune cell types and form tumor immune microenvironments (TIMEs). The interactions in these TIMEs affect tumor proliferation, metastasis, and clinical outcomes. To develop effective strategies for cancer prognosis and treatment, it is crucial to understand how immune cells are spatially organized to regulate tumor progression and respond to therapeutic interventions. Previous studies discovered important immune hubs of interacting cell types based on expression covariation from spatially-unaware single-cell RNA sequencing data. Recent advancement in spatial transcriptomics (ST) techniques such as multiplexed error-robust FISH (MERFISH) and Visium enable direct spatially-resolved quantification of many genes. These techniques prompt the development of computational methods to study TIMEs.

Topic models are powerful statistical methods used to identify latent patterns in data. Topic models have been used for cell-type deconvolution by considering cells as samples and genes as features. Spatial-LDA, developed recently, modeled multicellular microenvironment by treating multicellular environments as samples and genes as features. Spatial-LDA requires specification of the numbers of topics, and was only applied to protein-based image data with fewer features compared to those in spatial transcriptomic (ST) data. In this study, we developed HubIDP, a hierarchical Dirichlet process (HDP)-based approach to systematically identify immune hubs in ST data without the need to specify the numbers of topics.

We applied HubIDP on four MERFISH and Visium datasets from human colorectal tumor samples. After raw data from the two ST platforms were preprocessed, the resulting cell-by-gene count matrices and the spatial coordinates of each cell were used as the inputs to the model. HubIDP iteratively estimates the topic proportions for each cell and the distribution over genes for each topic. For each cell, HubIDP assigns topic weights based on its transcriptomic profile, distance to its neighboring cells, and inferred topic proportions of neighboring cells.

Signal pooling from neighboring cells and tying nearby cells with prior distributions are two popular strategies to handle signal sharing between neighboring cells in ST data analysis. However, we note that pooling strategies removed substantial complexity in existing cell type heterogeneity and oversimplified the results. We also compared these methods to our results.

We estimated topics in the four colorectal tumor ST datasets and visualized results through the topic proportions across cells. HubIDP successfully selected biologically-relevant topics based on our assessment of the most highly weighted genes in the topics identified by HubIDP, e.g. topics associated with specific immune or metabolic environments. By assessing the distribution of the activity scores, we found that many of the topics were spatially organized. Some but not all of these topics overlapped with previously identified multicellular immune hubs. In particular, we found hubs characterized by genes associated with anti-tumor responses that formed focal structures within the tumor, and hubs characterized by genes related to inflammatory reactions that were enriched at the luminal surface of the tumors in areas of epithelial damage.

We developed HubIDP, a statistical method to model and select multicellular tumor-immune cell hubs in spatial genomic data. HubIDP identifies immune hubs that consist of biologically-relevant cellular and gene-gene interactions in ST datasets from colorectal tumor samples. We show that applications of HubIDP to high-throughput tumor image data identify known TIMEs and identify novel characteristics of TIMEs.

P403

PD1 AND TIM3 EXPRESSION AS PREDICTOR OF EARLY HCC RECURRENCE AFTER PERCUTANEOUS THERMAL ABLATION

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Percutaneous thermal ablation is a cornerstone in the management of early hepatocellular carcinoma (HCC), but intrahepatic distant recurrence occurs up to 80% at 5 years. Combined approaches with immunotherapies are being studied to prevent recurrence. We evaluated the expression of immune checkpoint molecules (ICM) on intrahepatic and peripheral lymphocytes and investigated the association with early intrahepatic distant recurrence after local ablation.

This monocentric study was conducted between 2018 and 2023. After percutaneous thermal ablation for HCC, patients were followed and later divided into two groups, a “very early recurrence” group in case of intrahepatic distant recurrence within 12 months after percutaneous thermal ablation, and a “prolonged recurrence-free” group in case of no recurrence before 12 months of follow-up. Freshly harvested intratumoral and non-tumoral liver tissue biopsies and circulating blood samples were obtained before percutaneous thermal ablation and were explored with multiparametric flow cytometry.

We observed that the frequency of PD1+CD4+ T cells was higher in the very early recurrence group compared with the prolonged recurrence-free group in circulating blood ($p < 0.0001$), in the non-tumoral liver ($p = 0.0004$), as well as in the tumor ($p = 0.0140$). Similarly, the frequency of Tim3+CD8+ T cells was significantly higher in the early recurrence group in peripheral blood ($p = 0.0040$), in the non-tumoral liver ($p = 0.0012$) and in the tumor ($p = 0.0237$).

This study highlights the importance of the baseline systemic immunosuppressive status for tumor recurrence. The expression of immune checkpoint molecules on T cells, such as PD1 and TIM3 (mainly in the blood and in the non-tumoral liver) identifies HCC patients at risk of very early intrahepatic distant recurrence after percutaneous thermal ablation and gives a rationale for evaluating anti-PD-1 and/or anti-TIM3 to prevent tumor recurrence in pre-selected HCC patients.

P404

EOMES+ TR1-LIKE CELLS SUPPRESS THE CYTOTOXIC ANTI-TUMOR T-CELL RESPONSES VIA IL-10

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Understanding regulatory mechanisms, which limit tumor destruction by the adaptive immune system, has been proven to be crucial for obtaining unprecedented therapeutic response. In this respect, the abrogation of the immunosuppressive function of FoxP3+ T regulatory cells (Tregs) has been of particular relevance. A second key population of immunosuppressive T cells in cancer are EOMES+ type 1 regulatory T (Tr1) cells, which secrete the anti-inflammatory cytokine IL-10. Notably, IL-10 has a dual role in cancer, since it acts as a checkpoint receptor agonist upon cytotoxic T cell priming, but may also directly stimulate established anti-tumor CD8+ T cell responses. EOMES+ Tr1-like cells are enriched and clonally expanded in human tumors, suppress CD8+ T cell responses and are associated with poor survival of cancer patients. In melanoma patients treated with anti-PD1 antibodies, high levels of an EOMES+ Tr1-like cell gene signature was associated with responsiveness to immunotherapy. Consistently, human EOMES+ Tr1-like cells failed to efficiently suppress antigen-experienced CD8+ T cells in the presence of anti-PD1 antibodies in vitro.

At day 0, C57Bl/6 mice were subcutaneously engrafted with B16-OVA melanoma cells.

Mice were treated with 100 ug of anti-PDL1 antibody starting from day 7, every 3 days for 3 times. Lymphocytes from B16 OVA tumors were purified and stained for flow cytometry analysis.

Here, we investigated the molecular mechanisms through which EOMES+ Tr1-like cells regulate anti-tumor T cell responses in vivo in C57Bl/6 mice subcutaneously engrafted with B16-OVA melanoma cells. IL-10 producing EOMES+Tr1-cells were enriched also in this tumor model. Adoptive transfer experiments with IL-10-deficient FOXP3-CD4+ T cells unveiled a critical role of Tr1-derived IL-10 in inhibiting cytotoxic anti-tumor T cell responses. Moreover, reduced tumor growth induced by blocking PD-1/PD-1L interaction was associated with reduced IL-10 production by EOMES+ Tr1-like cells. Finally, preliminary data suggest that EOMES+ Tr1-like cells, but not FOXP3+ Tregs, responded to the model tumor neoantigen OVA.

Collectively, these findings suggest a key detrimental role of IL-10 producing EOMES+ Tr1 cells within the cytotoxic immune response against tumor antigens.

P405

EXPLORING THE PHENOTYPIC AND FUNCTIONAL DIVERSITY OF G-NK CELLS IN PERIPHERAL AND UMBILICAL CORD BLOOD

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NK cells play a crucial role in innate immunity, contributing significantly to immune surveillance and antitumor defenses. Recent research has unveiled adaptive characteristics in NK cells. FcR γ -deficient NK cells (g-NK cells), also known as adaptive NK cells, have garnered considerable attention due to their heightened responsiveness through the CD16a pathway and association with human cytomegalovirus (HCMV) infection. In contrast to conventional NK cells (c-NK cells), the g-NK subset in peripheral blood (PB) exhibits high levels of cytokine secretion, including IFN- γ and TNF- α , upon activation of the CD16a pathway. Remarkably, g-NK cells combined with daratumumab exhibit significant tumor reduction and enhanced durability in a murine model of multiple myeloma (MM). Additionally, g-NK cells efficiently infiltrate solid tumors, such as non-small cell lung cancer (NSCLC) and colorectal cancer (CRC), though their precise role in these tumors remains unclear. These findings underscore the potential of g-NK cells as promising immunotherapeutic agents. However, research has predominantly focused on g-NK cells derived from PB, leaving g-NK cells in umbilical cord blood (UCB) largely unexplored. This study aims to comprehensively investigate the distribution, phenotype, and functional characteristics of g-NK cells in both PB and UCB.

PB and UCB were collected from healthy individuals as the object of the study. Flow cytometry was utilized to analyze the phenotype and cytokine secretion of NK cells from different sources. NK cells were isolated and expanded from PB and UCB samples. The CD16a signaling pathway was activated by immobilized 3G8 antibodies (Abs) or Fc segments from specific mAbs binding to tumor cells with high antigenic expression. Flow-based killing assay and MTT assay were employed to assess target cell viability and cytotoxicity mediated by NK cells. Additionally, NK cell expansion and functional assays were conducted using HCMV and plasma from donors with a g-NK positive phenotype.

Our study identified a unique subset of NK cells in UCB, termed UCB-g-NK, that lacked FcR γ expression. While UCB-g-NK cells showed some similarities with PB-g-NK cells in phenotype, they responded differently to CD16a pathway activation. UCB-g-NK cells exhibited a classic adaptive NK cell phenotype with FcR γ -NKG2C+NKG2A- expression and minimal CD57 expression. In contrast, UCB-g-NK cells did not show stronger cytokine secretion than c-NK cells upon CD16a pathway activation, unlike PB-g-NK cells. Additionally, both PB-g-NK and UCB-g-NK cells demonstrated responsiveness to HCMV and plasma from g-NK-positive individuals. The *in vitro* expansion of NK (eNK) cells demonstrated promising potential for adoptive transfer therapy. The robust cytokine secretion observed in PB-derived NK cells was inherited from their parental NK cells. Notably, the expanded PB-g-NK (PB eg-NK) cells still exhibited superior CD16 pathway responsiveness compared to the expanded PB-c-NK (PB ec-NK) cells. Furthermore, UCB-derived NK cells demonstrated potent cytotoxicity, benefiting from the training provided by the acquired expansion system.

This study emphasizes the critical influence of origin and microenvironment on the functional differentiation, phenotypic diversity, and functional heterogeneity of NK cells. A comprehensive understanding of c-NK and g-NK cells in both PB and UCB holds great significance for harnessing their therapeutic potential and advancing adoptive therapy strategies in diverse clinical contexts.

P406

ELUCIDATING THE MECHANISM OF ACTION OF IL-5 AS A PROMOTER OF IFN EXPRESSION BY MAST CELLS IN THE TUMOR MICROENVIRONMENT

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Compelling evidence indicates that the efficacy of tumor targeted therapies relies on tightly regulated crosstalk between the adaptive and innate immunity of the host. Mast cells are innate immune sentinel cells resident in most tissues and readily identified by tryptase containing granules, expression of CD117 (c-kit) and high affinity IgE receptors. Although their role in allergic reactions is well established, the physiological and beneficial roles of mast cells in cancer have been recently recognized. In response to IgE/antigen mediated activation, mast cells release an array of acute (tryptase, histamine, leukotrienes, prostaglandins) and delayed (cytokines: TNF, IL-1 β , IL-6, IL-13 GM-CSF, and chemokines CCL2, CCL3, CCL4, CCL5, and CXCL8) inflammatory mediators. Notably, mast cells are an important source of interferons (IFNs), crucial mediators of inflammatory responses. Through a range of signaling molecules, we and others have shown that mast cells can activate the endothelium and promote recruitment of immune effector cells such as cytotoxic T cells and neutrophils. Importantly, through production of type I IFN, mast cells can recruit and activate NK cells enhancing their tumor killing capacity. We have recently discovered that the Th2 cytokine interleukin-5 (IL-5) primes human mast cells to produce significantly more IFN in response to oncolytic viral infection compared to mast cells not exposed to IL-5. In the current study we investigate the impact of IL-5 treatment on mast cell biology, on their transcriptional landscape and on IFN production.

Human umbilical cord blood-derived mast cells (CBMCs) were cultured for 42h with or without 10 ng/ml of human IL-5. Subsequently, CBMCs were infected with oncolytic reovirus, respiratory syncytial virus or transfected with poly(I:C), a synthetic analogue of dsRNA that mimics viral infection. Production of IFNs and relevant downstream targets was measured by qPCR, ELISA or Luminex Assay. Similar experiments were performed under IL-3 and GM-CSF treatment (cytokines that belong to the same family of IL-5). Expression levels of IL-5 receptor complex (IL-5R) during viral challenge and treatment with IL-5, IL-3 and GM-CSF were measured by qPCR. Total RNA-sequencing (RNA-seq) was performed on CBMCs (from 8 donors) following 42h of IL-5 priming. The ability of IL-5 to directly enhance transcription and protein production of specific genes was assessed by qPCR, western blot and ELISA. Further experiments to assess the effect of IL-5 treatment on mast cell survival, proliferation and mediators production were performed.

IL-5 pre-treatment of mast cells enhanced gene expression and secretion of type I and III IFNs (such as IFNA2, IFNB1, IFNL1) following oncolytic viral infection, without altering the degree of viral infection. Interferon-stimulated genes such as CXCL10 were also upregulated. IL-3 and GM-CSF, that share part of the IL-5R complex signaling pathway, were able to prime mast cells in a similar manner to IL-5. To investigate the effect of IL-5 on the transcriptional landscape of mast cells, we performed RNA-seq. The genes upregulated by IL-5 include those involved in cell survival and inflammatory response, which offer the potential to (i) dissect the novel IL-5/IFN signaling axis we identified and (ii) determine IL-5's role in NK cell recruitment and activation at the tumor site.

IL-5 pre-treatment of mast cells selectively regulated the expression of multiple genes including pro-survival factors. An environment with abundant IL-5 seems to improve mast cell overall fitness and survival, enhancing their ability to produce mediators such as IFNs. Results from our study shed light on the antitumorigenic properties of mast cells and their therapeutic potential to improve current immunotherapy approaches.

P407

QUALITY AND QUANTITY: WHAT WE NEED TO PREDICT CANCER NEOANTIGENS

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Enhancing neoantigen-specific T-cell responses against tumours has emerged as an effective strategy in the treatment of solid malignancies. Efficient and accurate selection of immunogenic neoantigens is a critical determinant of therapeutic success, however the number of possible neoantigens identified in a sequenced tumour sample often exceeds the limits of current treatment modalities. Computational approaches to neoantigen ranking are used widely in the field; however, current public datasets have low quantities of high-quality immunogenicity data for training and benchmarking, which limits modelling approaches. This makes generalising to new datasets problematic.

In this study, we demonstrate unbiased benchmarking results comparing 6 published peptide-MHC (pMHC) based immunogenicity models and 3 TCR-specificity based immunogenicity models on previously unseen data. Along with this, we demonstrate our new hybrid model which considers pMHC and TCR-sequencing inputs while being trained to predict neoantigen immunogenicity in unseen T-cell epitopes. Our new model utilises the transformer architecture along with transfer learning and broad negative data generation to improve on performance. Negative data is engineered to sample from a broad set of possible negative scenarios, rather than any singular approach. Negatives derived from immunogenicity screening, wild type peptides and background TCRs are combined to provide a wider negative distribution for model training.

We found high variability in the performance of previously published models on unseen T-cell epitopes. All 3 published TCR models tested did not generalise to unseen epitopes and would be incompatible with current neoantigen immunogenicity prediction strategies. pMHC prediction models demonstrated variable performance, with our model achieving improved performance in both ROC AUC and average precision: AUC=0.844, AP=0.18. These results show that while we have achieved an improvement in sensitivity and precision at this task, high precision was not found in any model tested. Analysis of the publicly available datasets used to train and test these models shows skews in the data affect the performance of individual models. Epitope-bias in screening assays used for benchmarking is also shown to cause differential performance between the models tested. Additionally, how negative data is generated is shown to produce advantageous performance in our new model.

This study underscores the lack of high-quality immunogenicity datasets available to train robust models, and highlights a crucial need for independent model benchmarking. Whilst including additional features and engineering more complex models alone may yield marginal improvements, these are unlikely to overcome the data shortage. Thus we demonstrate a clear need for publicly available data of higher quality and quantity to drive meaningful progress in this important field.

P408

TARGETING MITOCHONDRIAL BIOGENESIS AND ANTIOXIDANT PATHWAYS TO COMBAT T CELL EXHAUSTION

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T cells are major targets of cancer immunotherapy because of their critical role in killing cancer cells. However, due to chronic antigenic stimulation in the tumor microenvironment, T cells often acquire an exhausted phenotype that is characterized by loss of self-renewal capacity, decreased production of cytotoxic effector molecules, upregulation of inhibitory immunoreceptors, and distinct metabolic, transcriptional and epigenetic alterations. Thus, finding ways to alleviate this could greatly improve the efficacy of cancer immunotherapies. T cell activation is intrinsically linked to mitochondrial function, as T cells undergo programmed changes in mitochondrial metabolism, dynamics and biogenesis to support the transitions from naïve to effector to memory fates. Furthermore, reduced mitochondrial biogenesis and increased oxidative stress, caused by generation of mitochondrial reactive oxygen species (ROS), are major drivers of T cell exhaustion. Mitohormesis, or adaptive preconditioning to acute stress, has been shown to promote longevity and confer a number of other beneficial physiological effects, including enhanced mitochondrial biogenesis and antioxidant capacity.

Using a unique mouse model of mitochondrial oxidative mitohormesis we developed, we are investigating if increased mitochondrial biogenesis and a heightened global antioxidant state will help to alleviate T cell exhaustion. This model is based on an inducible and reversible knock down of mitochondrial superoxide dismutase (iSOD2 mice) to transiently increase mitochondrial superoxide stress. We showed previously that adult liver from adult mice that experience this form of mitochondrial stress only during embryogenesis exhibit a mitohormetic response including increased mitochondrial mass, lower ROS production and basal upregulation of cellular antioxidant capacity, driven by NRF2 and PGC-1 α . Similar mitohormetic responses were observed in iSOD2 mouse embryonic fibroblasts (MEFs) that underwent transient SOD2 knockdown and recovery in vitro. In parallel, we are also examining if mice that have permanently enhanced mitochondrial antioxidant capacity assuage T cell exhaustion.

We crossed the iSOD2 mice to P14 TCR transgenic mice and confirmed that SOD2 can be successfully knocked down in CD8⁺ T cells using this approach. Preliminary results indicate that these iSOD2 CD8⁺ T cells exhibit enhanced TCR activation and alterations in cytokine production.

We will next determine if adoptive transfer of these mitohormetically adapted cells (and cells with permanently enhanced mitochondrial antioxidant capacity) will help alleviate T cell exhaustion in infection and cancer models. These studies will increase our knowledge of the role of mitochondria and ROS in T cell activation and exhaustion and possibly pave the way toward using mitohormetic approaches to improve tumor immune responses and immunotherapy.

P409

MYELOID CELLS INTERACT WITH ANTIGEN-SPECIFIC ADOPTIVELY TRANSFERRED T-CELLS TO SUPPORT CYTOTOXICITY.

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In recent years, cell-based targeted immunotherapies such as Chimeric Antigen Receptor T-cells have revolutionized cancer therapeutics, especially against hematologic neoplasms. Nevertheless, these treatments have several drawbacks such as short lifespans due to exhaustion and high toxicity due to cytokine storm. Furthermore, although direct CD8+ cytotoxicity via perforin and granzyme is the main mechanism of action, the role of antigen specific CD4+ T-cells and other immune cells in the global antitumoral response is controversial. All these considerations can be elucidated through a better understanding of the complex interactions that occur among the engineered T-cells, the tumor cells, and the endogenous lymphoid, myeloid, and stromal cells that compose the TME.

Labeling Immune Partnerships by SorTagging Intercellular Contacts (LIPSTIC) and universal LIPSTIC (uLIPSTIC) are recently developed enzymatic labeling methods based on the ability of a genetically encoded Sortase A (SrtA) to covalently bind an LPXTG motif-containing substrate to an N-terminal pentaglycine tag (G5). In LIPSTIC, a known ligand and receptor of interest are genetically fused to either a SrtA (donor cell) or G5 (receptor cell). Upon administration of a biotinylated or fluorescent SrtA substrate (LPETG), SrtA transfers the substrate to G5 if an interaction occurred, which is revealed by flow cytometry analysis. The difference between the two is that LIPSTIC is restricted to a specific ligand and receptor of interest, whereas uLIPSTIC is a proximity-based approach that can record the history of interaction between all kinds of cells even without prior knowledge of the interacting molecules simply by driving very high expression levels of SrtA and G5 in opposing cell membranes, which would trigger the LIPSTIC mechanism if they come in close proximity to each other.

In this project we are using LIPSTIC technologies to understand how immune interactions elicited upon cellular immunotherapies shape the antitumoral response. To do so, a uLIPSTIC or LIPSTIC murine host is inoculated with OVA-expressing lymphoma cells and treated with ex vivo activated OTI or OTII cells from a uLIPSTIC CD4-Cre donor. A Biotin-LPETG Sortase A substrate is injected peritumorally and intratumorally at different time points after the adoptive cell transfer and the tumor and draining lymph nodes are harvested and dissociated for flow cytometry analysis.

After testing different approaches, we have established that a hybrid approach (a CD40-G5-LIPSTIC host with a uLIPSTIC SrtA donor) is the best strategy to visualize interactions in this model. Furthermore, we have optimized the protocol for ex vivo activation of donor T-cells and established robust gating strategies for the lymphoid and myeloid compartments of the tumor microenvironment. Our preliminary results indicate that 72 hours after ACT, transferred T-cells interact with different components of the myeloid compartment (Classical Dendritic Cells, Macrophages, and Ly6C high Monocytes), which is in line with the fact that all these populations express CD40, which is the receptor of interest in our LIPSTIC cassette. These results suggest that myeloid cells might contribute to the global antitumoral response of cellular immunotherapies. So far, no interactions have been detected within the lymphoid compartment. Further analyses are needed to sustain this claim. It is worth mentioning that through this approach we are unable to distinguish between tumor cells and endogenous immune cells since the tumor itself consists of CD45+ lymphoblasts. Furthermore, because our timepoints are so restricted, tracking tumor growth through caliper measurement is suboptimal. To address these issues, we are currently working on transducing a

non-immunogenic reporter protein (NGFR) and firefly luciferase into our cell line.

OT-I cells interact with components of the myeloid compartment to support cytotoxicity.

P410

MV130 AS A NEW TRAINED IMMUNITY-BASED CANCER IMMUNOTHERAPY

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Trained immunity (TI), known as innate immune memory, is described as the ability of innate cells of responding to an homologous or heterologous secondary challenge with an enhanced proinflammatory response upon the exposure to certain TI-inducing stimuli. The most studied TI-inducers are the BCG vaccine and the fungal β -Glucan. However, BCG and β -Glucan are of difficult clinical application. MV130 is a mucosal immunotherapy based on heat-inactivated bacteria indicated for recurrent respiratory infections. MV130 was demonstrated to induce TI in pre-clinical models against respiratory viruses and SARS-Cov-2 infections. However, the role of MV130 in conferring cancer protection is still under investigation.

C57BL/6J mice were intranasally immunized with 3 doses per week, for two weeks, with MV130. After at least 7 days of resting, mice were subcutaneously challenged with Lewis Lung Adenocarcinoma cells (LLCs) in order to study the short-term (7 days) and long-term (60 days) ability of MV130 to protect against tumor. Innate lung immune populations were rechallenged with the prototypical stimulus LPS and LLCs supernatant in order to identify the innate populations trained by MV130 that act with a boosted proinflammatory response.

We observed that when C57BL/6J mice are subjected to the prophylactic regimen of six doses of intranasally administered MV130, they exhibited delayed tumor development and improved tumor control against subcutaneously injected LLCs. The improved tumor response is maintained when tumor cells are injected 60 days after the last MV130 administration, indicating that MV130 induces a long-lasting memory, which is a main feature of TI. Upon ex vivo restimulation of lung innate populations with LPS or tumor cells-derived supernatant, we observe that alveolar macrophages and neutrophils present an enhanced proinflammatory phenotype (TNF production).

In conclusion, our preliminary data indicate that MV130 induces TI in innate immune cells by potentiating a proinflammatory antitumor response, paving the way for the induction of innate memory as a novel cancer immunotherapy strategy.

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TARGETING THE EXTRACELLULAR MATRIX TO IMPROVE CAR-T CELL THERAPY AGAINST SOLID TUMORS

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Adoptive cell therapy (ACT) with T lymphocytes expressing Chimeric Antigen Receptors (CAR-T) has emerged as a promising immunotherapy to treat hematological malignancies. However, CAR-T cell therapy in solid tumors remains challenging, mainly because the immunosuppressive tumor microenvironment (TME) limits proper T cells functionality. In particular, the tumor extracellular matrix (ECM), which is rich in collagen and hyaluronic acid (HA), blocks the infiltration of immune cells. Hence, we aim to improve the efficacy of CAR-T cells in those solid tumors characterized by a dense ECM and mostly unresponsive to ACT, such as prostate and pancreatic cancer. In this study, we engineered T cells to co-express the CAR molecule and either a collagenase (MMP8 and MMP14) or a hyaluronidase (PH20), in order to dismantle the tumor ECM and therefore to improve the CAR-T cells' infiltration and invasion. Since the ECM actively regulates the immune cell motility and functionality, its disruption during CAR-T cell therapy could alter the interactions between the effector and immune cells of the TME. Therefore, we aim to gain a deep understanding of the immune cells' interactions affected by ECM remodeling.

First, we cloned the ECM-degrading enzyme downstream of the human prostate-specific membrane antigen (PSMA) CAR transgene into a clinically relevant lentiviral vector. Thus, we transduced either Jurkat (PSMA CAR.E-J cells) or primary T cells (PSMA CAR.E-T cells) with a MOI of 5 and 30, respectively. The co-expression of transgenes was assessed by flow cytometry and Western blotting analysis. The PSMA CAR.E-T cells' cytotoxic activity was evaluated by in vitro Calcein-AM assay. Lastly, we assessed the enzyme functionality by in vitro assays. The PH20 activity was evaluated by particle exclusion assay, which allows us to measure the pericellular HA deposition by PC3 cells and its subsequent degradation upon PSMA CAR.PH20-J cells co-culture. Meanwhile, the MMP14 activity was assessed by Transwell cells invasion assay in Matrigel, followed by z-stack 3D confocal imaging analysis of PSMA CAR.MMP14-J cells migrating through the Matrigel layer. To investigate the effects on the TME induced by the ECM remodeling fully immunocompetent mice will be used to model the ECM-rich pancreatic cancer and its immunosuppressive TME. Murine Mesothelin (mMSLN) CAR-T cells expressing either MMPs or PH20 will be produced, and finally whole transcriptomic analysis at single cell level will be performed on tumors undergoing therapy.

Two days following transduction, we observed a high percentage of Jurkat cells positive for both PSMA-CAR (85.7%), and PH20 (39%) or MMP14 (74.3%) molecules, as confirmed by Western blotting analysis. On day 9 after transduction, PSMA CAR.E-T cells co-cultured with PSMA-positive PC3-PiP cells showed higher cytotoxicity ($34.5 \pm 3.00\%$, 1:25 T:E ratio), when compared to the PSMA CAR.E-T cells co-cultured with PSMA-negative PC3 cells ($12.36 \pm 1.37\%$, 1:25 T:E ratio). Lastly, preliminary results showed a remarkable difference in the infiltration capacity of PSMA CAR.MMP14-J cells (230 μm in depth) when compared to non-transduced Jurkat cells (60 μm in depth). Concerning the mMSLN CAR-T cells production, we successfully optimized a protocol to retrovirally transduce murine CD8⁺ T cells.

Here, we present a promising strategy to enhance CAR-T cells' therapeutic efficacy in solid tumors. Indeed, we observed that the PSMA CAR.E-J cells are endowed with high invasion capacity thanks to the efficient expression of ECM-degrading enzymes. In parallel, a deep characterization of the TME during the therapy in a fully preserved immune system will entangle unknown immune cells relationship, allowing for a better tumor therapy design.

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TARGETING TOLL-LIKE RECEPTOR 2 AND THE CYSTINE-GLUTAMATE ANTIPORTER XCT FOR ENHANCED IMMUNOTHERAPY IN PRECLINICAL MODELS OF BREAST CANCER

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Despite significant progress in its treatment, breast cancer remains the second leading cause of cancer death in women, due to relapses and metastases. Therefore, the development of combination therapies able to target key cancer-inducing or cell-sustaining pathways is needed. We have previously demonstrated that breast cancer cells express Toll-Like Receptor (TLR)2, which plays a key role in mammary cancer stem cell (CSC) self-renewal and promotes tumor growth through both tumor cell-intrinsic mechanisms and the suppression of T regulatory cells. Notably, TLR2 binds HMGB1 and other danger-associated molecular patterns actively secreted by CSCs or released during chemotherapy-induced cell death, activating NF-κB-dependent pro-tumorigenic signaling pathways. The intratumoral administration of TLR2 inhibitors effectively reduced immunosuppressive cells and synergized with chemotherapy in hindering tumor growth in preclinical models of mammary cancer.

The aim of this study was to further investigate the mechanisms underlying TLR2 pro-tumorigenic activity, and assess whether its targeting may potentiate immunotherapy by reducing immunosuppression. In particular, we focused on the cross-talk between TLR2 and the cystine-glutamate antiporter xCT, a CSC oncoantigen against which we have developed several vaccines that prevent metastasis in breast cancer models.

The effects exerted by TLR2 activation on xCT expression and function were analyzed in vitro in mouse and human breast cancer cell lines in which TLR2 was either activated with endogenous or bacteria-derived ligands or silenced. xCT expression was also analyzed in a mouse model of HER2/neu-driven mammary carcinogenesis on a TLR2 WT or KO background (TLR2WT-neuT and TLR2KO-neuT mice), and on cell lines derived from these tumors. The efficacy of the combined inhibition of TLR2 and xCT, in association or not with chemotherapy, was tested in vitro on these cell lines. In vivo, 4T1 tumor-bearing BALB/c mice were treated intratumorally with TLR2 inhibitors, in combination with doxorubicin and Bovine Herpes virus (BoHV)-4-based vaccines targeting xCT, and tumor growth and the immune response were monitored.

We observed that TLR2 activation upregulated xCT in breast cancer cells, while its silencing or deletion decreased xCT both in vitro and in the neuT mice. Since xCT regulates the intracellular redox balance, TLR2 downregulation enhanced intracellular reactive oxygen species. TLR2 and xCT inhibitors synergized in hindering breast cancer cell viability and inducing their apoptosis, and these results were further increased by their association with doxorubicin. In vivo experiments demonstrated that TLR2 inhibitors significantly decelerated mammary tumor growth and reduced lung metastases. Their combination with chemotherapy and xCT-targeting vaccines further improved these effects, augmenting the immune response activated by xCT vaccination.

In summary, we demonstrated that TLR2 plays a critical role in promoting breast cancer progression and that its activation upregulates xCT expression in breast cancer cells, providing protection against oxidative stress. We propose that combining TLR2 inhibitors with xCT inhibition or immunotargeting, along with chemotherapy, could offer more effective combination therapies for breast cancer. Furthermore, our findings raise concerns about the use of TLR2 agonists as adjuvants in anticancer immunotherapy.

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A NOVEL ANTI-PD-L1/PD-L2 ANTIBODY IN COMBINATION WITH A POTENT AGONIST OF STIMULATOR GENES (STING) INDUCES A ROBUST SYSTEMIC ANTITUMOR EFFICACY

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Immunotherapy with immune checkpoint inhibitors (ICIs) has changed the treatment landscape of many tumors. However, response rate remains relatively low in most cases, and in particular in tumors with a low T cell infiltration. Thus, combination with therapies able to revert this immune excluded phenotype may ameliorate ICI efficacy. Activation of stimulator of interferon genes (STING) has shown great potential to enhance antitumor immunity. Several synthetic STING agonists have been tested preclinically and in the clinic. However, these molecules are susceptible to enzymatic degradation leading to low bioavailability in target tissues, unwanted toxicities, and narrow therapeutic windows. We have previously reported a novel STING agonist, ISAC-8803, with marked anti-cancer activity in mouse tumor models and in canine companion animals with spontaneous glioblastoma. Here, we tested the ability of ISAC-8803 to increase the efficacy of an antibody against both PD-L1 and PD-L2 with effector function (IMGS-27907), diminishing the immune suppression in immune excluded tumors.

The activation of the components of the STING pathway was analyzed in cells treated with ISAC-8803 in vitro by Western blotting and using reporter cell lines. The anti-cancer activity of ISAC-8803 was tested in vivo, alone and in combination with IMGS-27907, in mouse models of melanoma (B16-PDL2, expressing PD-L1 and PD-L2) and mammary adenocarcinoma (TS/A, expressing low PD-L1 and no PD-L2). Mice with established tumors were treated with ISAC-8803 intratumorally at 10 µg/dose twice (days 11 and 14 for B16F10-PDL2 and days 23 and 26 for TS/A), and with IMGS-27907 intraperitoneally at 10 mg/kg twice a week for 3 weeks, starting with the first ISAC-8803 treatment. Tumor growth was monitored, and some tumors were removed and analyzed via H&E, IHC and FACS to assess overall necrosis, T cell infiltration and activation, and macrophage content.

ISAC-8803 more potently activated the human and mouse STING pathways (STING, TBK1, IRF3 and NF-κB) relative to clinical benchmarks. In vivo, the combination of ISAC-8803 with IMGS-27907 resulted in 70% overall survival in B16F10-PDL2 tumor bearing mice, compared to ≤10% in monotherapy and control groups, and significant extension of survival in the TS/A model. In the TS/A model, the combination therapy resulted in a large necrotic area compared to the respective control and individual treatments. CD3+ and CD8+ cells were more numerous in the tumors treated with the combination, while macrophages showed a decrease in comparison with the controls.

In conclusion, we demonstrated that ISAC-8803 is a potent STING agonist that, when used in conjunction with a novel anti-PD-L1/PD-L2 monoclonal antibody, induces curative responses in checkpoint-refractory tumor models. This better tumor control is associated with an increase of T cells and decrease of macrophages at the tumor site, suggesting that delivery of STING agonists intratumorally can potentiate the systemic activity of a novel checkpoint inhibitor.

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ROLE OF CXCR4 IN TRIGGERING IMMUNE SURVEILLANCE OF THE MALIGNANT MESOTHELIOMA TUMOR ENVIRONMENT

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High-mobility group box 1 (HMGB1) is a nuclear protein acting as a Damage Associated Molecular Pattern and alarmin when released by cells undergoing stress and/or death. HMGB1 is released into the extracellular space, forms a complex with CXCL12 and binds its receptor CXCR4, creating an HMGB1-CXCL12-CXCR4 axis. This axis is upregulated on the surface of tumor cells, promotes tumor progression, and correlates with a poor prognosis. Moreover, CXCR4 alone appears as monomers, dimers, and nanoclusters on the cell surface and CXCR4 cluster size affects receptor dynamics and function. Recently, we discovered an anti-tumor immunosurveillance mechanism, ImmunoGenic Surrender (IGS), which is induced by the N-terminus of HMGB1 (BoxA; aa 1-82) or CXCL12. This mechanism involves the engagement with CXCR4 of the “don’t-eat-me” signal CD47, its removal from the tumor cell surface and the triggering of tumor cell phagocytosis by macrophages. However, since BoxA cannot currently be used for therapy, novel drugs which promote IGS need to be discovered. Moreover, the full extent of how CXCR4 clustering modulates receptor function/s in IGS is not fully understood. Therefore, we aimed at: 1) unravelling the mechanism(s) by which CXCR4 promotes anti-tumor immunity, beginning with CXCR4 clustering, and 2) test a candidate drug for Immunogenic Surrender, called Diflunisal (DFL), which interferes with the heterocomplex formed by HMGB1 and CXCL12.

To determine the therapeutic effect of DFL in vivo, BALB/c mice were inoculated with Malignant Mesothelioma (MM) cells and treated or not with increasing concentrations of DFL. Tumor progression was monitored using BioLuminescence imaging. Proximity ligation assays (PLA) were utilized to determine complex formation between pairs of proteins and dimerization or multimerization of single proteins. We tested the effects of various drugs in in vitro phagocytosis assays, where MM cells were co-cultured with macrophages. CRISPR/Cas9 technology was utilized to selectively knock-out CXCR4 from MM cells. *Cxcr4*^{-/-} cells were transfected with fluorescently tagged wild-type and mutant CXCR4 and tested for the effect on CD47 surface expression of either blocking CXCR4 internalization or dimerization.

Quantitation of PLA images confirms previous NMR studies indicating that DFL breaks the HMGB1•CXCL12 complex on the surface of MM cells. At a high concentration DFL suppresses tumor growth in vivo and promotes survival in comparison to vehicle treated control mice. DFL also reduced: 1) the clustering of CXCR4, 2) the interaction between CXCR4 and CD47, and 3) the overall expression of CD47 on the surface of MM cells, as determined by PLA. This translated to an increase in phagocytic activity by macrophages when co-cultured with DFL-treated MM cells. To show that clustering of CXCR4 is crucial for reducing surface CD47 expression we used various cell lines or drugs that either reduce CXCR4 cluster size, prevent its dimerization or prevent its internalization.

We show that DFL, an already FDA approved drug, promotes crucial processes of Immunogenic Surrender: in vitro it reduces CXCR4 clustering and CD47 expression on the cell surface, and increases phagocytic activity by macrophages; In vivo it promotes survival. Moreover, we highlight CXCR4 clustering (as opposed to expression level) as an essential regulator of this immunosurveillance mechanism in the tumor milieu, which should be the focus of future studies when using CXCR4 as a therapeutic target.

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PROTEOMIC ANALYSIS IDENTIFIES ASSOCIATION OF PERIOSTIN, A MATRICELLULAR PROTEIN, WITH HIGH TUMOR STROMA AND IMMUNE EXCLUSION IN TRIPLE NEGATIVE BREAST CANCER

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Triple-negative breast cancer (TNBC) has been historically challenging to treat due to limited therapeutic options. Immunotherapy has emerged as a promising alternative for TNBC treatment. However, within the immune subtypes (inflamed, excluded, desert) of tumors, excluded/desert tumors exhibit diminished effectiveness of immunotherapy compared to inflamed tumors. One of the factors contributing to immune evasion in tumors is the tumor stroma produced by cancer-associated fibroblasts (CAFs). In this study, we aimed to identify overexpressed proteins in tumors with high tumor-stroma ratio (TSR, amount of tumor-associated stroma) among the immune excluded TNBC using proteomics analysis.

A total of 403 TNBC patients who received neoadjuvant chemotherapy (NAC) were classified into immune subtypes based on the Klintrup criteria and tumor-infiltrating lymphocytes (TILs) using pre-NAC needle biopsy samples. The evaluation of stroma was assessed using the TSR, with a threshold of 50% used to classify tumors as either high stroma or low stroma. Proteomic analysis was performed using the formalin-fixed paraffin-embedded blocks of pre-NAC needle biopsy samples. We analyzed differentially expressed proteins between inflamed and excluded tumors. We also compared high and low stroma tumors within the excluded tumor. We investigated the correlation between level of identified protein expression and other clinicopathologic features. To validate expression of identified protein, immunohistochemical (IHC) staining and single cell transcriptomic analysis using public data were conducted. Survival analysis was performed.

After removal of cases that failed quality control and had low protein diversity, a total of 247 samples with inflamed or excluded immune subtype constituted the final cohort for analysis. Excluded tumors had 32 upregulated proteins compared to inflamed tumors and those were associated with extracellular matrix-related pathways. Within the excluded tumors, periostin was the only extracellular matrix-related protein that showed significantly higher expression in high stroma tumors. The expression of periostin demonstrated a positive correlation with TSR (%) ($r=0.51$, $p < .001$) and a negative correlation with TILs ($r=-0.30$, $p < .001$). IHC staining showed that periostin was expressed in the tumor stroma not in tumor cells. Through the analysis of single cell data, a distinct subtype of CAFs specifically expressing periostin was identified. High periostin expression had unfavorable recur-free survival in univariate Cox analysis (hazard ratio 1.422, 95% confidence interval 1.115-1.814, $p=.005$).

Periostin, a matricellular protein, was highly expressed in immune excluded subtype with high stroma in TNBC. IHC staining and single cell analysis revealed that periostin was expressed in CAFs. Moreover, high periostin expression was associated with poor prognosis. Consequently, the development of targeted agents against periostin+ CAFs to inhibit immune evasion of TNBC may improve the effectiveness of immunotherapy.

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CMTM6 REGULATES PROTEIN EXPRESSION OF CD58 AND PD-L1 AND ANTITUMOR T CELL RESPONSE

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The immune response is intricately regulated by coinhibitory and costimulatory immune checkpoints to eliminate foreign invaders and abnormal cells while minimizing collateral damage. Dysregulated expression of immune checkpoints is exploited by cancer to evade immune destruction. Therapeutics that block inhibitory immune checkpoints, such as PD-L1, form the foundation of current cancer immunotherapy. However, the lack of costimulatory signals can render these therapies ineffective. Substantial evidence has shown that the expression of a costimulatory molecule, CD58, is crucially required in the antitumor immune response. Nonetheless, the underlying mechanisms that regulate CD58 expression remain unclear.

FACS-assisted functional genetic screening and comparative proteomics were employed to identify regulators of CD58 and proteins influenced by CMTM6. Co-immunoprecipitation experiments were conducted to assess protein-protein interactions. Protein labeling and tracking were carried out to assess the stability of CD58. In vitro human primary T cell-tumor cell coculture models and an in vivo xenograft mouse tumor model were used to evaluate antitumor T cell response. Finally, immunohistochemistry (IHC) analysis of 88 melanoma samples and 102 colon cancer samples was performed to determine CMTM6 and CD58 expression in human cancer. Moreover, the association between clinical responses of melanoma patients to anti-PD-1 or anti-PD-1+anti-CTLA-4 treatment and the expression levels of CMTM6 and CD58 were evaluated.

Through comparative proteomic analysis and a large-scale haploid genetic screen, we found that CMTM6, a transmembrane protein that we and others previously identified as a positive regulator of PD-L1, plays a significant role in maintaining CD58 expression. Genetic disruption of CMTM6 led to reduced expression of both immune checkpoint ligands, while reconstitution restored their expression. These effects were validated in various cancer cell types as well as in primary dendritic cells.

Mechanistically, we found that CMTM6 maintains CD58 protein stability by protecting it from lysosome-mediated degradation. Inhibition of lysosome activity decreases the rate of surface CD58 degradation in CMTM6-deficient cells to a level comparable to that observed in CMTM6-proficient cells. Co-immunoprecipitation experiments demonstrated interactions between CMTM6 and both CD58 and PD-L1. These and previous results support a model where CMTM6 accompanies the immune checkpoint ligands and maintain their expression on cell membrane.

Notably, through in vitro and in vivo experiments assessing the antigen-specific primary T cell-tumor cell interactions, we discovered the following: 1) while the residual CD58 and PD-L1 at the surface of CMTM6-deficient tumor cells continue to actively modulate T cell responses, indicating a role of CMTM6 in fine-tuning the immune response, 2) the loss of CD58 upon CMTM6 deficiency significantly dampens antitumor T cell responses.

Furthermore, to assess the potential clinical significance of CMTM6 and CD58 expression, we performed IHC analysis on tumor samples from melanoma and colon cancer patients. The analysis revealed widespread expression of CMTM6 and CD58 in tumor cells, with a significant positive correlation between their expression levels. Additionally, we analyzed tumor biopsies obtained from melanoma patients who were treatment-naive for anti-PD-1 and later received either anti-PD-1 or anti-PD-1+anti-CTLA4 therapy. The results showed a significant association between higher levels of CMTM6 or CD58 expression and a favorable response to immune checkpoint therapies.

In summary, our study i) provides fundamental insights into CD58 regulation, ii) identifies CMTM6 as a shared regulator and interaction partner of the CD58 and PD-L1, and iii) underscores the importance of CMTM6-mediated CD58 regulation in antitumor T cell response and its potential impact on T cell-based immunotherapies.

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THE MARINE CAROTENOID DIATOXANTHIN DISPLAYS ANTI-INFLAMMATORY, IMMUNOMODULATORY, CHEMO-PREVENTIVE AND CARDIO-PROTECTIVE PROPERTIES IN VITRO, AND BY RNA-SEQ ANALYSIS.

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Microalgae represent one of the most promising eco-sustainable sources of natural bioactive pigments, very attractive for human health. Diatoxanthin (diato), a xanthophyll produced by diatoms, showed several functional activities with beneficial potential (such as chemo-preventive, anti-inflammatory, antioxidant, photoprotective, immune-modulatory).

The anthracycline doxorubicin (doxo) is still used in breast cancer (BC) chemotherapy, however it can cause dose-dependent cardiotoxicity, mainly by oxidative stress. We propose that diato can both exert anti-cancer activities, while not being cardiotoxic, and that it could not increase doxo toxicity on cardiomyocytes.

The effect of diato was investigated in vitro, by MTT and in combination with doxo by 3D-spheroid assays, on the MDA-MB-231 triple negative BC (TNBC) cells. The ability of diato to modulate secretome was evaluated in vitro on the Human Umbilical-Vein Endothelial cell line (HUVEC) by angiogenesis arrays. Gene expression analysis was performed by qPCR, measuring different inflammation and angiogenesis gene expression in cells treated with diato. RNA-Seq transcriptome analysis were performed to verify wheatear diato regulate pathways involved in TNBC progression and in endothelial cell inflammation.

We observed that diato reduces MDA-MB-231 cell proliferation. Furthermore, we found that diato treatment in combination with doxo inhibits TNBC spheroid growth, reduces their 3D-integrity and decreases their viability. Diato, also, shown a down-regulation of inflammation and angiogenesis associated genes, as angiostatin, IL2, IL4 and PECAM-1 in antibody array and TGFβ1, TGFβ2, TIMP1 and TIMP2 gene in qPCR analysis on TNFα stimulated HUVEC.

Finally, RNA-Seq analysis demonstrated that diato was able to reduce several biological processes involved in inflammation and tumour development and metastasis. In particular, in TNBC cells diato down-regulated ANGPT1, GPR37, ANG, QRICH2, MUC1 genes involved in angiogenesis process; EPHA7 that was found upregulated in MDA-MB-231. Moreover, following diato treatment, IL-6R and IL-7 are found to be down-regulated. Instead, in TNFα stimulated HUVEC, we observed a down-regulation of angiogenin, a powerful inducer of angiogenesis, and HSPA12A and EDNRB involved in regulation of macrophage cytokine production.

Taken together, our data suggest promising chemo-preventive and angiopreventive activities of diato in TNBC therapy. Microalgae derived drugs can be a potential and relevant source of novel nutraceutical and preventive compounds, which can be considered as adjuvant in cancer prevention treatments approaches.

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MOLECULAR MECHANISMS OF PD1-INDUCED SHP2 ACTIVITY IN T CELLS

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The inhibitory receptor PD1 reduces T cell activation by recruiting and activating SHP2 tyrosine phosphatase. PD1 blockade using antibodies restores T cell responses against tumors, and is one of the most successful immunotherapy strategies. However, the efficacy of antibody therapies is often limited due to inadequate pharmacokinetics and tissue penetrance. Small molecules targeting PD1/SHP2 axis could overcome these limitations and become alternatives to current antibody-based immunotherapies. Development of such novel therapeutic strategies is critically dependent on understanding PD1 inhibitory pathway. Thus, we aim to discover novel molecular mechanisms that regulate SHP2 activity downstream of PD1.

SHP2 is tyrosine phosphorylated at its C-tail upon recruitment to PD1, but the functional consequences of this post-translational modification are unknown. We used a combination of biochemistry and imaging approaches to determine the roles of specific tyrosine phosphorylation sites in regulation of SHP2 activity.

Using in vitro phosphatase assays, we have determined the roles of specific tyrosine phosphorylation sites in regulation of SHP2 activity. Phosphorylation of SHP2 C-tail induced its catalytic activity in the absence of receptor binding. However, in the presence of the phosphorylated PD1, SHP2 C-tail phosphorylation decreases the activation through PD1, most likely by reducing the SHP2 binding to PD1. We investigated in vitro SHP2 binding kinetics to the phosphorylated cytosolic domain of PD1 using biolayer interferometry (BLI). SHP2 phosphorylation decreases its binding affinity to PD1, with a critical contribution of C-tail tyrosine 542. We analyzed PD-1/SHP2 interaction in a human T cell line using fluorescence recovery after bleaching (FRAP). This analysis showed that phosphorylation of SHP2 controls its PD1 binding kinetics, with C-tail phosphorylation-deficient SHP2 mutants showing increased PD1 exchange rates.

Our results provide evidence for the role of SHP2 C-tail phosphorylation in regulation of SHP2 catalytic activity and PD1 binding.

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AN OLIVE MILL WASTEWATER POLYPHENOL RICH EXTRACT EXHIBIT ANTI-INFLAMMATORY IMMUNOMODULATORY PROPERTIES ON CANCER CELLS ACTING ON CYTOKINE AND CHEMOKINE PATHWAYS

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Several diet derived compounds, like extra virgin olive oil, have been reported to exert anti-oxidant, anti-proliferative, anti-angiogenic and pro-apoptotic effects in a variety of cancers. Olive oil has been used for health purposes since antiquity. The presence of different phenolic molecules, including hydroxytyrosol, has been demonstrated to prevent the occurrence of a variety of pathological processes, including cancer. The strong antioxidant potential of these molecules has been extensively investigated, we focussed on their anti-inflammatory, immune-modulating, anti-angiogenic and cancer chemopreventive properties. We have investigated an extract from olive mill wastewaters (OMWWs), named A009, which represents a waste product from the olive oil industry, on endothelial cells and several tumour cell lines, both in vitro and in vivo.

The ability of A009 to affect cell proliferation and survival has been evaluated on human umbilical endothelial vein cells (HUVECs), numerous different tumour cell lines, MCF-7 and MDA-MB-231 (breast), HT-29 and HCT-116 (colon) and the murine CT26 CRC cells, PC-3 and DU-145 (prostate) and lung (A549 and H1650) cancer cells, by MTT and crystal violet assays. The induction of apoptosis and reactive oxygen species (ROS) were assessed. By using morphogenesis and Boyden chamber assays, functional studies evaluated the capacity of OMWWs to interfere with endothelial cell tube formation, migration and invasion. Finally, the inhibition of angiogenesis and tumour cell growth was investigated in vivo, by the matrigel sponge assay and tumour xenograft.

The A009 extract was able to inhibit the growth of both HUVECs and tumour cell lines, exerting a stronger inhibitory effect as compared to the pure hydroxytyrosol alone. This effect was directly associated with the induction of apoptosis. The CXCR4/CXCL12 axis receptors, which are involved in cell migration and invasion, pro-angiogenic factors (VEGF, CXCL8, CCL2), and a number of immune-modulating molecules were the main pathways that the A009 extracts modulated in endothelial cells.

Furthermore, several molecular targets, including IL8, Angiogenin, mTOR, VEGF, CXCR4, CXCL12 and CXCL8, were inhibited by A009 in several cancer cells.

Conclusions: Our results suggest that the polyphenol-enriched extracts from the olive oil processing (OMWWs) A009, show promising angio-preventive, immune-modulatory, anti-inflammatory and chemo-preventive potentials. The richness of the extract results in a stronger anti-angiogenic and anti-tumor properties compared to hydroxytyrosol alone, making it a promising nutraceutical product.

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P420

AN IMMUNOLOGICAL SYNAPSE FORMATION BETWEEN T REGULATORY CELLS AND CANCER ASSOCIATED FIBROBLASTS PROMOTES TUMOR DEVELOPMENT

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Cancer-associated fibroblasts (CAFs) have emerged as a dominant non-hematopoietic cell population in the tumor microenvironment (TME), serving diverse functions in tumor progression, invasion, matrix remodeling and resistance to therapy. Extensive molecular characterization revealed an increased heterogeneity in the CAF compartment and proposed an interaction between CAFs and tumor-infiltrating immune cells, which may shape tumor immune evasion. However, the precise mechanisms via which CAFs imprint on anti-tumor immunity remain poorly understood.

Mouse models that allow depletion of α SMA⁺ CAFs were used for assessing tumor development. In vitro and in vivo assays for the establishment of the crosstalk of α SMA⁺ CAFs with Treg cells were employed. Conditional knock-out animals in which autophagy ablated specifically in α SMA⁺ CAFs were also used. Tumor growth experiments, proteomic and transcriptomic analysis and immune checkpoint inhibitor immunotherapy protocols were also performed.

Herein, we describe a synapse formation between alpha smooth muscle actin (α -SMA) expressing CAFs and T regulatory cells (Tregs) in the TME. Specifically, Foxp3⁺ Tregs were located close to α -SMA⁺ CAFs in diverse types of tumor models as well as in biopsies from melanoma and colorectal cancer patients. Notably, α -SMA⁺ CAFs demonstrated the ability to phagocytose, process and present tumor antigens and exhibited a tolerogenic phenotype which instructed a Treg cell movement arrest with Treg cell activation and proliferation, in an antigen-specific manner. Of interest, α -SMA⁺ CAFs were characterized by the presence of double-membrane structures, resembling autophagosomes, in their cytoplasm, while analysis of single-cell transcriptomic data pointed autophagy and antigen processing/presentation pathways to be enriched in α -SMA-expressing CAF clusters. In a mechanistic view, conditional knockout of the autophagy pathway in α -SMA⁺ CAFs promoted an inflammatory re-programming of CAFs, reduced infiltration and antigen-specific of Treg cells, attenuated tumor development, and potentiated the efficacy of immune checkpoint inhibitor immunotherapy.

Overall, our findings reveal a novel immunosuppressive mechanism operating in the TME, which entails the formation of synapse between α -SMA⁺ CAFs and Tregs in an autophagy-dependent fashion and raises the potential for the development of CAF-targeted therapies in cancer.

P421

THE ANTI-APOPTOTIC MEMBERS OF BCL-2 FAMILY INTERFERE WITH IMMUNOTHERAPY RESPONSE AND PROMOTE THE PD-L1 EXPRESSION IN SOLID TUMORS

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Immune-checkpoint inhibitors (ICI) targeting the PD-1/PD-L1 pathway have substantially changed the treatment of some solid tumors, significantly improving the survival of cancer patients. Nevertheless, immunotherapy provides benefits only in a subset of patients and most of them develop resistance over time. The outcome of ICI treatment is affected by tumor-intrinsic factors, such as tumor mutation burden, deficiencies in antigen presentation, PD-L1 expression, as well as the complex and dynamic tumor microenvironment. In this scenario, we and others previously reported that the anti-apoptotic proteins belonging to the Bcl-2 family and, in particular Bcl-2 and Bcl-xL, play a pivotal role in orchestrating the crosstalk between tumor cells and those of the surrounding microenvironment in different tumors, ultimately promoting tumor progression. Importantly, we also demonstrated that Bcl-2 and Bcl-xL proteins promote a pro-inflammatory tumor niche fueled by M2 tumor-associated macrophages.

On this basis, the aim of our study was to investigate whether the anti-apoptotic members of the Bcl-2 family may regulate pathways involved in the resistance to ICI, though the regulation of PD-L1 expression.

GEPIA 2.0 and Kaplan Mayer plotter Immunotherapy softwares were used to explore whether the Bcl-2 family proteins (Bcl-2, Bcl-xL and Mcl-1) correlated with PD-L1 expression and influenced the progression free survival of patients treated with ICI. Established and patients-derived cancer cell lines from different tumor histotypes and expressing different levels of Bcl-2 proteins (silencing and overexpressing). Flow cytometry, western blot and qRT-PCR analyses were used to evaluate the correlation between the Bcl-2 family proteins and PD-L1 expression. Monocytes derived macrophages (M-DM) exposed to conditioned media (CM) from Bcl-2 overexpressing melanoma cells were used to investigate changes in PD-1 expression level.

Mining RNA-seq data deposited in the TCGA through the online software GEPIA 2.0, we found that the levels of the anti-apoptotic members of Bcl-2 family (Bcl-2, Bcl-xL and Mcl-1) positively correlate with those of PD-L1 in different solid tumor histotypes datasets, i.e skin cutaneous melanoma (SKCM), uveal melanoma (UVM), non small cell lung cancer (NSCLC) and prostate (PRAD), colon (COAD) and pancreatic (PAAD) adenocarcinomas. Next, as a result of flow cytometry analysis we obtained a positive significant correlation between PD-L1 and Bcl-2 levels both in cutaneous melanoma ($R=0.89$, $p=0.04$), NSCLC ($R=0.93$, $p=0.01$) and COAD ($R=0.9$, $p=0.03$), but not with Bcl-xL ($R=0.14$, $p=0.7$ for melanoma; $R=0.02$, $p=0.8$ for NSCLC; $R=0.7$, $p=0.49$ for COAD) or Mcl-1 ($R=0.2$, $p=0.6$ for melanoma; $R=0.05$, $p=0.94$ for NSCLC; $R=0.4$; $p=0.2$ for COAD). We also found that silencing Bcl-2 or Mcl-1 in melanoma, NSCLC and pancreatic adenocarcinoma cell lines, reduced PD-L1 protein levels. Conversely, Bcl-xL modulation failed to affect PD-L1 protein levels. In addition, we observed an upregulation of PD-1 in M-DM exposed to CM from Bcl-2 overexpressing melanoma cells compared with control ones. Finally, by using the Kaplan Mayer plotter Immunotherapy software and focusing on the pre-treatment biopsies, we identified that Bcl-2 and Bcl-xL levels, but not those of Mcl-1, are associated with a worse progression free survival in melanoma patients treated with anti-PD-1 antibodies.

Our results indicate that anti-apoptotic Bcl-2 family members may play a different role in determining the ICI response and suggest a possible correlation between expression level of these proteins and that of PD-L1 in solid tumors.

P422

EVALUATING COMPLETE AND MAJOR PATHOLOGIC RESPONSE AS SURROGATE ENDPOINTS IN NEOADJUVANT IMMUNOTHERAPY CLINICAL TRIALS FOR EARLY-STAGE NON-SMALL CELL LUNG CANCER: A SYSTEMATIC REVIEW AND META-ANALYSIS.

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The use of neoadjuvant immunotherapy alone or in combination has rapidly evolved in the last 5 years and has been able to show complete and major pathologic responses in a substantial percentage of patients. There is significant debate as to whether pathologic response (pCR and MPR) can be considered a surrogate endpoint. We therefore performed a systematic review and meta-analysis to evaluate the surrogacy of pCR and MPR for event free survival (EFS) in neoadjuvant clinical trials for early-stage NSCLC.

We performed a systematic literature search on PubMed and reviewed abstracts of the most relevant international conferences until June 2023. Relevant information relative to response rates, Odds Ratios (ORs) of response, 2-years EFS rates and Hazard Ratios (HRs) were retrieved from all eligible clinical trials and the association were analyzed. To evaluate patients level surrogacy the association between response rate and 1- and 2-year EFS was evaluated. In paper presenting EFS rates by achievement of pCR and MPR a forest plot with random effect model was also produced. For trial level surrogacy, ORs for pCR and MPR and HRs for EFS were retrieved and analyzed using a linear regression model weighted by sample size. R² and linear regression slope were used respectively to estimate the proportion of variation in EFS effect explained by pCR effect and the magnitude of change in EFS effect as a function of the magnitude of change in pCR effect. The R² with 95% CI were calculated by bootstrapping approach.

Five RCTs and ten single-arm trials were identified for a total of 2343 patients. At patient level, the R² of pCR and MPR with 2-years EFS were 0.79 (0.44-0.99) and 0.85 (0.57-0.99), respectively, while OR of 2-year EFS rates according to response achievement was 0.12 (0.05-0.30) and 0.04 (0.02-0.08), respectively. At trial level, R² for association of OR for response and HR for EFS was 0.64 for both analyses but with 95% CI ranging from 0.01 to 1.00.

While we were able to show a strong correlation between pCR and EFS, trial level surrogacy was not proven. The lack of surrogacy can be related to the high heterogeneity among clinical trials and the still scarce number of data available. More research to reduce the heterogeneity (e.g., pathological evaluation, surgery, and staging harmonization) must be done to introduce response rate as a primary measure to predict EFS.

P423

INTERFERON-INDUCED METABOLIC REWIRING PROMOTES THE SURVIVAL AND IMMUNOEVASIVE PROPERTIES OF MELANOMA CELLS

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Uncovering how metabolic rewiring contributes to immune evasion in cancer may lead to new ways to target tumor metabolism to bolster anti-tumor immunity and increase the efficacy of immunotherapy. During tumor development, type I and type II interferons (IFNs) are released by the innate and adaptive immune systems as a pro-inflammatory signal to induce cell-surface presentation of antigens. This allows cytotoxic T lymphocytes (CTLs) to recognize foreign antigens presented on malignant cells and target them for killing. In a process known as immunoediting, however, chronic stimulation with IFNs and other cytokines results in the selection of tumor subclones that have acquired immunosuppressive properties to enable disease progression. In the background of immunoediting, tumor cells also undergo metabolic adaptations which promote their malignant phenotype. Metabolic rewiring and immune evasion are both cancer hallmarks, however, their interdependence remains poorly understood.

To model chronic IFN stimulation in vitro, YUMM1.7 cells (Yale University Mouse Melanoma; BrafV600E/Pten-/-/Cdkn2a-/-) were treated with type II IFN (IFN γ) for 28 days. Oxygen consumption rates were subsequently measured using an Agilent Seahorse XF Analyzer. ¹³C-labeled glucose and glutamine trace metabolomics were performed to assess changes in glucose and glutamine utilization in chronic IFN-treated cells, and cell proliferation following glutamine starvation was measured using the Incucyte Live Cell Analysis System. Control and chronic IFN-treated cells were transferred into C57BL/6 mice to determine their tumorigenic and metastatic potential. Lastly, RNA sequencing was performed to profile differences in the transcriptome of control, acute IFN-treated and chronic IFN-treated melanoma cells.

Chronic stimulation of YUMM1.7 cells with type II IFN in vitro causes downregulation of oxidative phosphorylation and an increase in lactate production, indicating a shift toward aerobic glycolysis. Metabolic tracing with ¹³C-labeled glucose and glutamine also revealed an increase in glutamine uptake and incorporation into tricarboxylic acid (TCA) cycle intermediates. Subsequent glutamine starvation experiments confirmed that chronic IFN γ -treated cells are more dependent on glutamine consumption for survival. Coinciding with these metabolic changes, chronic IFN γ -treated melanoma cells exhibit increased tumor growth and metastasis in vivo. Further, RNA sequencing has given insight into potential mechanisms of metabolism-driven immune suppression.

Together, these data show that type II IFN, one of the key cytokines involved in immunoediting, induces metabolic rewiring events that promote the unique survival and immunoevasive properties of melanoma cells. Ongoing integrative transcriptomic and metabolomic analyses are being used to identify metabolic vulnerabilities of chronic IFN γ -treated cancer cells that can potentially be targeted to impair their proliferative capacity and/or ability to evade the immune system.

P424

NOVEL OVARIAN CANCER PD-1+ NK CELL SUBSETS, TARGETABLE BY COMBINED IMMUNE-CHECKPOINT BLOCKADE

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Ovarian cancer (OC) is the fifth leading cause of cancer-related death and High-Grade Serous Ovarian Carcinoma (HGSOC) is its most common histotype. Current therapies are ineffective and most patients develop a recurrence within a few years. Therefore, the identification of new efficient therapeutic approaches is crucial to improve OC patient outcomes. Outstanding results can be obtained with immune-checkpoints(ICs)/ligands blockade strategies (mainly PD-1/PD-L axis) in immunotherapeutic protocols for aggressive tumors. Although this treatment comes as a T-based approach, it is now evident that also Natural Killer (NK) cells represent a promising immunotherapy tool and that PD-1 can be found on NK cells. Here we deeply characterized the role of PD-1 in HGSOC-infiltrating NK cells.

We performed multiparametric cytofluorimetric analysis of PD-1+ NK cells derived from peripheral blood and peritoneal fluid from a large cohort of HGSOC patients (n=60) subjected to primary surgery before chemotherapy. We characterized their anti-tumor activity by analyzing CD107a expression on PD-1+ NK cells in the presence of PD-Ls+/- HLA-I+ autologous HGSOC cells by blocking ICs with specific mAbs. We also analyzed PD-1 on NK cells derived from primary and metastatic tumor tissues by combining cytofluorimetric analysis and immunohistochemistry to correlate phenotype with spatial features.

Here, we identified two new PD-1+NK subsets occurring in HGSOC patients but absent in healthy donors, characterized by a CD56dimNKG2A+KIR+/-NKp46+CD57low phenotype and inefficient in producing an effective anti-tumor response against autologous OC cells. This impairment could be rescued with anti-PD-1/PD-Ls mAbs, in combination with mAbs blocking both NKG2A and KIRs, co-expressed with PD-1 primarily on tumor-associated NK cells. Furthermore, we found significant enrichment of the PD-1+NK cell subsets in the metastatic niche, suggesting a role for PD-1 in metastatic promotion/progression. Reflecting this, we found that high levels of tumor-infiltrating PD-1+NK cells correlate with an overall poor outcome.

Our data demonstrate the existence of novel tumor-infiltrating PD-1+ NK cell subsets in HGSOC patients, which are inversely related to patient outcome, showing the importance of these NK subsets. These subsets show indeed impaired anti-tumor activity, which can be rescued by

combined ICs blockade, paving the way for more successful NK cell-based immunotherapy in OC patients.

P425

TIME-SPECIFIC DELETION OF IL-17A-PRODUCING CD4+ T CELLS LEADS TO COLORECTAL CANCER REGRESSION WITHOUT INDUCING PATHOLOGICAL INTESTINAL INFLAMMATION

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Inflammation is a known driver of colorectal cancer (CRC). Yet, targeting immune drivers of inflammation has yielded limited efficacy. IL-17A-producing CD4+ T cells are present in the inflamed intestine and enriched in CRC but their role remains unclear, amid conflicting findings.

By pairing multi-omics with functional approaches in human and mouse, we found that the functional heterogeneity of IL-17A-producing CD4+ T cells reflects in opposite tumor outcomes.

While IL-17A^{high}CD4+T (TH17) cells correlate with better overall survival, IL-17A^{low}FOXP3+CD4+T cells correlate with CRC severity and worse overall survival in patients. Computational modeling suggests that IL-17A^{low}FOXP3+CD4+ T cells can originate from intestinal TH17 cells and are on a conversion path toward FOXP3+TREG cells. We confirmed this trajectory using fate-mapping mouse models during inflammation-induced intestinal carcinogenesis. In addition, constitutive deletion of IL-17A^{low}FOXP3+CD4+T cells reduces tumor burden but exacerbates colon inflammation. Instead, the conditional deletion of all IL-17A-producing CD4+T cells in mice with established tumors leads to tumor regression but does not exacerbate colonic inflammation.

Our data suggest the therapeutic relevance of considering the whole spectrum of IL-17A-producing CD4+T cells for a timely intervention. We propose that this intervention can impair tumor progression and prevent off-target inflammation in established CRC, and therefore represent a safer and more effective means to treat CRC.

P426

TARGETING OF LACTATE DEHYDROGENASE C AS A NOVEL APPROACH TO SIMULTANEOUSLY TARGET TUMOR CELLS AND MODULATE THE IMMUNE MICROENVIRONMENT.

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Cancer treatment has witnessed remarkable progress over the last few decades, with immunotherapy showing durable anti-tumor activity in several types of cancer. Nevertheless, the success of immunotherapy, in particular immune checkpoint inhibition, is limited to a subset of patients, which in part is driven by an unfavorable tumor microenvironment. Hence, the development of novel cancer strategies should consider the immune contexture and tumor cell-immune cell interaction in addition to the tumor specificity of a candidate target. We and others previously demonstrated that Lactate Dehydrogenase C (LDHC) is a promising therapeutic target as it is a highly tumor specific and immunogenic antigen that exhibits multiple pro-tumorigenic functions and when targeted sensitizes tumor cells to common anti-cancer drugs. However, it remains to be determined whether tumoral LDHC expression may influence tumor cell-immune cell interaction and anti-tumor immunity.

We generated three LDHC loss-of-function breast cancer cell line models (MDA-MB-68, BT549 and HCC1954), and assessed the effect of depleting tumoral LDHC expression on immune cell functionality in co-culture systems.

Knocking down the LDHC expression in all three breast cancer cell lines significantly increased IFN- γ release and T cell-mediated cancer cell killing, indicating that targeting LDHC has the potential to enhance T cell activation and functionality. Next, analysis of 23 tumor-derived inflammatory cytokines revealed that silencing LDHC decreased the soluble levels of IL-6 and Galectin-9 while increasing the secretion of GM-CSF, IFN- γ and MCP-1. Notably, a reduction in IL-6 alongside IL-1b and IL-4 was also observed when LDHC silenced cells were incubated in direct, but not in indirect, contact with lymphocytes. This prompted us to further investigate the expression of immune checkpoint molecules which are involved in direct tumor cell-immune cell interaction. Direct co-culture significantly reduced the expression of CTLA-4, TIM3 and VISTA on CD8+ cytotoxic immune cells. In addition, we found that indirect co-culture reduced the expression of PD-1, CTLA-4, LAG3, TIGIT and TIM3 on immune cells and of PD-L1 on the tumor cells. Furthermore, transcriptomic and flow cytometric analysis confirmed a downregulation of PD-L1 expression across all three cell lines, supporting a likely dysregulation of the PD-1/PDL1 axis following LDHC silencing.

Our findings highlight the potential role of tumor associated antigens, such as LDHC, in shaping the tumor immune microenvironment. Hence, targeting LDHC may have a dual anti-cancer benefit by directly impairing tumor cell survival while supporting a favorable tumor immune microenvironment.

P427

ZEBRAFISH AVATARS FOR PERSONALIZED MEDICINE & DISCOVERY OF NEW INNATE-BASED MECHANISMS OF IMMUNE EVASION

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Immunotherapy with immune checkpoint blockers is a revolutionary approach to cancer treatment, however only a fraction of patients responds to therapy. Therapy may fail due to additional immune-suppressive mechanisms present in the tumor microenvironment. To generate effective responses, it is critical not only to discover the mechanisms underlying immune evasion as well as new molecules that can revert this immune-suppression to fully activate the immune system to fight cancer.

Our Lab has been at the forefront of the development of an unique model for personalized medicine – the zebrafish Patient Derived Xenograft-“zAvatar” model to perform rapid in vivo screens. This assay relies on the injection of fluorescently labelled patient tumor cells into zebrafish embryos. zAvatars are then treated with the available therapy options and response to therapy is assessed by single cell confocal microscopy to evaluate induction of apoptosis. Overall, tumor behavior and response to anti-cancer therapy are accessed in 10 days, a time frame compatible with oncology clinical decisions.

In the last years we have been validating the model and performed co-clinical studies to determine its predictive value. In these co-clinical studies, we compared the patient’s response to therapy to the anti-cancer response in their matching zAvatar test. The correspondence is as high 90% in colorectal (55 patients, Costa et al, in prep) and ovarian cancer (22 patients, Estrada et al, in prep) and 100% in breast cancer (18 patients, Mendes et al, in prep).

However, we found that this model can also be used to unravel cancer-innate immune interactions. We found that while some human cancer cells are eliminated, others are able to evade innate-immunity. We demonstrated that clearance is dependent on the host innate-immunity and that this innate-rejection phenotype is extremely efficient, allowing for 70-80% tumor clearance in just 4 days. We observed that while some human cancer cells implant very efficiently others disappear from the host, and defined them respectively as progressors and regressors, following Schreiber’s terminology. We hypothesized that progressors could be evading the immune system, whereas regressors would generate an innate-reactive TME and be cleared by innate immune cells. To explore this hypothesis, we focused on a pair of human colorectal cancer (CRC) cells derived from the same patient with these opposing phenotypes. Indeed, progressors recruited less neutrophils and macrophages, and polarized macrophages towards a M2-like pro-tumoral state. Genetic and chemical depletion of myeloid cells confirmed that macrophages and neutrophils play a crucial role in this clearance process. Finally, single cell RNAseq (scRNAseq) revealed the dynamics of the different subclones: while some get cleared others expand upon in vivo selection. The “cleared” subclones were associated with immune inflammatory pathways and the expanded subclones with suppressor signalling. This study illustrates how our assay, coupled with scRNAseq, allows tracing of subclonal selection and identification of the signals that mediate the TME cross-talk. This discovery opened the opportunity to use the zAvatar model as a new discovery platform to find new innate-immune evasion mechanisms and molecules that can boost this innate-tumor rejection. We already found some candidate genes for new evasion molecules and started a FDA-phenotypic screen.

We developed an unique in vivo fast model for personalized medicine with outstanding capacity to predict patient outcome and therefore with potential to truly tailor treatments in a personalized manner, increasing efficacy rates and avoiding unnecessary toxicities. We also show that this model

can be used to further understand the role of innate immunity on cancer immunoediting and lead to new avenues of anti-cancer therapies, ultimately to be combined with immune-checkpoint-blockers, engaging both immune arms to fight cancer.

P428

A FIBER-RICH DIET PERTURBS THE MICROBIOTA-IMMUNE AXIS AND THWARTS MULTIPLE MYELOMA PROGRESSION

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Through degradation of dietary fibers, gut commensal bacteria generate short-chain fatty acids (SCFAs), metabolites with local and systemic anti-inflammatory activity that modulate the function of dendritic cells (DCs) and limit the expansion of Th17 cells. Clinical and experimental evidence supports the beneficial effects of SCFAs in multiple myeloma (MM) patients, but knowledge of the molecular mechanisms linking diet, gut microbiota and cancer is limited. We have previously demonstrated a direct link between intestinal microbiota, gut-born Th17 cells and MM progression in transgenic Vk*MYC mice developing de novo MM. Similarly, in asymptomatic smoldering MM (SMM) patients, higher levels of BM IL-17 predicted accelerated progression to full blown MM. We hypothesized that administration of a high-fiber diet, by expanding SCFA-producing bacteria, could modulate the immune response and restrain Th17 expansion, thus preventing MM evolution.

C57BL/6J mice challenged with Vk*MYC-derived MM cells (a model of aggressive MM; t-Vk*MYC) and transgenic Vk*MYC affected by asymptomatic MM (mimicking SMM; Early-MM) were orally fed high-fiber or standard diet. Gut and BM T cell infiltration and effector functions, along with effects on disease progression and overall mouse survival were taken as indicative of diet mediated effects. To assess the impact of dietary fibers on gut microbiota composition, 16S ribosomal RNA sequencing was performed on stool samples. SCFAs were quantified by nuclear magnetic resonance (NMR) spectroscopy.

As expected, t-Vk*MYC mice fed a high-fiber diet lost weight when compared to t-Vk*MYC mice fed a standard diet. Mice on high-fiber diet also showed delayed M-spike appearance and prolonged survival. Early-MM VK*MYC mice have been enrolled in a trial investigating the capacity of fiber-rich diet to delay progression from asymptomatic to symptomatic MM (Late-MM) and data are being collected. Mechanistically, t-Vk*MYC mice fed a high-fiber diet showed reduced representation of IL-17 producing cells and more favorable Th17/T regulatory cell (Treg) ratios. DCs exposed in vitro to SCFAs produced less pro-Th17 cytokines. T-Vk*MYC mice treated with butyrate experienced prolonged survival that associated with decreased Th17/T reg ratio.

Modulation of the gut microbiota by prebiotics and postbiotics may substantially impact the immune response, thus intercepting the trajectory of MM evolution.

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SEX HORMONE LEVELS IMPACT THE EFFICACY OF CANCER IMMUNOTHERAPY IN OBESE MALES WITH MELANOMA.

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Recent studies in melanoma patients suggest that obese patients have a better outcome than non-obese patients when treated with immune checkpoint inhibitors (ICI), and that this effect is predominantly observed in men. However, whether and why obesity is a determinant factor for response to ICI in a sex-dependent manner is unknown.

Here, we investigated the impact of obesity on antitumor immune responses and efficacy of ICI in males, both using mouse models and a cohort of ICI-treated patients.

Male mice fed with a high-fat diet to induce obesity or with a control diet were subcutaneously injected with B16-F10 melanoma cells and treated with anti-PD1 or control antibodies. Obesity was associated with increased tumor growth, in accordance with previous studies. In obese mice, ICI were able to reduce tumor growth to levels observed in lean mice, in which we observed no effect of ICI. These observations are supported by an enhanced infiltration of gp100-specific CD8⁺ T cells and a higher expression of immune-related pathways in obese males.

Adipose tissue plays an important role in the modulation of sex hormones by converting androgens to estrogens through the action of aromatase. We observed that obesity increases the plasmatic estrogen/androgen ratio in male mice and reshapes the tumor gene expression profile in males, with an enrichment of genes involved in the response to estrogens. To examine how estrogens produced by adipose tissue modulate anticancer immunity, we exposed adipocytes derived from human adipose stem cells to testosterone. We confirmed that these cells produced estrogens in the presence of testosterone and that this was dependent on aromatase expression. We further showed that activation of murine bone marrow-derived dendritic cells (BMDCs) was impaired in the presence of supernatant from testosterone-treated aromatase-deficient adipocytes compared to that from wild-type adipocytes. Furthermore, estrogen treatment during BMDC differentiation enhanced their capacity to present antigen, boosted antigen-specific CD8⁺ T cell priming, and improved tumor cell killing.

Lastly, we explored the translational aspect of these findings in a pilot cohort of melanoma patients receiving ICI (n=37). No relationship was found between body mass index (BMI), estrogen levels and clinical outcome in women. However, in men, overweight and obese patients responded better to ICI, based on RECIST criteria. Interestingly, a high estrogen level was associated with a higher chance of ICI response in men but not in women, supporting the hypothesis that estrogens impact ICI efficacy in males.

Overall, our results suggest that the obesity-mediated increase in estrogen levels through aromatase activity in the adipose tissue is a determinant factor for response to ICI in males. We propose that sex hormone levels may serve as new biomarkers to predict response to ICI in patients. This work may open the path to develop new therapeutic strategies targeting the sex hormone pathways to improve the efficacy of cancer immunotherapies.

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MANNANOSE RECEPTOR C-TYPE II (MRC2): A NOVEL POTENTIAL STROMAL TARGET IN NSCLC

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The advent of immunotherapy in the clinical practice of non-small cell lung cancer (NSCLC) has highlighted the impact of the tumor immune microenvironment (TIME) in dictating the clinical response. Among stromal cells, CAF subtypes participate in generating a hostile TIME inhibiting T cell recruitment and activation.

To identify stromal-derived molecules that might be targeted to convert an immunosuppressive to an immunopermissive TIME we have applied a bioinformatics pipeline, developed upon a machine learning framework for RNA-seq data analysis to transcriptional data of Lung Adenocarcinoma (LUAD) and Lung Squamous Cell carcinoma (LUSC) patients, available on the TCGA public database. The pipeline aimed at identifying putative lung-cancer stromal-related antigens for an innovative CAR-T therapy.

We assumed that tumors with low cytotoxic T cell (CTL) infiltration and with a stroma enriched in the ECM-myCAF subtype of immunosuppressive CAFs, express high levels of molecules involved in the physical barrier and immunosuppressive factors that impede the anti-tumor function of T cells. Among the targets identified in the signature of ECM-myCAFpositive/CTL negative tumors, we focused on Mannose Receptor C Type 2 (MRC2)

Immunohistochemical analysis on multi-tumor tissue TMA revealed MRC2 overexpression, compared to the normal matched tissue counterpart. Focusing on lung cancer, the majority of the 64 cases we analyzed overexpress MRC2 in the tumor stroma surrounding the cancer islets, frequently distributed in a fibrous ring that protects the tumor cells from CD3⁺ T lymphocytes entry. In accordance, CAFs obtained from freshly explanted NSCLC specimens, although heterogeneous, express MRC2 protein at high level. Looking at MRC2 expression on a panel of NSCLC cell lines, we found a different level of positivity in various adenocarcinoma and in none of the squamous cell lines. Accordingly, MRC2 IHC staining was evident also on tumor cells of 8 out of the 64 evaluated NSCLC tissues, including 5 adenocarcinomas but none squamous cell carcinoma. These data suggest that MRC2 could represent a valid molecule for the targeting of both tumor cells and stroma. MRC2 transfection in normal lung fibroblasts induces an increased expression of well-established markers of CAF activation (FAP and PDPN). In line, NSCLC-CAFs depleted for MRC2 showed a reduction of collagen gel contraction ability, of the expression level of ECM-related molecules (COL1A1, COL1A2, and COL6A1, FN1 and ITGB1) and an altered topography of CAF derived ECM. Considering that TGFβ1 is crucial in activating resident fibroblasts to CAFs, we demonstrated that TGFβ1 stimulation upregulates MRC2 in normal lung fibroblasts. Conversely, MRC2 is significantly involved in the regulation of all three TGFβ isoform expression and secretion. Hence, we hypothesized a complex mechanism involving TGFβ-MRC2, which generates an autocrine loop that sustains TGFβ1 expression and secretion. Furthermore, our results showed that in NSCLC-derived CAFs, PD-L1 and PD-L2 expression is significantly modulated by MRC2, as demonstrated by loss and gain of functions experiments performed in CAFs and in normal lung fibroblasts, respectively.

Overall, these data indicate that MRC2 expressed in NSCLC-derived CAFs could contribute to a hostile, immunosuppressive TIME by modifying the ECM composition and architecture, the secretion of the immunosuppressive TGFβ cytokine and the expression of the immune checkpoint ligands, PD-L1 and PD-L2. In conclusion, our data pave the way for the definition of novel strategies that might include the MRC2 targeting to favour the endogenous and therapy-mediated anti-tumor

immune response.

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BOOSTING TCR-EDITED T CELLS FOR THE TREATMENT OF GASTROINTESTINAL TUMORS BY DISRUPTING TIGIT WITH CYTOSINE BASE EDITING

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Among gastrointestinal tumors, colorectal cancer (CRC) and pancreatic ductal adenocarcinoma (PDAC) are still major unmet medical needs. CRC and PDAC mortality is related to metastases, that hinders the therapeutic efficacy of conventional treatments. Adoptive cell therapy (ACT) with TCR-edited T cells is a promising therapeutic strategy for advanced patients. However, the paucity of TCRs targeting relevant tumor antigens and the immunosuppressive tumor microenvironment limit ACT applicability.

We combined the analysis of published datasets with a multidimensional investigation of CRC and PDAC samples collected at Ospedale San Raffaele to select a set of tumor-associated antigens (TAAs) to be targeted by ACT. To retrieve novel tumor-specific TCRs, we repetitively stimulated healthy donors' and cancer patients' PBMCs with immunogenic peptides belonging to the selected TAAs. We generated TCR-edited T cells by combining lentiviral transduction of a tumor-specific TCR with the disruption of the endogenous one. To boost the therapeutic efficacy of TCR-edited T cells we coupled TCR editing with TIGIT disruption, a key immunosuppressive molecule engaged by PDAC and CRC cells. To ameliorate the safety profile of our cellular products, we optimized the genome editing approach of T cells by relying on a cytosine base editor (CBE). We analyzed the occurrence of sgRNA-dependent and sgRNA-independent off-target events in base edited T cells by performing targeted NGS and ultra-deep whole exome sequencing, respectively. To validate our TCR-edited TIGIT disrupted T cells we challenged them in vitro with PDAC cell lines, primary and liver metastases CRC and PDAC patient-derived organoids. Moreover, we tested these T cells using an orthotopic mouse model of CRC liver metastases.

Our analysis identified 19 relevant TAAs to be targeted by T cells. By clonal tracking of TCR repertoire of T cells stimulated with autologous APCs loaded with the selected antigens, we isolated 5 TCRs specific for different TAAs, including MSLN and MET. Importantly, TCR-edited T cells displayed anti-tumor activity against PDAC cell lines, CRC and PDAC patient-derived organoids while sparing HLA unmatched and antigen negative controls. To improve the efficacy of ACT for gastrointestinal tumors, we combined TCR-editing with TIGIT disruption. CRISPR/Cas9 activity induces important off-target events at the genomic level, affecting the safety profile of therapeutic cellular products. To overcome these limitations, we employed a cytosine base editor. We generated TAAs-specific T cells deprived of the endogenous TCR and TIGIT exploiting CRISPR/Cas9 and the CBE. Both editing procedures proved highly efficient in simultaneously disrupting TRAC, TRBC1, TRBC2 and TIGIT without impairing T cell memory phenotype differentiation nor their expansion abilities. Remarkably, we detected gene translocations only in CRISPR/Cas9 treated cells (up to 0.44%). Also, CBE has the potential to act at off-target loci in the genome, mediating sgRNA-dependent and -independent DNA deamination. To assess sgRNA-dependent DNA off-target base editing, we sequenced the top off-target loci for each sgRNA mapping on coding regions. We confirmed that CBE shows high on-target activity with minimal off-target editing. To assess sgRNA-independent DNA spurious deamination, we performed ultra-deep whole exome sequencing on samples derived from 4 different donors and collected at the end of our manipulation procedure. We observed that CBE efficiently mediated gene disruption without introducing biologically relevant off-target mutations. TIGITKO TCR-edited T cells engineered with CBE showed superior abilities, compared to TIGITWT T cells, in controlling tumor growth in an orthotopic model of CRC liver metastases.

Our findings suggest that T cell products generated by CBE have a better safety profile and that TIGIT disruption could improve the therapeutic efficacy of TCR-edited T cells for gastrointestinal tumors.

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TYPE I IFN RECEPTOR STABILIZATION AT HEPATIC METASTATIC SITE

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Liver metastases from colorectal carcinoma (CRC) and pancreatic ductal adenocarcinoma (PDAC) are unfavorable prognostic factors and largely incurable diseases. The liver metastatic microenvironment (TME) is uniquely predisposed to induce a state of immune insensitivity involving a series of distinct and temporally coordinated cellular and molecular signaling pathways that impede the antitumor potential of the innate and adaptive immune response in the liver.

In this context, the type I IFN system is evolving into a complex system that can lead to immune dysfunction through mechanisms based on the expression of immune checkpoint proteins and downregulation of the type I interferon receptor IFNAR1 in the TME. Whether CRC and PDAC metastases induce similar immune dysfunction is currently unknown.

To test whether metastatic liver TME deregulates IFNAR1 and promotes a state of immune dysfunction, we used cohorts of patients with synchronous primary and liver CRC metastases, patient-derived organoids from liver CRC metastases, and mouse models of CRC and PDAC liver metastases.

We found that several type I IFN subtypes are upregulated in CRC metastatic liver lesions, consistent with an increase in IRGs, checkpoint inhibitors, genes associated with pathogenic inflammation, and degradation of IFNAR1. To clarify the cellular source of type I interferons, we examined mouse CRC cell lines and tumor organoids (MTO) and mouse PDAC cells and found that CRC cells expressed the same type I IFN subtypes in vitro, whereas PDAC cells and corresponding liver lesions did not. Similar results were also observed in patient derived organoids from liver CRC metastases. We then treated mice with established intrahepatic CRC or PDAC tumors expressing different type I IFNs with continuous IFN α and found that IFN α did not control CRCs but efficiently inhibited PDAC metastasis, consistent with differential expression of IFNAR1 by these tumors. Finally, we found that stabilization of IFNAR1 by p38/PDK inhibitors in the hepatic microenvironment improved the therapeutic outcome of IFN α therapy.

Characterization of type I IFN immune dysfunction in CRC liver metastases and strategies to stabilize IFNAR1 in the CRC liver microenvironment represent a promising new therapeutic approach to improve IFN α immunotherapies.

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HFE POLYMORPHISM IMPACTS ON TUMOR AND IMMUNE CELL BEHAVIOR IN PANCREATIC CANCER ECOSYSTEM

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HFE is the most frequently mutated gene in hereditary hemochromatosis, a disease in which iron accumulates in organs leading to toxicities. Worldwide, two main polymorphisms in HFE protein have been observed, namely C282Y and H63D. Even if associated to milder iron accumulations, H63D polymorphism increases the risk of cancer development and aggressiveness. As observed in pancreatic cancer patients, a great percentage of those that underwent to surgery also present H63D polymorphism but displayed a worse survival.

To better characterize how the iron accumulation affects anti-tumor response in the context of the pancreatic ductal adenocarcinoma (PDA), we crossed genetically engineered mice (GEM) that spontaneously develop PDA with mice engineered to express H67D mutation (HfeH67D), the orthologue of H63D in human. Tumor burden, metastasis and perineural invasion were evaluated through the histological analysis. In vivo immunization assay was used to investigate the memory response in terms of antigen-specific cytokine secretion and antibody production.

H67D polymorphism accelerated PDA progression of GEM, increased the number of metastases, and altered the composition of infiltrating immune cells. Accordingly, these mouse models also displayed a reduced overall survival compared to controls. By exploiting OVA as a well-known antigen, we injected OVA-expressing PDA cells subcutaneously in mice carrying or not the H67D mutation to assess their ability to mount a normal memory response. OVA-expressing PDA cells grew faster in the presence of H67D polymorphism, but immunized mice displayed a reduction in tumor volume similar to the WT counterpart. HfeH67D mice displayed an increased production of anti-OVA IgG antibodies, especially IgE, but a decreased in OVA-specific IFN γ -secreting T cells.

Overall, H67D polymorphism impacts on the activation of anti-tumoral immune response and accelerates EMT, which consequently worsen PDA progression

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COLORECTAL-CANCER INFILTRATING BACTERIA DIFFERENTIALLY MODULATE RECRUITMENT AND FUNCTIONS OF TUMOR-ASSOCIATED NEUTROPHILS

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Colorectal cancer (CRC) is a major cause of cancer related death worldwide. Tumor infiltration by immune cells is critically associated with favorable clinical outcome. Surprisingly, at difference with other tumor types, infiltration by tumor-associated neutrophils (TANs), also predicts improved prognosis. However, mechanisms underlying TAN-mediated beneficial effect are still unclear.

During CRC oncogenesis alterations of gut mucosal barrier favor bacteria translocation from the gut lumen into the mucosa and their direct interaction with epithelial and immune cells. Although neutrophils represent a front-line arm of immune responses to bacteria, the outcome of their interplay with bacteria infiltrating CRC tissues has not been thoroughly explored yet.

Here, we investigated the impact of CRC-associated bacteria on TAN's recruitment and functional modulation.

Human CRC cells from established cell lines were injected intra-cecum (i.c.) into immunodeficient NSG mice and chemokine gene expression was assessed in i.c xenografts by quantitative PCR. Microbiome analysis of human CRC samples was performed based on 16S gene sequencing and effects of the two most abundant bacterial species, defined as Operational Taxonomic Unit (OTU)5 and OTU2, were tested in vitro. Bacteria were expanded under anaerobic conditions and incubated on human CRC cell lines and on neutrophils from peripheral blood of healthy donors and patients. Chemokine gene expression and protein production by CRC cells in vitro was assessed qPCR, and by ELISA on culture supernatants, respectively. Ability of CRC culture supernatants to promote neutrophils' recruitment was tested in migration assays. Bacteria-induced neutrophil activation was evaluated based on expression of surface activation markers, as assessed by flow cytometry, and morphological changes as detected by confocal imaging. Release of cytotoxic factors was tested by assessing the viability of CRC cells cultured with neutrophils' supernatants. Neutrophil-derived factors with tumoricidal activity were identified by proteomic analysis of neutrophil supernatants.

CRC cell exposure to gut bacteria in vivo, as occurring in i.c. tumors, boosted the expression of genes encoding neutrophil-recruiting chemokines, including CXCL1, CXCL2, CXCL5 and CXCL8. Interestingly, in vitro OTU5 promoted release of these chemokines by CRC cells and neutrophils' migration more efficiently than OTU2.

Furthermore, CRC-associated bacteria differed in their ability to induce neutrophils activation. Remarkably, OTU5 induced neutrophil activation more effectively than OTU2, as indicated by a stronger modulation of CD66b, CD16, CD54 and CD62L surface markers and by an increased motility and cluster formation. Most importantly, OTU5, but not OTU2, triggered in neutrophils the release of cytotoxic factors with tumoricidal activity. Proteomic analysis of culture supernatants identified distinct clusters of proteins specifically secreted by neutrophils, most notably an up-regulation of some proteins upon OTU5 stimulation.

Our data unravel a critical role of CRC-associated bacteria on neutrophils' recruitment and functional modulation and suggests gut microbiota conditioning might represent as an innovative therapeutic approach to elicit neutrophil-mediated anti-tumor effects.

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EXPLOITING EXOSOMES TO CIRCUMVENT IMMUNOTHERAPY RESISTANCE IN TUMORS: A NOVEL THERAPEUTIC APPROACH

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The emergence of resistance to immunotherapy is a formidable challenge in cancer treatment, particularly in non-small cell lung cancer (NSCLC). This research was designed to investigate a novel approach that leverages the potent immune-modulatory properties of exosomes, which are nano-sized extracellular vesicles secreted by various cell types. We focused on exosomes derived from T cells, engineered to carry the immune-activating cytokine, Interleukin-2 (IL-2), to overcome resistance in NSCLC.

CD8⁺ T cells were cultured, and exosomes were isolated from the supernatants using differential ultracentrifugation, followed by purification through a size-exclusion chromatography step. These T cell-derived exosomes were then loaded with IL-2 via a proprietary electroporation protocol. Nanoparticle tracking analysis, transmission electron microscopy, and Western blotting for exosomal markers (CD63, CD9) and IL-2 confirmed successful loading and verified the exosomal size and morphology.

We established an in vitro model using NSCLC cell lines (A549 and H1975), known for their resistance to PD-1/PD-L1 inhibitors. These cells were treated with IL-2-loaded exosomes. Post-treatment assessments included cell viability (MTT assay), proliferation (BrdU incorporation assay), and the expression of immune markers (flow cytometry and quantitative RT-PCR).

The exosomes displayed the characteristic size range (30-150 nm) and marker expression of exosomes, with successful IL-2 incorporation. Treatment with IL-2-loaded exosomes led to a 40-60% reduction in cell viability and proliferation compared to untreated controls. Furthermore, significant upregulation in the expression of immune response markers, including PD-L1, granzyme B, and IFN- γ , was observed, suggesting an enhanced immune response.

The present study provides compelling evidence that T cell-derived exosomes carrying IL-2 represent a promising theranostic strategy to overcome immunotherapy resistance in NSCLC. Their capacity to modulate the tumor microenvironment and potentiate immune responses could revolutionize current paradigms in cancer immunotherapy. Furthermore, the potential synergistic effect of using PD-1 inhibitors in combination with these exosomes could provide a more effective treatment regimen for NSCLC. Our findings warrant further validation in in vivo models and subsequent clinical trials.

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EXPANDING THE IMMTAC PLATFORM FOR CANCER IMMUNOTHERAPY TO UNCONVENTIONAL T CELL RECEPTORS

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ImmTAC molecules are T Cell Receptor (TCR)-anti-CD3 bispecific fusion proteins that can redirect and activate polyclonal T cells to kill tumor cells in an MHC-restricted manner. Tebentafusp, a gp100-directed ImmTAC, is the first TCR therapeutic to demonstrate survival benefit in a solid tumor and has been approved in 2022 by the FDA and the EMA for the treatment of HLA-A*02:01+ adults with unresectable or metastatic uveal melanoma (mUM).

The use of TCRs as therapeutics allows access to a multitude of intracellular targets not available for conventional antibody therapeutics. To overcome moderate binding affinities of natural cancer specific TCRs (μM to nM range) we developed a platform to affinity enhance TCRs to pM values, before fusing them to an anti-CD3 scFv effector arm, with nM affinity. The long half life of target-bound molecules favours engagement of T cell in the tumor microenvironment, creating an inflammatory milieu and promoting recruitment of rejuvenated T cells from the circulation.

To overcome the limitations of MHC restriction, we are expanding the ImmTAC platform to ab and gd TCRs targeting monomorphic unconventional antigen presenting molecules, including CD1 and MR1. Tumor associated unconventional T cells, including MAIT, iNKT cells and gd T cells, are positively associated with clinical outcome in several cancers. Unconventional T cells recognize cancer dysregulated metabolites, lipids and stress molecules which offer a unique window of specificity over normal tissues.

We have successfully engineered three unconventional TCRs: a metabolite specific MR1-restricted TCR; a CD1c-lipid restricted TCR and a butyrophilin-specific Vd2 Vg9 TCR. We demonstrate selective recognition of antigen positive targets, with minimal off target effect.

This important proof of principle paves the way for harnessing unconventional T cell receptors in cancer immunotherapy.

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AN INFLAMMATORY STATE REMODELS THE BONE MARROW MICROENVIRONMENT AND T-CELL LANDSCAPE IN HUMAN CLONAL HEMATOPOIESIS AND RELATED MYELODYSPLASIA

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The bone marrow (BM) niche is crucial for hematopoietic stem/progenitor cell (HSPC) functions. Mesenchymal stromal cells (MSCs) are essential for HSPC maintenance, bone repair, and immune responses. However, functional dysregulation of BM-MSCs and their impact on the active state of the T cell Landscape in clonal hematopoiesis (CH) and myelodysplastic syndromes (MDS) remains largely unknown.

Using single-cell RNA-seq and high-resolution multispectral imaging, we analyzed the single cell pathological interplay between CD34+ HSPC, CD271+ MSC, and CD3+ T cell populations from a curated cohort of matching BM liquid and trephine biopsies of a prospective longitudinal CH/MDS study (>300 samples, NCT02867085).

we found significant BM remodeling in CH (n=9) compared to healthy non-mutation carriers (n=10), which was dependent on VAF% of mutant HSPCs and associated with emergence of inflammatory-biased MSCs and expansion of sinusoidal endothelium. These stromal changes were exacerbated in MDS (n=16) by a buildup of exhausted regulatory T cells. These findings were supported by scRNA-seq (n=10) with the identification of stress-induced MSC subsets in BM-CH and enrichment of inflammatory IFN-responsive cytotoxic T cell subsets in BM-MDS, especially those harboring SF3B1-Mutation. Lastly, proteomics analysis from BM-MDS co-culture model replicated these inflammatory and pro-angiogenic processes, emphasizing the importance of these cues for stroma remodeling.

Collectively, our study provides the basis to investigate this inflammatory BM phenotype as a potential risk-stratifying tool of disease evolution in CH/MDS and identify potential targets for immunotherapy applications.

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UTILIZING RNA DEVICES TO MODULATE PD-1 EXPRESSION IN MAMMALIAN CELLS FOR CANCER IMMUNOTHERAPY

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Immune checkpoint blockade, a major medical breakthrough in recent years, has become one of the most effective approaches in immunotherapy against a broad spectrum of cancers, including those in late metastatic stages. The agents against either the PD-1 receptor or the PD-L1 receptor-ligand have high efficacy and long durability. However, one of the problems associated with the current immunotherapy approach is the exhaustion of T lymphocyte cells.

PD-1 is an important receptor to maintain peripheral tolerance and cellular homeostasis and hence, treatment procedures mediated by checkpoint inhibition are reported to impose deleterious effects. We propose a different approach. Instead of checkpoint blockade, we specifically control expression of genes key to checkpoint inhibition by using riboswitch devices to control the expression of PD-1 directly at the levels of transcription, splicing and translation. This approach bypasses the host cell regulatory networks and pathway altogether.

We have developed a system for rapid testing of rationally designed tetracycline regulated RNA devices to control PD-1 expression by CRISPR knock-in (in mouse EL4 lymphocyte cells) and have established the proof of concept that RNA devices work efficiently in mammalian cells and demonstrated their reversibility and recoverability to regulate the gene expression.

In summary, our system clearly indicates the robustness, efficiency, and reversibility of the functional RNA devices to control PD-1 expression for the development of a proper treatment regimen for cancer and viral infections.

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IMMUNOSUPPRESSIVE TUMOR MICROENVIRONMENT OF OSTEOSARCOMA

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Osteosarcoma (OS) is the most common malignant bone tumor in the children, accounting for 6% of all pediatric cancers. It is characterized by a high degree of genomic instability, resulting in numerous copy-number alterations and genomic rearrangements but with no disease-defining recurrent activated mutations. Despite multiple clinical trials using various combination chemotherapy, there has been no significant improvement in prognosis for nearly four decades. Given the diverse genomic landscape of OS and the difficulty of identifying druggable therapeutic targets, the use of immunotherapy appears to be a reasonable alternative approach. However, results from recent clinical trials based on immune checkpoint inhibitors (ICIs) were disappointing. This suggests that there may be additional immune suppressive mechanisms in the tumor microenvironment (TME) in OS other than the PD-1/PD-L1 mechanism that could be interfering with the effects of immunotherapy. Therefore we carried out a detailed analysis of the TME of a small number of OS cases using a combination of single cell RNA sequencing and spatial transcriptomics techniques.

To better understand the immune microenvironment in OS and guide future therapeutic development, we performed single-cell RNA sequencing (scRNA-seq) using the 10x Genomics Chromium platform on six fresh tumor biopsy samples from pediatric OS patients. Raw data was processed using 10x Cell Ranger to produce transcript read counts for each cell. After further data filtering based on mitochondrial/ribosomal RNA content and doublet removal using DoubletFinder, counts were normalized using Seurat, and cells were integrated across samples with Harmony. Data was combined with a previously published OS scRNA-seq cohort of six samples (GSE162454). Two additional OS samples were profiled using 10x Genomics Visium spatial transcriptomics for validation of discovered subtypes and to add spatial context.

Initial clustering identified 16 major cell types based on high expression of canonical cell markers, including macrophages, OS cells, T cells, and granulocytes. Copy number changes in OS cells (identified with inferCNV) revealed multiple subclones in each tumor. Several immunosuppressive cell types were identified via subclustering of major cell types, including neutrophil myeloid-derived suppressor cells (MDSCs), regulatory T-cells, exhausted T-cells, and LAMP3+ dendritic cells. Markers for the cell types found in OS were identified for further validation and cohort expansion using imaging techniques, including Visium spatial transcriptomics. We discovered 9 total spot clusters in the Visium data and performed deconvolution using the scRNA-seq cell identities to examine colocalization of discovered cell types. Many of the discovered clusters were common between patients and showed consistent cell type proportions. However, we did discover patient-specific differences in the frequency of some cell types, with one sample showing a higher proportion of T-cells along with increased presence of colocalized IFN-stimulated macrophages, and the other sample with a greater presence of neutrophils and MDSCs.

Using single-cell transcriptomics, we were able to discover the presence of multiple distinct immunosuppressive cell subtypes of neutrophils, T-cells, and dendritic cells. Additionally, spatial transcriptomics revealed multiple similar clusters between samples, and common colocalization of

the discovered cell types within those clusters. However, differences in T-cell presence and interferon induction may be indicative of patient-specific immunogenicity of OS. Taken together, these preliminary results confirm the hypothesis of significant immunosuppression in the TME of OS caused by different mechanisms. These findings suggest potential novel immunotherapy targets that could be considered in future clinical trials. Functional validation of some of these targets are in progress.

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PROSTAGLANDIN E2 (PGE2) CONTROLS INFLAMMATORY ACTIVATION OF MACROPHAGES

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PGE2 is a lipid mediator with pleiotropic functions in tissue homeostasis and cancer. It drives acute inflammation, but it also modulates tissue remodeling and suppresses the anti-tumor immune response by hijacking cytotoxic activities of NK and CD8 lymphocytes and by hampering macrophage activation. Previous studies in our laboratory demonstrated the ability of PGE2 to antagonize the expression of type I IFN in macrophages exposed to inflammatory stimuli, by acting on chromatin remodeling. However, how PGE2 controls additional inflammatory responses in macrophages remains completely uncharacterized.

We performed transcriptomic analysis of mouse Bone Marrow-derived Macrophages (BMDMs) exposed to multiple inflammatory stimuli (LPS, TNF α and IL-1b), in the presence or absence of PGE2. These experiments were combined with functional assays to dissect the mechanisms of action of PGE2.

We found that co-exposure of BMDMs to PGE2 and inflammatory stimuli resulted in increased induction of a set of genes encoding for key inflammatory molecules and tissue-reparative mediators, exemplified by Il1b. PGE2-mediated synergistic effects with inflammatory stimuli resulted in increased IL-1b intracellular levels and higher IL-1b release, upon NLRP3-inflammasome activation, in co-stimulated BMDMs. Mechanistically, PGE2 co-stimulation was not associated with increased activation of NF- κ B signaling. Moreover, a permeable analog of cAMP, second messenger downstream PGE2 receptors EP2 and EP4, is able to phenocopy the PGE2-mediated synergistic effects with inflammatory stimuli on gene expression.

These analysis showed that PGE2 exerts divergent activities on macrophages. While suppressing type I IFN and Interferon-stimulated genes, co-stimulation with PGE2 increases the expression of a set of inflammatory and tissue reparative mediators, including Il1b. By identifying a set of genes differently regulated by PGE2, our findings reconcile with the seemingly opposing functions of PGE2 in macrophages. Overall, our data highlight the critical role of PGE2 in driving immune-modulation and dysfunction in the tumor microenvironment, further supporting the therapeutic potential of PGE2 blockade alone or in combination with immunotherapeutic approaches. Mechanistically, how PGE2 controls inflammatory responses in macrophages is currently under investigation.

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FINAL CLINICAL AND BIOMARKER OUTCOMES OF A PHASE II STUDY OF PRE-OPERATIVE INDUCTION PEMBROLIZUMAB WITH RADIATION THERAPY IN EARLY-STAGE TRIPLE NEGATIVE AND HIGH-RISK HORMONE RECEPTOR POSITIVE BREAST CANCER (PEARL)

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Hypofractionated radiation therapy (RT) delivered to an in-situ breast tumor in combination with anti-PD(L)1 therapies has shown potential in pre-clinical studies to stimulate anti-tumor immune responses and induce long-term, tumor-specific memory. Herein, we report the mature results of the first, phase 1b/II study on combining focal RT with induction pembrolizumab in the pre-operative treatment setting for TNBC and high-risk hormone receptor-positive (HR+) breast cancer (BC).

Between 12/2017 to 12/2021, 66 patients with stage I-III TNBC (54) or high-risk, HR+ BC (12) were treated with pembrolizumab cycle 1 (200 mg iv q3wks), followed by pembrolizumab cycle 2 with the addition of focal RT (8Gy x3) delivered to a breast primary tumor, then NAC (MD choice), breast and axillary surgery, postoperative RT to the chest wall and regional lymph nodes, and adjuvant systemic therapy per treating physician. Serial tumor and blood specimens were collected at 3 timepoints: 1) pre-treatment; 2) after pembrolizumab cycle 1; 3) after pembrolizumab + RT. Dual primary endpoints were 1) feasibility, defined by the number of patients who did not require a delay in starting NAC; 2) change in tumor infiltrating lymphocytes (TILs) score between pre- and post-pembrolizumab timepoints using Salgado criteria. Secondary endpoints included pCR, defined as ypT0/TisypN0, change in PDL1 status (pre- versus post-pembrolizumab), adverse effects (AEs), and cosmesis evaluations.

The median age of the cohort is 53y (range 26-94) with median follow-up of 27.5mo (range 3-47.5). The majority (84.8%) were clinical stage II; 3% stage I and 12.1% stage III. 42.4% of patients presented with clinically node-positive disease. The majority (79%) received anthracycline-containing NAC with 18.1% receiving a taxane. In the TNBC cohort, 5.6% received carboplatin. The primary endpoint of feasibility was met, with only 2 (3%) experiencing delays >4wks in initiating NAC. All patients received breast surgery without delay. No patients experienced grade 4 irAEs, but 3 experienced grade 4 AEs attributable to chemotherapy (sepsis 1; neutropenia 2). In the entire cohort, the rate of pCR was 68.2% (45), and 2-year event free survival (EFS) was 90%. Eight patients (12.3%) had disease recurrence (7.7% local recurrence, 4.6% distant recurrence), all were TNBC. Fifty-seven patients (48 TNBC, 9 HR+) had evaluable baseline tumor and blood biopsies for PDL1 and TIL analysis. At baseline, 45(79%) were PDL1+ (40 TNBC, 5 HR+) with a combined positive score (CPS) >1. A higher percentage of responders (83.9%) were PDL1+ at baseline compared to non-responders (70.4%). Relative to baseline/pre-treatment biopsy, the proportion of PDL1+ tumors increased after 1 cycle of pembrolizumab (from 79% to 87% PDL1+, p=0.02) but not after pembrolizumab + RT. Following pembrolizumab cycle 1, the average TIL score increased slightly in the TNBC cohort (22.4 to 30.6, p=0.162), however decreased significantly after the addition of RT + pembrolizumab (to 17.7, p=0.002). In contrast, the average TIL score in the HR+ cohort remained low and with minimal change across timepoints (9.3 to 7.6 to 9.2, p=0.58). Blood correlatives will be presented at the meeting.

Focal breast RT with pembrolizumab followed by NAC in the preoperative setting, the combination is safe and feasible. Efficacy was similar to KEYNOTE-522 in the TNBC cohort despite the absence of pembrolizumab administration during NAC. As expected, pembrolizumab increased PDL1 expression. The addition of RT did not change baseline PDL1 status, but significantly reduced TILs. These results are hypothesis-generating that the strategy of combining pre-operative RT with pembrolizumab may offer opportunities to de-escalate NAC in properly selected patients, and emphasizes the significance of sequencing of pembrolizumab with RT in optimizing anti-tumor responses. Three year follow-up results will be reported at presentation.

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THERAPEUTIC TARGETING STING-MEDIATED TUMOR INFLAMMATION INDUCED BY CHROMOSOMAL INSTABILITY

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Activation of the immune system is an important consequence of normal and tumor tissue exposure to cancer treatments such as chemotherapy, ionizing radiation and immunotherapies. Recent evidence suggests that activation of the cytosolic double-stranded DNA (dsDNA) sensing cGAS-STING pathway plays an important role in intrinsic antitumor immunity and that this pathway is required for the therapeutic effects of radiotherapy and immune checkpoint inhibitors. However, while acute cGAS-STING-mediated inflammatory response impart an anti-tumor effect, chronic tumor inflammation has been associated with disease progression, therapeutic resistance, immune suppression and metastasis.

To better define the temporal nature of the cellular response to STING activation, we developed a tractable model system using non-immortalized IMR90 human lung fibroblasts, which have an intact cGAS-STING pathway that is unstimulated at baseline yet primed to respond upon cGAMP treatment. We treated IMR90 fibroblasts with cGAMP for 5 consecutive daily doses and used quantitative PCR to assess time-dependent transcription of key Interferon Stimulated Genes (ISGs) and endoplasmic reticulum (ER)-stress response target genes after the 1st and 5th daily dose of cGAMP. Next, we developed a model of IMR90 fibroblasts expressing an inducible interferon-stimulated-responsive elements (ISRE) coupled with GFP. Then, we performed a pooled CRISPR screening using GFP-fluorescent protein as a functional readout in IMR90 fibroblasts to identify genes whose depletion negatively alter the induction of type I interferon program.

This work is based on the findings that Chromosomal Instability, a genomic source for tumor cell-derived chronic inflammatory signaling, promotes immune evasion and metastasis through chronic activation of cGAS-STING pathway. This highlights an important paradox: how do chromosomally unstable tumors, which are characterized by persistent sensing of cytosolic dsDNA, evade immune surveillance given the ability of cGAS-STING to activate anti-tumor immune response?

Here we show that repetitive – or chronic – stimulation from elevated cGAS activity can lead to rapid desensitization of type I IFN signaling, increased STING turnover and upregulated ER-stress in untransformed fibroblasts and in chromosomally unstable cancer cells. This reduction in response to repeated stimulation – otherwise known as tachyphylaxis – was specific to STING activation as transfection with Poly(I:C), an activator of the dsRNA sensing pathway, led to a robust induction of ISGs. Next, using our chronically stimulated fibroblast model we performed a genome-wide CRISPR screening for an unbiased discovery approach. We hypothesize that guide-RNA based disruption in key mediators of attenuated type I IFN signaling will rescue signaling and restore IFN response at “acute” level.

We identified potential targets involved in ER stress response and TLR-mediated inflammatory innate response which could negatively regulate the activation of type I program by promoting STING translocation to ERGIC compartment with a rapid STING degradation and turnover. These target genes will be subjected to further investigations.

To date these findings represent an important opportunity to target potential drivers of STING-mediated IFN signaling suppression and test if inhibition of the ER stress sensors or regulators of TLR-mediated inflammatory innate response can prime the Tumor Microenvironment into an inflamed state that is likely to respond immunotherapy treatment offering an exciting opportunity for

therapeutic intervention in chromosomally unstable tumors for which there are currently few effective therapeutic options.

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BIFIDOBACTERIUM SUPPLEMENTATION IMPROVES ONCOLYTIC ADENOVIRUS EFFICACY IN A MURINE SYNGENEIC MODEL OF MELANOMA

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In the past two decades, cancer immunotherapy has demonstrated tremendous efficacy in many cancer types. However, the efficacy of immune checkpoint inhibitors (ICIs) therapy has a high variability due to genetic and environmental sources. Several studies demonstrated the influence of gut microbiota on ICIs efficacy; in particular, commensal Bifidobacterium was identified as a key determinant of antitumor immunity in vivo, increasing anti-PD-L1 efficacy. Recently, novel strategies of active immunotherapy, based on oncolytic viruses (OVs) are achieving preclinical and clinical success with a relevant contribution to the treatment of several types of solid tumors.

We hypothesized that modulation of gut microbiota could also affect oncolytic virotherapy efficacy; therefore, we decided to investigate whether the effect of OVs on melanoma progression could be altered by manipulation of the intestinal microbial community.

To this end, we pre-treated a group of C57BL/6J mice with an oral administration of vancomycin and, subsequently, inoculated subcutaneously syngeneic B16.OVA melanoma cells to the groups. We then treated both groups with an intratumoral injection of oncolytic adenovirus (OAd). To confirm that perturbation of the intestinal microbiota was involved in this phenomenon, we performed the rescue of the group pre-treated with vancomycin, by cohousing them with an OAd control group. Subsequently, we evaluated whether a probiotic containing Bifidobacterium spp., already effective in improving response to ICIs, could enhance the response to OAd therapy.

We observed that OAd efficacy was extremely reduced in mice pre-treated with vancomycin, showing a faster tumor progression and a reduced number of tumor-infiltrating lymphocytes (TILs) compared to the control group. The combined therapy with Bifidobacterium supplementation had a higher efficacy; in addition, it reduced tumor-infiltrating Tregs and stimulated an enrichment of bacterial genera belonging to Firmicutes phylum. Using an innovative bioinformatic tool, Homologous Evaluation Xenopeptides (HEX), we identified Bifidobacterium-derived epitopes highly similar to MHC class I-restricted melanoma epitopes. As confirmed by IFN- γ ELISPOT assay, these peptides were able to trigger a robust CTL response and cross-reacted with melanoma peptides.

Our data indicate that gut microbiota affects the immune responses elicited by OAds and Bifidobacterium enhanced their antitumoral activity in a melanoma model and molecular mimicry is potentially involved

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DEVELOPMENT OF A NOVEL NEOANTIGEN-REACTIVE CD8+ T CELL ADOPTIVE CELL THERAPY MODEL IN POORLY IMMUNOGENIC B16F10 MELANOMA

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Adoptive cell therapy (ACT) for cancer, which typically involves large-scale transfer of tumor-targeting lymphocytes to patients, is a promising avenue for immunotherapy treatment. ACT has demonstrated remarkable, but limited, success in the clinic to date. One issue impeding the universality of ACT is target antigen selection; not all tumors express empirically validated tumor antigens. Further, it is not clear whether there are substantial benefits in targeting neoantigens (which are uniquely expressed by tumors, albeit sometimes at a low expression level) vs. robustly expressed tumor associated antigens [TAAs] (which exhibit varying degrees of exclusivity to tumor tissue).

To answer these questions, we sought to develop a preclinical mouse model. B16F10 melanoma is a murine implantable tumor model that is widely used in the cancer immunotherapy field. ACT models in B16F10 have historically been comprised of TAA (pmel) and B16F10-OVA (foreign antigen) models; while these have been useful, neither model neoantigen-reactive T cell immunity. We conducted whole exome sequencing and RNA sequencing of B16F10 and strain-matched splenocytes and utilized our previously developed OpenVax pipeline to predict Class I-restricted neoantigenic epitopes in B16F10. Using peptide-based TLR7/8-conjugated vaccines (in collaboration with Vaccitech), we vaccinated mice with predicted neoantigenic peptides and performed tetramer staining to identify neoantigen-reactive CD8+ T cells. T cell receptor (TCR) sequencing was performed and subsequently we engineered neoantigen-reactive T cells in-house via transduction with identified neoantigen-reactive TCRs to enable intensive study.

Of interest, we identified a high-affinity TCR reactive against an anchor residue (p5)-modified H2-Db-restricted peptide from Heat Shock Transcription Factor 2 (Hsf2); T cells transduced with this TCR efficiently recognized cognate peptide at low concentrations, but without targeting the corresponding wild type peptide. Further, co-culture of B16F10 with Hsf2-reactive T cells induced IFN γ and Nur77 production by the T cells, indicating recognition of cognate peptide in the context of direct presentation by tumor cells. Encouraged by these data, we obtained high-resolution crystal structures of the binary peptide-MHC (pMHC) and ternary pMHC-TCR complexes. We observed a hydrophobic arch in H2-Db that enables solvent accessibility at peptide positions 4,6 and 7, and thus, intermolecular contact between the pMHC and TCR. Interestingly, this structure is nearly identical to that reported for a H2-Db-restricted influenza peptide (Young et al. 1994, Cell), indicating similarities between neoantigen and viral peptides' modes of recognition by TCRs.

To assess the viability of Hsf2-reactive T cells as an ACT model for neoantigen-reactive T cell immunity in B16F10, we transferred 20 million T cells into irradiated C57BL/6 mouse hosts; notably, ACT efficacy was only observed when Hsf2 neoantigenic peptide level was highly expressed. Furthermore, the tumor-killing ability of multiple neoantigen-, and, for comparison, pmel, Tyrp1 and Trp2 (TAA)-reactive T cells, were assessed in the in vitro IncuCyte system, revealing that comparatively low neoantigen expression level in tumors or low T cell TCR avidity are impediments to T cell killing.

Altogether, we have identified a novel neoantigen and corresponding high-affinity neoantigen-reactive TCR that enables robust tumor recognition and tumor growth control in the commonly used murine melanoma model B16F10. We have developed an ACT model in which Hsf2-reactive T cells can be compared alongside traditionally studied TAA-reactive T cells. Further, in our system, we have a variety of tools available to understand the impact of parameters such as TCR avidity, tumor antigen abundance, and cross-reactivity to wildtype pMHC, on ACT efficacy. We anticipate that this model will enable discovery of novel mechanisms impacting ACT success.

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EXTRINSIC AND INTRINSIC ROLE OF THE INHIBITORY RECEPTOR PIRB IN PANCREATIC CANCER PROGRESSION

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Paired Immunoglobulin-like Receptor B (PIRB) is the murine ortholog of the human Leukocyte Immunoglobulin-like Receptor B (LILRB) 2 and 3, inhibitory receptors widely expressed with multiple tissue-dependent functions. PIRB and LILRBs are expressed on the surface of hematopoietic cells, namely macrophages, granulocytes, mast cells, dendritic cells and B lymphocytes. The peculiarity of these molecules is the presence of 3 Immunoreceptor Tyrosine-based Inhibitory Motives (ITIM), activated by contacting with high affinity their principal ligand, class I major histocompatibility complex (MHC) molecules. Previous studies have linked PIRB loss to the presence of hypersensitive B cells, which display a higher production of antibodies, increased cytotoxic T cells and less suppressive myeloid cells polarized toward an M1-like anti-tumoral phenotype. Furthermore, these receptors have been also identified as players in directly sustaining progression and invasiveness of several types of tumor.

The potential involvement of PIRB in the adaptive immune response prompted us to better characterize its role in modulating the anti-tumoral immune response in models of pancreatic ductal adenocarcinoma (PDA). To this, mice lacking PIRB gene (*Pirb*^{-/-}) were crossed with genetically engineered mice (GEM) spontaneously developing pancreatic cancer. Histological and immunohistochemical analyses of pancreatic tissues were performed to measure tumor lesions and characterize the immune infiltrate. To investigate the ability to mount a memory response, non-proliferating ovalbumin (OVA) expressing PDA cells were injected subcutaneously in C57/BL6 WT and *Pirb*^{-/-} mice. After three weeks, mice were challenged with alive cells, with tumor growth measured twice a week for 28 days. At time of sacrifice, ELISPOT assay was performed to assess the secretion of Interferon g (IFN γ) and ELISA assay was performed on sera to investigate the anti-tumor humoral response. On the other side, the pro-tumoral role of PIRB inside PDA cancer cells was investigated through the generation of a *Pirb*^{-/-} murine PDA cell line (K-PIRB) and the assessment of its proliferative and invasive behaviour with a MTT, wound healing and soft agar clonogenic assay

PIRB deletion significantly improved survival rate of GEM compared to the *Pirb* proficient counterpart, and this correlated with a reduced percentage of transformed ducts in both early and late-stage disease. Immunohistochemical analyses of pancreatic tissues demonstrated increased frequency of tumor infiltrating T lymphocytes (TIL), as well as of intra-tumoral B cells in GEM *Pirb*^{-/-} mice. Moreover, the absence of PIRB significantly improved immunization against a well-known experimental antigen, namely ovalbumin (OVA). Indeed, immunized *Pirb*^{-/-} mice showed a reduced growth of subcutaneous living tumor cells, together with an enhanced number of IFN γ -secreting T cells and higher levels of OVA-specific IgG. Furthermore, preliminary in vitro experiments showed a slower proliferation and invasiveness of K-PIRB cells in comparison to wild-type counterpart.

Overall, PIRB represents a promising target to improve the anti-tumor immune response and deserves further characterization to design novel immunotherapy strategies for the treatment of PDA.

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ENHANCING NEOANTIGEN EXPRESSION IN SMALL CELL LUNG CANCER VIA INHIBITION OF NONSENSE-MEDIATED DECAY

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Cancer immunotherapy has revolutionized the field of oncology and is one of the most promising treatments for lung cancer. Small cell lung cancer (SCLC) is a highly aggressive tumor type with poor prognosis and limited response rates to current immunotherapeutic approaches. Paradoxically, SCLC is characterized by a remarkably high tumor mutational burden, but not all mutations are equally immunogenic. Frameshift (fs) mutations could create proteins which may serve as strongly immunogenic neoantigens, but due to the fs-induced generation of premature termination codons, their mRNAs are often degraded via Nonsense-Mediated Decay (NMD). Given the high frequency of fs mutations observed in SCLC, we aimed at enhancing tumor immunogenicity by targeting the NMD pathway.

We first employed whole exome sequencing to detect tumor-specific mutations in SCLC samples – including immortalized SCLC cell lines and clinical samples – and predicted candidate neoantigens based on the ability of mutant peptides to interact with sample-specific HLA-I molecules. So far, we have profiled tumor-specific mutations for more than 400 patients. Next, we validated the expression and presentation of neoantigens in control versus NMD-inhibited samples via transcriptome sequencing, whole cell proteomics and HLA-I-immunopeptidomics. Of note, NMD inhibition was achieved via chemical and genetic approaches. Importantly, we estimated tumor-specific NMD activity based on transcriptome sequencing data and NMD reporter assays to predict which patients could better profit from a NMD inhibition treatment. Based on these predictions, tumor samples with a high NMD activity and a high number of fs mutations were selected for co-cultures with PBMCs derived from healthy donors. Immunogenicity of NMD-regulated neoantigens is being currently investigated in vivo using murine and human SCLC xenograft models humanized with autologous or HLA-I-matched PBMCs. To this end, our lab has so far established more than a hundred patient-derived SCLC xenografts.

NMD inhibition in several SCLC cell lines resulted in the upregulation of global bonafide NMD targets as well as sample-specific fs-mutated mRNAs. Interestingly, the changes in neoantigen landscapes observed upon NMD inhibition extended beyond fs-mutations, indicating that this approach might be even more powerful than anticipated. Pathway analysis revealed an enrichment of multiple tumor-relevant biological processes upon NMD inhibition, including a decreased proliferation and an enhanced apoptosis of tumor cells, among others. Several in vitro assays confirmed that NMD inhibition reduced proliferation and enhanced apoptosis in SCLC cell lines, suggesting that NMD activity per se contributes to SCLC pathophysiology which further supports the therapeutic value of NMD inhibition for this aggressive cancer type. Proteomics and HLA-I-immunopeptidomics results are pending but tumor-PBMC co-cultures revealed an increased tumor immunogenicity upon NMD inhibition. Specifically, siRNA-mediated inhibition of NMD factors in tumor cells resulted in enhanced T cell-mediated tumor killing in vitro, an effect found to be HLA-I-dependent (i.e., antigen presentation-dependent), as it was abrogated when using B2M knockout tumor cells.

NMD inhibition in SCLC cell lines decreased proliferation and induced apoptosis in a cell-autonomous manner. Additionally, inhibition of NMD led to the upregulation of multiple neoantigen-encoding mRNAs and enhanced tumor immunogenicity in vitro. Our strategy not only provides a novel immunotherapy approach for the treatment of low immunogenic tumors, but also enables

patient stratification for an informed personalized care. In the future, NMD inhibition could be combined with immune checkpoint blockade to enhance immunotherapy efficiency. Our approach could furthermore be used for the rational design of personalized fs-neoantigen-based vaccines.

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CHEMOTHERAPY BOOSTS TUMOR ASSOCIATED ANTIGENS INDUCED-T LYMPHOCYTE EFFECTOR RESPONSE IN PANCREATIC CANCER PATIENTS

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Pancreatic Ductal Adenocarcinoma (PDA) is one of the most aggressive malignancies with a 5-year survival rate of 11%. Less than 20% of patients have a resectable disease at the diagnosis due to the lack of distinctive symptoms and reliable biomarkers. PDA is resistant to chemotherapy (CT), thus understanding how to gain an anti-tumor effector response following tumor associated antigen (TAA) stimulation is critical to set up an efficient tailored immunotherapy.

Peripheral blood mononuclear cells (PBMC) from PDA patients, before and after gemcitabine-based CT, were in vitro stimulated with four TAA (ENO1, FUBP1, K2C8 and GAPDH). The proliferative response and the cytokine release by T cells were evaluated, by BrdU incorporation assay and ELISA respectively, as well as the TAA-specific clonality of TCR. The V-J rearrangement of TCR β repertoire was profiled with next-generation sequencing (NGS). Besides, PBMC gene expression analysis through mRNA NGS was performed in a subset of PDA patients before and after gemcitabine-based CT.

Proliferation and cytokine release from PDA patients' T lymphocytes, before and after CT, were analyzed in vitro in response to TAA. CT led to an increased number of TAA recognized by patient T cells, and this positively correlated with survival. After CT the high-rate of TAA-induced proliferative responses were maintained and high IFN γ production responses induced by TAA were significantly increased. The hierarchical clustering analysis of the effector versus regulatory tone (IFN- γ /IL-10 ratio) before and after CT stratified patients into different groups (responder, exhausted, non-responder). Transcriptional analysis revealed that after CT, PBMC from responding patients (those who have higher number of responses to TAA) displayed more differentially expressed genes compared to non-responding patients, suggesting that CT modulates gene expression on immune cells by highlighting predictive molecular signatures. Of note, non-responding patients have an increased expression of the IRAK1/IL1R signaling axis, whereas responding patients display a fatty acid signature (LDAH). The clonotypic analysis of TAA-stimulated T lymphocytes from CT-treated patients showed that some clonotypes were expanded or de-novo induced, and that some clonotypes detectable before CT were reduced or even disappeared after CT.

Overall, these data indicate that CT shifted the immunological tone toward an effector phenotype, with a significant gain of TAA-induced effector T cell response, enhancing the expansion of TCR rearrangements, suggesting a stronger reactivity of precise TAA-specific T cell clones after CT. Notably, CT led to a marked fatty acid signature in TAA-responding patients, supporting the development of precision immunotherapy, in selected effector patient groups, based on CT combined with TAA vaccination.

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DEVELOPMENT OF TCR-NK CELLS FOR ANTIGEN-SPECIFIC IMMUNOTHERAPY OF CANCER

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The currently celebrated success of Chimeric Antigen Receptor modified T (CAR-T) cells in cancer immunotherapy remains limited to targeting cell surface antigens that constitute about ¼ of all known human genes. For the remaining potential antigens, T cell receptor (TCR) gene transfer can be used to redirect the activity of cytotoxic T cells towards selected epitopes of intracellular antigens presented on the target cell surface by Major Histocompatibility Complex (MHC) molecules. In this second approach, the heterodimeric nature of the TCR molecule poses a risk where TCR α and TCR β chains introduced by gene delivery mispair with the endogenously expressed β or α chains in the T cell, giving rise to mixed TCR dimers with unpredictable specificity. Recent studies by us and others have put forward the use of Natural Killer (NK) cells that are inherently void of TCR expression to circumvent this problem and effectively target intracellular antigens for cancer immunotherapy.

We previously reported the proof of principle for functional TCR expression on the NK cell surface using a co-receptor-independent TCR α/β specific for the HLA-A2-restricted tyrosinase-derived epitope (Tyr368-377) that was delivered via lentiviral transduction to NK cell lines along with the CD3 chain genes. Since NK cells already express CD3 ζ , its ectopic overexpression in TCR-NK cells did not increase antigen-specific triggering. On the contrary, increased background activity against non-specific targets was observed in TCR-NK cells with CD3 ζ overexpression. The underlying mechanism of this effect remains unknown.

In this study, we compared effector functions such as degranulation and cytokine secretion of TCR-NK cells manufactured with or without CD3 ζ ectopic overexpression. Moreover, we aimed to analyze in detail the gene expression profiles of TCR-NK cells to understand the transcriptomic changes associated with this complex genetic modification. CD3 complex (CD3 δ , CD3 γ , CD3 ϵ) with or without CD3 ζ and TCR α/β genes were lentivirally delivered and pure populations of TCR-NK cells were used for bulk RNA sequencing to determine differentially expressed genes that relate to CD3 ζ overexpression. Additionally, we also sought to identify the interaction of TCR signals in TCR-NK cells with the CD8 coreceptor and Killer-cell Immunoglobulin-like receptors (KIRs) by overexpressing CD8 subunits or KIR genes to NK-92 cells. Western blot analyses were carried out to observe differentially effected signaling pathways upon target engagement.

Our initial observations indicate that the CD8 $\alpha\alpha$ homodimer could have some suppressive effects in TCR-NK cells while the presence of CD8 $\alpha\beta$ and CD8 $\beta\beta$ is correlated with increased cytotoxic activity. Furthermore, we have shown in a limited setting that the antigen-specific cytotoxic activity of TCR-NK cells is unaffected by the presence of a particular KIR expression. RNAseq analysis revealed that the use of CD3 ζ overexpression dramatically changes gene expression profile and might lead to aberrant behavior of TCR-NK cells. Additionally, our analysis shows that genes which have roles in antiviral defense mechanism pathways are significantly altered in all genetically modified cells due to the side-effects of gene delivery by lentiviral vectors.

We conclude that the inclusion of CD3 ζ in vector design may be deleterious to the antigen-specificity of TCR-NK cells. Further development of TCR-NK cells should rely on expression of only CD3 $\delta\gamma\epsilon$ components of the CD3 complex.

P449

PHOSPHORYLATED FAT10 INTERFERES WITH IFN-I SIGNALING

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FAT10, is an ubiquitin like modifier which target its substrates for 26S proteasomal degradation independent of ubiquitin. It is expressed in the organs of the immune system and its expression can be strongly induced in all cell types by co-stimulation with the proinflammatory cytokines TNF and IFN γ . FAT10 has been shown to be overexpressed in multiple types of cancer, which may be attributed to the proinflammatory tumor microenvironment. Moreover, the pro-malignant capacities of FAT10 suggest its direct involvement in tumorigenesis. Recent studies have shown that FAT10 is involved in the downregulation of RIG-I mediated type I interferon secretion (IFN-I); however, the molecular mechanism for this regulation is yet to be understood. We aimed to understand how FAT10 is involved in influenza A virus (IAV) induced RIG-I mediated type I interferon secretion.

Through phospho-proteomic analysis we found that FAT10 is phosphorylated at 5 serine and threonine residues. FAT10 phosphorylation is strongly enhanced by stimulation with TNF and upon IAV infection. By doing in vitro and in cellulo overexpression and knockout experiments we found that FAT10 is phosphorylated by members of JNK and IKK kinase families. Experiments with phospho-mimetic and phospho-deficient mutants of FAT10 showed that phosphorylation of FAT10 is important to downregulate RIG-I mediated type I interferon signaling.

Mechanistically, we could show that phosphorylation of FAT10 increases its binding to the TRAF3-deubiquitylase OTUB1 resulting in the stabilization and thus in the activation of OTUB1. Consequently, FAT10 phosphorylation results in deubiquitination of TRAF3, which is unable to maintain IRF3 phosphorylation and downstream induction of IFN-I.

Taken together, we reveal a mechanism how phosphorylation of FAT10 limits the production of tissue destructive IFN-I in inflammation.

P450

MORPHOLOGICAL ANALYSIS OF NEUTROPHIL MIGRATION IN 3D

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Neutrophils must migrate through complex interstitial environments to perform their functions as first responders at the sites of inflammation and tumors. Significant progress has been made toward understanding neutrophil migration in 2D by studying cell morphology and the distribution and dynamics of the cytoskeleton. However, the mechanism of neutrophil migration in 3D is not well understood. Recent advancements in image analysis have made it possible to objectively compare 3D morphology and the relative localization of intracellular structures among populations of cells. This new methodology first standardizes 3D shapes so that complex morphologies can then be simplified and compared using principal component analysis.

To visualize cell shape and the localization of intracellular components during migration, I created transgenic HL-60 cells that expressed fluorescent protein fusions labeling the cell membrane (GFP-CAAX), the actin cytoskeleton (halotag-actin), and non-muscle myosin (mApple-MLC). I imaged dHL-60 neutrophil-like cells migrating through 3D collagen gels in vitro, acquiring simultaneous time-lapse images of the cell membrane paired with actin, myosin, or the cell nucleus (labeled with SPY650-DNA). To investigate different modes of migration in 3D, I observed neutrophils during random migration, and, subsequently, during directed migration in response to a light-activated gradient of chemoattractant fMLP.

The shapes of neutrophils undergoing random versus directed migration were highly similar. Additionally, the location of the nucleus and actin cytoskeleton were also similar between modes of migration. To investigate differences in the dynamics of 3D migration, I tracked the shape transitions that individual cells made through time, and observed that shape transitions were significantly muted in cells undergoing chemically-directed migration compared to those performing random migration.

These results suggest that while there are no cell morphologies that are unique to these migratory modes, randomly migrating neutrophils change their shape more frequently, reflecting their relative lack of directional persistence.

P451

BILE SALT HYDROLASE-EXPRESSING ENGINEERED NATIVE BACTERIA FOR THE TREATMENT OF COLITIS-INDUCED COLORECTAL CANCER

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Colorectal cancer affects more than 60% of patients diagnosed with ulcerative colitis (UC). UC is a prevalent inflammatory disease of the colon and it associated with lower levels of secondary bile acids and bacterial bile salt hydrolase (BSH) activity. We have previously demonstrated that native bacteria can serve as chassis to functionally manipulate the gut microbiome. Intestinal *Escherichia coli* (*E. coli*) isolated from mouse feces engineered to express BSH can engraft for years after a single gavage, retain their function, alter luminal and serum metabolome, and affect host physiology without changing the microbiome composition in mice. Serendipitously, colon tumors isolated from patients are often colonized with *E. coli*. Other laboratories demonstrated that secondary bile acids can restrain T helper 17 (Th17) cells and promote regulatory T cell differentiation, thus potentially affect immune response to various stimuli. Our aim was to determine whether microbial bile acid biotransformation with engineered native bacteria can reduce tumor burden and inflammation in a mouse model of colitis-induced colon cancer.

We used a murine native strain of *E. coli* to knock-in the bacterial BSH enzyme in an intact and unperturbed microbiome and assessed its effect on disease severity in colitis-induced colorectal cancer using azoxymethane (AOM) and dextran sulfate sodium (DSS). Disease severity was assessed histologically after hematoxylin and eosin staining of colonic tissue, and lamina propria T cell analyses by flow cytometry.

Our engineered native *E. coli* successfully engrafted and colonized the gastrointestinal tract of 100% of the mice after a single gavage and maintained BSH activity throughout the duration of the experiment. Functional expression of BSH resulted in colitis amelioration and protection from severe disease, in both acute and chronic settings. Moreover, we found fewer inflammatory CD4 T cell populations, including interleukin-17A (IL-17A), tumor necrosis factor α (TNF α), and interferon γ (IFN γ -producing T cells, in the colonic lamina propria of mice receiving BSH bacteria. BSH-expressing bacteria accumulated mainly in the site of higher colitis activity.

Our findings demonstrate that modulation of bile acids by engineered native bacteria ameliorate colitis in a preclinical model and can serve a strategy for functionally curative treatment of colitis-induced colorectal cancer. Moreover, these results show the potential contribution of bacterial BA biotransformation to the progression and severity of UC and colorectal cancer.

P452

DECODING SPATIAL PATTERNS PROMOTING IMMUNE EXCLUSION IN TRIPLE NEGATIVE BREAST CANCER

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TNBC is an aggressive breast cancer subtype with high tumor heterogeneity, posing challenges in developing effective immunotherapies. Although bulk RNA-seq data has identified four subtypes, preliminary data suggests individual TNBC tumors are composed by more than one subtype. We hypothesize that spatial organization of tumor subtypes influences T cell infiltration and contributes to immune evasion.

Using spatial transcriptomics and highly multiplexed protein imaging, we analyzed treatment-naïve TNBC tumors and compared the spatial organization of tumor cell states in T cell infiltrated and excluded tumors. We quantified and mapped the T cell infiltrate and investigated the molecular programs leading to T effector cell exclusion.

Preliminary results suggest that the mesenchymal tumor cell state can form highly immunosuppressive spatial domains characterized by T cell exclusion and high-level expression of TGFB2, CXCL8 and SPP1. We aim to decipher the molecular programs leading to T effector cell exclusion, with a particular focus on epithelial-to-mesenchymal (EMT) gene programs in tumor cells, recruitment of immunosuppressive cell populations and immunosuppressive cytokines.

Our findings may provide insights into immune evasion mechanisms in TNBC and inform the development of effective immunotherapies.

P453

TRANSCRIPTIONAL LANDSCAPE OF THE IMMUNE NICHE OF SKIN EPITHELIAL STEM CELLS.

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Tissue homeostasis relies on the activity of stem cells (SC) that proliferate, differentiate, and repair the tissue on demand. Epithelial SC reside in specialized microenvironments (niches) composed by cells of different lineages adept at instructing SC activity. The interaction between SC and their niche must be tightly controlled as promoting excessive SC activity might lead to tissue outgrowth and tumor formation. After acquiring oncogenic mutations, transformed tumor SC (tSC) need to evade immune-mediated attack and simultaneously repurpose the immune niche to favor their outgrowth. To date, the tools available to investigate the spatial and transcriptional landscape of the SC immune niche are limited and present several restrictions (including lack of unbiased high-throughput transcriptional readout or insufficient resolution).

Here, we optimized a novel mouse model (“Universal Labeling Immune Partnerships by SorTagging Intercellular Contacts” or “uLIPSTIC”) to profile the immune niche of skin hair follicle stem cells (defined as Sox9-expressing cells). This tool uses enzymatic transfer of a labeled substrate to tag interacting cells *in vivo*. Using flow cytometry, we characterized the lymphocyte populations interacting with hair follicle stem cells.

A discrete subset within all the main lymphocyte populations (including effector CD4⁺ T cells, CD8⁺ T cells, regulatory T cells and innate lymphoid cells) was efficiently labeled using the uLIPSTIC technology. Immunofluorescence confirmed the close proximity of these lymphocyte subsets and SOX9⁺ hair follicle stem cells, with most of the interactions occurring in the upper region of the hair follicle. To unbiasedly unveil the transcriptional signature of the hair follicle immune niche, we sorted lymphocytes that were interacting or not interacting with SOX9⁺ cells and processed them for single cell RNA sequencing. For each lymphocyte subset, we identified genes and pathways correlating with their interaction with hair follicle stem cells, including known candidates necessary for their intra-epithelial retention (such as the integrin CD103).

These results serve as a proof of concept to investigate how the niche evolves during tumor progression. Using the same mouse model, we will be able to dissect how skin squamous cell carcinoma stem cells shape their immune niche to evade immune-mediated attack and repurpose it to favor their growth.

P454

THE LINK BETWEEN TUMOR METABOLIC STATE, TME AND IMMUNOTHERAPY RESPONSE IN BREAST CANCER

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Triple-negative breast cancer (TNBC) is one of the most challenging cancers to treat. The tumor microenvironment (TME), which includes malignant epithelial tumor cells, blood vessels, immune and other stromal cells, plays a major role in therapy resistance. We showed that elevated tumor glycolysis corresponds to poor T-cell infiltration (Zappasodi, Serganova et al., 2021) and greater vessels numbers (Serganova, Cohen et al., 2018) in murine triple negative breast cancer (TNBC) models. These tumor microenvironment (TME) features were found to be associated with unfavorable clinical outcomes to immune checkpoint blockade (ICB) therapy in other cancer types. The molecular mechanisms linking tumor metabolism with T-cell recruitment, vascular immune barrier, and therapeutic response to ICB therapy in TNBC are unknown. To gain mechanistic insight in these relationships, we developed and compared metabolic variants of syngeneic murine TNBC models with respect to TME architecture and response to ICB therapy. For clinical translation, we evaluated the relationship between these TME features in TNBC patients.

Glycolysis-defective (LDHA knock-down, KD) and mitochondria-defective (Rho0) murine 4T1 TNBC models were developed and described previously. These cell lines were engineered to express a HIF-1 bioluminescence reporter to measure HIF-1 response in vitro and in vivo. To simulate the clinical management of TNBC, mice were treated with neoadjuvant regimens of ICB, and followed for metastasis development by weekly bioluminescence. TME was assessed by immunofluorescence staining and/or flow cytometry. Vessel permeability was assessed by Evans Blue dye assay.

We observed alterations in the vasculature-immune TME compartment across TNBC metabolic variants. In LDHA-KD tumors, vessels were shorter, more linear, and less branched with a decrease in both CD31+ endothelial cells and NG2+ pericytes. Rho0 tumors presented similar phenotypes as control tumors with respect to CD31+ cells, but more abundant NG2+ cells that did not co-locate with vessel cells, as for normal pericytes. Changes in CD31+ and NG2+ cell numbers across these metabolic variants corresponded to changes in tumors vessel permeability. Evans Blue dye extravasation was significantly less in metabolic variants (LDH-A-KD, Rho0) vs. control tumors. We analyzed T-cell-vessel distance, as a surrogate parameter of vessel function to deliver immune cells to the tumor core. In advanced tumor settings (day 19), we observed that CD4+ and CD8+ T cells are differently distributed with respect to CD31+NG2+ co-labeled mature vessels, being proximal to vessels in Rho-0 tumors, indicative of poor intratumoral T-cell delivery. HIF-1 activity was higher in control and Rho0 tumors vs. LDH-A-KD tumors, according to their glycolytic phenotype. The analyses of immune compartment TME revealed increased T cells in LDHA-KD tumors and decreased myeloid cells populations in both LDHA-KD and Rho0 metabolic variants. Mice bearing these tumors experienced prolonged tumor-free survival upon a pre-operative ICB regimen. Initial TME analyses of human TNBC cases showed spatially separated clusters of LDHA+ tumor cells, immature CD31+ vessels not colocalizing with NG2+ cells, indicating abnormal angiogenesis, and poor T-cell infiltration, in contrast with normal adjacent tissue, which mostly lacked LDHA and HIF-1-alpha expression, displayed fewer vessels normally covered by pericytes, and T-cell infiltration in between normal epithelial cells.

Our data in TNBC models show that metabolic perturbations in malignant tumor cells can lead to changes in the TME via modulations of HIF-1 response, vessel architecture, permeability, and

leakiness, affecting T-cell infiltration and tumor cell extravasation, which can contribute to the response to ICB. Initial results in human TNBC suggest the presence of glycolytic tumor cells similarly reshape the TME.

P455

FUNCTIONAL DISCOVERY OF TARGETABLE DEPENDENCIES IN RECURRENT GLIOBLASTOMA

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A major challenge in the treatment of aggressive cancers is the presence of treatment-resistant tumor cells that eventually lead to disease recurrence, leaving patients with few options for effective second-line therapy. Although several studies have profiled the genomic and transcriptomic landscapes of aggressive cancers at diagnosis (primary disease) and relapse (recurrent disease) [Korber et al, Cancer Cell 2019; Lambo et al, Nature 2019; Shlush et al, Nature 2017; Abbosh et al, Nature 2017; Morrissy et al, Nature 2016; Johnson et al, Science 2014], they contain no functional genomic profiles of treatment resistance and post-treatment evolution to recurrence. Here we present the first set of genome-scale functional genetic CRISPR-Cas9 screens of patient-matched primary and recurrent glioblastoma (GBM) models, which yield the first functional genetic insights at tumor recurrence in any solid tumor, but also present the first set of functional genetic screens at tumor relapse post-standard of care. Through phosphoproteomic profiling, we introduce a novel PTP4A2-ROBO1 signaling axis that has not been described yet in GBM or other cancers, and demonstrate its targetability in patient-derived GBM models.

CRISPR-Cas9 whole genome screening, and mutational, proteomic and transcriptomic profiling were undertaken of a matched primary-recurrent GBM pair and multiple recurrent GBM patient lines. Phosphoproteomic profiling identified an interaction between top essential gene PTP4A2 and axonal guidance gene product ROBO1, as well as its downstream signaling pathways. Genetic and pharmacologic knockdown of PTP4A2 was accomplished using a small molecule inhibitor and CRISPR KO respectively. Using patient-matched primary and recurrent cancer models, as a proof-of-principle validation of our findings, we engineered and constructed both a small molecule inhibitor and a biologic against different targets in the same signaling pathway, and demonstrate efficacy for both with in vitro and in vivo patient-derived models of recurrent GBM.

We identify protein tyrosine phosphatase 4A2 (PTP4A2) as a top essential gene, and a novel modulator of self-renewal, proliferation and tumorigenicity at GBM recurrence. We show that convergence of PTP4A2 dephosphorylation activity and ROBO1-mediated signaling drives recurrent GBM invasion, stemness and growth. Mechanistically, genetic perturbation and a small molecule inhibitor of PTP4A2 repress tumor cell invasion and growth through a signaling axis with roundabout guidance receptor 1 (ROBO1) and β -Catenin. Importantly, engineered anti-ROBO1 single-domain antibodies mimic the effects of PTP4A2 inhibition. PTP4A2-targeted therapeutic strategy using a pan-PTP4A inhibitor was limited by poor penetrance across the blood brain barrier (BBB) in vivo. To overcome this limitation and leveraging our novel discovery of a PTP4A2-ROBO1 signaling axis, we engineered a novel second generation chimeric antigen receptor (CAR)-T cell therapy that elicits specific and potent anti-tumor responses in vivo. Not only does a single dose of anti-ROBO1 CAR-T cells double median survival in an in vivo patient-derived xenograft (PDX) model of recurrent glioblastoma, this therapeutic also eradicates tumors in ~50% of mice engrafted with PDX models of lung-to-brain metastases and pediatric medulloblastoma.

We present the first functional genetic characterization of patient-matched primary and recurrent cancer models. As a proof-of-principle validation of our findings, we engineered and constructed a novel and highly potent CAR-T cell therapy that is effective against multiple treatment-refractory brain tumor indications. We conclude that functional reprogramming drives tumorigenesis and dependence on a PTP4A2-ROBO1 signaling axis at GBM recurrence, and demonstrate its

targetability through small molecule inhibition, and ROBO1-CAR T cell therapy.

P456

INTRATUMORAL CD40 AGONIST SOTIGALIMAB IN COMBINATION WITH PEMBROLIZUMAB INDUCES RAPID ACTIVATION OF DENDRITIC CELLS AND MACROPHAGES AND DRIVES ANTI-TUMOR RESPONSES IN NONINJECTED DISTANT TUMORS IN METASTATIC MELANOMA

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The use of checkpoint inhibitors (CPI) has become an essential modality in the treatment of metastatic melanoma (MM). However, long-term survival and durable remission rates remain low and new treatment options are needed to improve clinical outcomes. CD40 promotes antigen presentation, stimulates T-cell responses, and reprograms immunosuppressive macrophages. Our pre-clinical studies have demonstrated that intratumoral (IT) CD40 activation induced systemic anti-tumor effects and synergize with anti-PD-1 therapy. This study was initiated to profile immune changes to therapy in both tumor tissue and blood samples from the CD40 agonist sotigalimab in combination with pembrolizumab in metastatic melanoma trial (NCT02706353)

Serial blood and tumor tissue samples were collected to perform in-depth analysis of immune cells using nCounter® gene expression assay, TCR sequencing, single-cell RNA sequencing (ScRNA-seq), single-cell assays for transposase-accessible chromatin (scATAC-Seq), and multiplex immunofluorescence imaging.

Efficacy analysis of thirty-two CPI treatment naïve MM with disease evaluations demonstrated an ORR of 47% in distant lesions and a disease control rate (DCR) of 81%. The ORR at the RP2D was 50% (12/24) and DCR was 96% (23/24). To determine the impact of the IT administration of sotigalimab alone on the local immune response, gene expression using NanoString nCounter gene Human PanCancer Immune Profiling panel was performed on 23 matched tumor biopsies taken before and 24 hours after sotigalimab administration. We observed that sotigalimab induced NF-κB and MAPK pathways, and significantly increased genes expressed in a variety of antigen presenting cells (APCs) as well as a significant enrichment in numerous gene sets related to immune activation and cell signaling pathways (TNF-α signaling via NFκB, interferon gamma response, inflammatory response, and IL6 JAK STAT3 signaling) consistent with CD40 activation. We also sought to compare differences in gene expression of specific immune cell populations and observed an upregulation of transcripts associated with DC (CD209, HSD11B1, CCL13, CCL1, CCL17, CCL22, NPR1, PPFIBP2, LAMP3, and EBI3) and macrophages (CD163, CD68, CD84, and MS4A4A). Multiplex immunofluorescence imaging confirmed a significant increase in the density of CD11c+DC-LAMP+CD40+ activated DC after sotigalimab treatment. Consistently, paired-ScRNA-seq analysis and scATAC-Seq in blood samples demonstrated an early increase in the fractions of DCs, classical monocytes and non-classical monocytes after treatment. These results demonstrate that sotigalimab engaged CD40 pathway and induced rapid APCs activation. To assess whether sotigalimab in combination with pembrolizumab can induce an inflammatory response and can turn cold tumors hot, gene expression profiling of the local and distant tumor lesions was performed at baseline and week 6 after treatment. Post injection biopsies showed robust upregulation of the genes associated with DCs, macrophages, and B cells and effector T-cells in local lesions accompanied by an increase in T cell activation genes at distant lesions. Additionally, TCR sequencing analysis showed an increase in T-cell infiltration and clonality after treatment in both the local and distant tumors. Analysis of clone sharing and dynamics between local and distant tumors showed an expansion of the number of shared clones between these tumors post treatment including clones with significantly greater

abundance. New clones with significantly higher abundance also were found at week 6 in both local and distant lesions. All these immunologic changes were correlated with clinical response.

Results from this trial demonstrated that combination of sotigalimab with pembrolizumab has a notable clinical response and generated robust innate and adaptive immunologic response that extended beyond the injected local lesions.

P457

THE DESIGN OF A NOVEL PRMT5 BISUBSTRATE INHIBITOR USING CLICK CHEMISTRY

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Significance: The protein-arginine methyltransferases (PRMT) family of proteins function to methylate numerous nuclear and cytoplasmic substrates and play a critical role in the regulation of important biological processes. PRMT5 specifically regulates certain immune response pathways including T-cell proliferation, T-cell activation-induced expansion, and B cell development through the prevention of p53-dependent and p53-independent blocks in Pro-B and Pre-B cells. Thus, PRMT5 has also been shown to be overexpressed in B cell lymphomas. The catalytic activity of PRMT5 is dependent on the enzymes catalytic binding site and s-adenosyl methionine (SAM) co-factor. Therefore, we believe the design of a novel bisubstrate PRMT5 through the use of Click chemistry could be a highly effective treatment in the modulation of B cell proliferation in B cell lymphoma.

We proposed the development of a bisubstrate PRMT5 inhibitor by appending an azo and alkyne functional group to various scaffolds, which recognize the allosteric site and SAM cofactor site individually. We then used a previously published fused bisubstrate inhibitor from Merck and to generate analogs of this inhibitor through the Click chemistry design. These molecules were built using ligand-based design functions in Molecular Operating Environment (MOE) and sequentially optimized by Gaussian, which were both accessed through the Ohio Supercomputer Center (OSC). These ligands were then docked using MOE and their potencies were analyzed. Following final optimization these ligands will be synthesized and tested so the potency can be compared to that of current PRMT5 inhibitors.

Through preliminary docking methods and computational analysis of PRMT5 we have worked to develop several target sites within PRMT5's catalytic pocket which could allow inhibitors to effectively undergo the click reaction when entering the catalytic pocket of PRMT5. In addition, we have begun optimizing several ligand combinations which are capable of effectively inhibiting the enzyme.

As we continue working on development and further testing, we aim to synthesize our inhibitors and conduct various biochemical assays testing their potency. The use of Click Chemistry to create a novel bisubstrate inhibitor of PRMT5 has shown promise in our development phase. With several residues supporting interactions which could facilitate the Click reaction and aid in inhibitor selectivity, we believe the use of a Click Chemistry driven bisubstrate inhibitor could be an appealing approach towards inhibition of PRMT5 and the treatment of B cell lymphomas.

P458

REPROGRAMMING MYELOID CELLS BY JAK INHIBITION TO ENHANCE CHECKPOINT BLOCKADE IMMUNOTHERAPY

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Despite the transformative effect of immunotherapy on cancer treatment, the majority of patients fail to respond. Among the key biological factors limiting immunotherapy response rates are the failure of myeloid cells to effectively present antigen and the myeloid-driven suppression of T and NK cell function. Reprogramming myeloid cells from a suppressive state into an immune-enhancing state is a goal of significant translational interest. Using preclinical and clinical studies, we investigated the potential of JAK inhibitors to enhance the efficacy of checkpoint inhibitors in a myeloid-dependent manner.

The combination therapy of systemic treatment with the JAK inhibitor ruxolitinib with anti-PD1 + anti-CTLA4 was evaluated for efficacy and biomarkers compared to checkpoint inhibitors (ICI) alone in four murine immunocompetent models of cancer. Tumor-infiltrating, blood and lymphoid organ immune cells were phenotyped using single-cell transcriptomics, functional assays and flow cytometry. The combination therapy was clinically tested in an investigator-initiated Phase I/II clinical trial of ruxolitinib with nivolumab in relapsed or refractory Hodgkin lymphoma (NCT03681561). Patients who previously failed to respond to ICI received ruxolitinib for 1 week then nivolumab every 4 weeks concurrent with ruxolitinib. Hematologic, transcriptomic and flow cytometric analyses were performed on peripheral blood collected at baseline and after ruxolitinib treatment.

In multiple immunocompetent mouse tumor models (A20, LLC1, MC38), ruxolitinib enhanced tumor control by checkpoint inhibitors and this effect was associated with loss of suppressive gene expression, acquisition of immunostimulatory markers and T cell stimulatory activity in myeloid cells. Ruxolitinib limited the upregulation of immunosuppressive genes in bone marrow cells in part by blocking G-CSF signaling and in combination with checkpoint inhibitors induced MHC-II expression in tumor-infiltrating myeloid cells. Depleting monocytic or granulocytic cells abrogated the beneficial effects of ruxolitinib.

Clinically, Hodgkin lymphoma patients who failed to respond to anti-PD1 therapy showed a best disease control rate of 63% (12/19) including 5 complete responses in evaluable patients treated with ruxolitinib and nivolumab. Ruxolitinib treatment did not impair T cell counts or T cell cytokine production but significantly reduced the neutrophil-to-lymphocyte ratio and the expression of myeloid derived suppressor cell markers in monocytes compared to pre-treatment. Complete responders showed a significantly greater reduction in the neutrophil-to-lymphocyte ratio and the percentage of myeloid derived suppressor cells in peripheral blood than other patients.

These results support the therapeutic potential of JAK inhibition in combination with checkpoint inhibitors in cancer and identify myeloid cell modulation as a key mechanism of efficacy.

P459

RACIAL DISPARITIES IN CLINICAL OUTCOMES OF METASTATIC RENAL CELL CARCINOMA (mRCC) PATIENTS RECEIVING IMMUNOTHERAPY: A POPULATION-BASED ANALYSIS

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Immune checkpoint inhibitors (ICIs) have emerged as a significant therapeutic strategy in the management of metastatic renal cell carcinoma (mRCC). Clinical trials demonstrated that first-line ICIs-based combination significantly improved the survival outcomes of mRCC compared to the standard regimens, such as tyrosine kinase or mTOR inhibitors. However, ethnic and racial minority patients are often underrepresented in these trials, leading to limited knowledge about ICI-specific survival outcomes for mRCC across different racial and ethnic groups. We aim to examine the impact of race and ethnicity on the ICI-specific survival outcomes of mRCC.

The data of 4858 metastatic renal cell carcinoma (mRCC) patients diagnosed between 2014 and 2019, who received ICI-based regimens, were obtained from the National Cancer Database (NCDB). The Kaplan-Meier method and Log-rank test were performed to analyze the survival outcomes. Cox Proportional Hazards regression was employed for multivariate analysis of factors associated with survival. Models were adjusted for age, sex, comorbidities, treatment centers, and grade. All statistical analyses were performed using SPSS version 29.0, and a p-value of less than 0.05 was considered statistically significant.

The majority of the patients were White (n =4180), followed by Black (n =383) and Asian (n =102). The median overall survival (mOS) of the mRCC patients treated with ICI was 22.6 (95% confidence interval [CI] 21 - 24.1) months. The 3- and 5-year OS rates were 37.5% (95% CI 35.5 - 39.7) and 25.9% (95% CI 23.4 - 28.7), respectively. The mOS of Whites (23.2 months, 95% CI 21.6 – 24.7, p<0.001) and Asians (22.2 months, 95% CI 16.4 – 55.1, p= 0.047) were higher compared to Blacks (14.8 months, 95% CI 11.9 – 19.2, p<0.001). The 3-year OS of Blacks, Whites, Asians were 22.6% (95% CI 16.7% - 30.6%), 38.2% (95% CI 36% - 40.5%), 41.3% (95% CI 29.9% - 57.1%) respectively. In multivariate analysis, Blacks had higher mortality compared to Whites (HR 0.70, 95% CI 0.58 – 0.84, p<0.001). The mortality of Asians was better when compared to Blacks, although was not statistically significant (HR 0.71, 95% CI= 0.48 – 1.04, p= 0.08). Private insurance (HR 0.7, 95% CI 0.53 - 0.94, p= 0.016) and cytoreductive nephrectomy (HR 0.77, 95% CI 0.62 - 0.96, p= 0.02) were independent predictors of better OS in mRCC patient who received ICI. Treatment at Comprehensive Community Cancer Program compared to academic centers (HR 1.46, 95% CI 1.14 - 1.85, p= 0.002), lymph node invasion (HR 1.29, 95% CI 1.05 - 1.58, p= 0.015), and radiotherapy (HR 1.34 [95% CI 1.08 – 1.68]; p =0.007) were independent predictors of worse OS.

Our real-world analysis shows that Black patients had significantly worse OS than other races, independent of sociodemographic, clinicopathologic, and treatment-related factors. This highlights the need for personalized treatment approaches and increasing the diversity of clinical trials population. Future research is required to understand the underlying reasons for these disparities, including potential genetic or biological differences, as well as social and environmental factors.

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UNDERSTANDING SECOND GENERATION CHIMERIC ANTIGEN RECEPTOR SIGNALLING USING PHOSPHOPROTEOMICS FOR THE TREATMENT OF GLIOBLASTOMA

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Chimeric Antigen Receptors (CARs) targeting of CD19 or BCMA proteins in late-stage haematological adult and paediatric cancers have shown significant increases in patient survival, leading to FDA approval and integration into clinical care. The FDA approved CARs are all second-generation and contain either CD28 or CD137 signalling domains. A previous study by Stan Riddell's group comparing CD28 and CD137 signalling using phosphoproteomic analysis, reported minimal differences between the two CARs. They went on to determine that this was due to the transmembrane domain choice rather than the co-stimulation domain. It remains to be determined if other co-stimulation domains, such as CD27, CD134 or CD278, also have conserved signalling in a CAR and thus the variation in phenotype between CARs is a measure of structural variations rather than differences in signalling pathways. These data are important in that it will help to determine if efforts should be focused on different signalling components or structural variations to better design CARs to enhanced efficacy in cancers such as glioblastoma, where CARs are presently less effective.

To examine differential signalling pathways we constructed a standardised monomer CAR library (CD3z, CD27CD3z, CD28CD3z, CD134CD3z, CD137CD3z, CD278CD3z) varying only the co-stimulation signalling domain. These CARs were transduced into a T cell lymphoma cell line to facilitate standardisation of CAR surface expression. Each CAR was stimulated via agonist antibody for the duration of normal synapse signalling and kinetic samples were generated and processed for Data Dependent Phosphoproteomic Analysis (DDA phosphoproteomics). Several bioinformatic strategies were then employed, including Kinase Substrate Enrichment Analysis (KSEA).

All six CARs were validated in primary T cells for antigen dependent cytotoxicity and cytokine production. Phosphoproteomic analysis shows that each CAR has a unique signalling program with only ~20% significantly changed phosphoproteins shared between each CAR. KSEA provided a number of potential pathways that could be targeted. A literature search provided six rational molecular inhibitors in preclinical and clinical development that warranted exploration in combination with CAR T cells. Primary CAR T cells were then combined with each inhibitor and examined for cytotoxicity and cytokine production in co-culture assays with antigen positive target glioblastoma cell lines.

That co-stimulation domain choice is an important consideration when developing combination CAR T cells with molecular therapies for glioblastoma.

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DEVELOPING NOVEL TARGETING CARS FOR GLIOBLASTOMA

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Glioblastoma is a devastating brain tumour with an alarmingly low survival rate of 5% beyond 5 years. Current treatments are insufficient, and new targeted therapies are urgently needed to improve patient outcomes. Chimeric Antigen Receptor (CAR) T cell therapy has revolutionized the treatment of some types of blood cancers by equipping a patient's immune cells with synthetic receptor that targets and destroys cancerous cells. However, there is a critical need for effective CAR T cell therapies for glioblastoma patients.

Our research team has developed a pipeline to identify novel targets using cell surface proteomics of primary glioblastoma tumours direct from surgical resection. As a proof of concept, we first developed a novel CAR targeting the Epidermal Growth Factor Receptor Variant III (EGFRvIII), a specific mutation expressed in a subset of glioblastoma cases, to generate and test novel CAR T cells for efficacy against glioblastoma.

Our new high-affinity EGFRvIII CAR demonstrated highly effective anti-tumour activity in two orthotopic in vivo models of glioblastoma. We mapped the epitope binding domain utilising deep mutational scanning and then we conducted comprehensive safety testing confirming that this CAR is likely to be safe for clinical translation, even in tissues with high EGFR expression such as keratinocytes.

Our high-affinity EGFRvIII CAR is effective and is ready for clinical translation. This CAR has established a versatile and robust pipeline for the development of novel CAR T cell therapies. With this pipeline in place, we aim to iterate and develop further targeted CAR T cell therapies for glioblastoma patients using the novel targets identified in our cell surface proteomics datasets.

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A NOVEL IMMUNE CHECKPOINT ROLE FOR THE FES NON-RECEPTOR TYROSINE KINASE

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Immunotherapies are a promising emerging pillar of cancer treatment, but they still face many barriers due to the immunosuppressive nature of cancer. Cancer immunotherapy relies on the interplay between innate and adaptive immune responses. One way of stimulating such responses, known as immunogenic cell death (ICD), involves release of tumour-associated antigens and damage associated molecular patterns (DAMPs). These DAMPs function to recruit and activate innate immune cells including antigen-presenting cells (APCs) through engagement of pattern recognition receptors (PRRs), subsequently leading to production of the pro-inflammatory Signal 3 cytokines required for activation of adaptive immune cells (e.g., cytotoxic T lymphocytes [CTLs] and natural killer [NK] cells). The non-receptor tyrosine kinase Fes suppresses innate immune responses in APCs by inhibiting components of the PRR signaling cascade. In non-cancer contexts, this negative regulation of APCs by Fes may guard against consequences of overactive innate immunity, including endotoxic shock or autoimmune disease. However, this same inhibitory effect on APC function may also serve as a checkpoint to successful anti-cancer immunotherapy, by obstructing efficient priming of cancer specific CTLs by APCs. Therefore, by inhibiting Fes, we hypothesize there will be greater Signal 3 cytokine production, resulting in greater CTL activation, and therefore improved tumor control.

Using bone marrow derived macrophages (BMDMs) from wildtype (WT) or Fes knockout (*fes*^{-/-}) mice, we assessed PRR signal transduction cascades regulated by Fes using immunoblot analysis following stimulation with Toll-like receptor 4 agonist, lipopolysaccharide. We also assessed transcription of key Signal 3 cytokine genes by qRT-PCR, and analyzed the ability of SIINFEKL peptide loaded BMDMs to prime CTLs from OT-1 mice (which express a T cell receptor that recognizes the SIINFEKL peptide) in an antigen cross-presentation co-culture assay. Using syngeneic orthotopic mouse engraftment models of triple negative breast cancer (EO771) and melanoma (B16-F10) we compared the ability of treatment with doxorubicin (which induces ICD) or anti-PD-1 (immune checkpoint inhibitor) plus doxorubicin to control tumor growth and prolong survival in WT and *fes*^{-/-} mice. Tumors and spleens were also harvested, and immune profiles analyzed by flow cytometry.

In vitro, *Fes*^{-/-} BMDMs displayed stronger PRR signaling and increased IFN β , IL-12, IL-1 β , and TNF α RNA transcripts following LPS stimulation compared to WT BMDMs; and in antigen cross-presentation co-culture assays, LPS stimulated *fes*^{-/-} BMDMs had improved CTL priming capabilities compared to WT BMDMs. In vivo, tumor growth was slower in *fes*^{-/-} mice, which survived longer in both the EO771 and B16F10 engraftment models relative to WT mice. Doxorubicin treatment slowed tumor growth and extended survival, and the combination of doxorubicin and anti-PD-1 was more effective in *fes*^{-/-} compared to WT mice. This was especially striking in the B16F10 model which has been shown to be resistant to PD-1 therapy. Immune profiling of tumors showed correlative increases in CTL activation and PD-1 positivity in *fes*^{-/-} mice, as well as increased activation and PD-1-positivity of NK cells. Additionally, we observed a shift from predominately M2-polarized (pro-tumorigenic) tumor-associated macrophages to predominantly M1-polarized (anti-tumorigenic) in *fes*^{-/-} compared to WT mice.

These results implicate Fes as a potential novel immune checkpoint whose inhibition may enhance anti-cancer immunotherapy by suppressing its role in dampening inflammatory Signal 3 cytokine production by APCs.

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SOCS1 DELETION ENHANCES ANTI-TUMOUR IMMUNE RESPONSES

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Suppressor Of Cytokine Signaling (SOCS) 1 is a critical negative regulator of cytokine signalling, selectively regulating interferon (IFN) and interleukin (IL)-2 family signalling. Growing evidence suggests SOCS1 is an important immune checkpoint in cancer.

To investigate the tumour cell intrinsic contribution of SOCS1 we have deleted it from 6 different mouse cancer cell lines and evaluated their tumour growth in syngeneic mouse models.

SOCS1 knockout (KO) consistently resulted in reduced tumour burden in metastatic models of melanoma and breast cancer. The decrease in metastatic growth of the SOCS1 KO B16F10 melanoma cell line, correlated with an increase in the number of immune cells in the tumour microenvironment in the lung.

As expected, IFN γ treatment of SOCS1 KO cells in vitro resulted in an enhanced and prolonged signalling response. To identify changes in expression of genes and proteins relevant to tumour biology, a global secretome, proteome and transcriptome analysis was performed. IFN γ treatment of B16F10 cancer cells revealed an enhanced IFN γ signature with upregulation of key pathways that promote anti-tumour immunity. Upregulation of MHC class I and CXCL chemokine family proteins were independently confirmed in vitro and are now being investigated as the in vivo mechanism for enhanced control of SOCS1 KO cancer cells.

P464**DECIPHERING THE DETERMINANTS OF IMMUNOTHERAPY RESPONSE IN “HOT” TUMOR MICROENVIRONMENTS**

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Despite the clinical success achieved with checkpoint inhibitors, immunotherapy (IT) still remains ineffective for many cancer patients. This is especially true for immunologically “cold” tumors, which lack a significant number of tumor-infiltrating T cells. Conversely, “hot” tumor microenvironments (TME), yet adaptive immune-rich ecosystems, have been significantly associated with better prognosis as well as favorable IT response. Inflamed tumors typically contain ectopic tertiary lymphoid structures (TLSs), which mimic lymph nodes. However, the degree of the pre-existing immunity might be variable in terms of type and density, and “hot” tumors, even comprising TLSs, but not responding, or developing secondary resistance to IT are counted. Here we explore the complexity of TLSs, as well as the intra-tumoral pattern and role of CXCL13 and its companion receptor CXCR5, one of the major TLS-associated chemokines.

Our study comprises retrospective and prospective cohorts of melanoma patients receiving anti-PD1/PDL1, irrespective of setting (metastatic, adjuvant, neoadjuvant), and pre-treatment Head and Neck Squamous Cell Carcinoma (HNSCC) samples from patient progressing or non-progressing to first- or second-line anti-PD1 therapy. Moreover, a cohort of pre and post-therapy lesions from dendritic cell-vaccinated melanoma patients has also been analyzed. Pathologist-guided immunohistochemistry (IHC) screening approach was established on Formalin-Fixed-Paraffin-Embedded (FFPE) sections to identify the abundance and reciprocal distribution of 4 major TME markers (CD3/CD20; CD8/CD163 double IHC). Marked TLS presence was followed by staining with CD21 and CD23 characterizing TLS maturity. The same FFPE is analyzed by whole-exome and transcriptome sequencing. Moreover, 5' single-cell RNA sequencing (scRNAseq) on prospectively collected IT-resistant (ITr) lesions was conducted. To spatially validate major signature leading genes, an in-house sequential IHC approach was developed to interrogate up to 8 markers on the same FFPE slides coupled with the in situ RNA transcript analysis. Cancer cell lines from both pathologies are also screened for CXCL13/CXCR5 and applied in in vitro assays.

We discovered a nuance of B cell infiltration in lesions collected from both melanoma and HNSCC patients, spanning from scattered to well-organized intra-tumoral and/or peri-tumoral TLSs. We are characterizing these tumor-infiltrating B cells in context with other TME elements, such as T cells, myeloid cells, and cancer cell states, and correlating the data with the clinical outcomes. Notably, paired pre- and post-vaccine melanoma transcriptomes highlight the upregulation of many TLS signature genes, which reached statistical significance when grouping lesions according to CD8+ T cell expansion measured by IHC (post/pre CD8 ratio>1): MS4A1 (0.0318), CCL19 (0.0072, CXCL13 (0.0163) SELL (0.0252). Remarkably, in the context of anti-PD1 setting, scRNAseq revealed the occurrence in ITr progressing lesions of CXCL13-expressing CD8 clusters resembling dysfunctional

and exhausted (the latter lacking IFNG and CD69 expression) CD8+ T cells, and a CD4+ T regulatory cluster (FOXP3, TNSFRF4). CXCL13 production upon in vitro modeling of long (>5days) T cell:tumor antigen exposure was also confirmed.

Whether CXCL13 production, not derived from canonical CD4+ T follicular helper cells and CD21+ follicular dendritic cells, but rather from dysfunctional/exhausted intra-tumoral CD8+ T cells or CD4+ regulatory cells, is still functioning as B cell attractant or might cover other roles is still unknown. We found CXCR5 expression, albeit mainly intracellularly, in both HNSCC and melanoma cell lines and we are now applying 3D tumor models and Zebrafish embryos to dissect the tumor direct function of intra-tumoral CXCL13.

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IDENTIFICATION OF SMALL MOLECULAR COMPOUNDS SELECTIVELY TARGETING REGULATORY T CELLS

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Tumor progression in various cancer types is often accompanied by tumor immune evasion acting through a variety of parallel mechanisms, one of which is suppression of anti-tumor immunity by regulatory T cells (Tregs). This phenomenon is associated with a poor prognosis, highlighting the significance of exploring Treg-targeted therapies. While previous clinical trials have predominantly focused on targeting Treg surface markers to modulate their function, it has resulted in undesired immune-related side effects. Consequently, there is a growing interest in identifying and validating alternative Treg targeting strategies. Here, we explored the possibility of perturbing the expression or function of the key lineage-defining transcription factor for Treg differentiation and function, known as Forkhead box P3 (FoxP3).

To identify small molecules interfering with FoxP3 expression or function, we started on a comprehensive screening approach. A library comprising 1522 approved drugs was evaluated using a unique and efficient high-throughput flow cytometry assay that employed a phenotypic, cell-based methodology. Through this screening process, we successfully identified some promising candidate compounds that underwent initial validation tests, satisfying our selection criteria. To further expand our exploration, we employed *in silico* prediction techniques to identify drug-like analog compounds. Subsequently, sub-libraries were constructed, and the effects of these compounds on Tregs were investigated.

Our findings regarding one of the drugs and its analogue compounds demonstrate their ability to significantly reduce FoxP3 levels and impair the suppressive function of Tregs. Additionally, an analogue compound selectivity targeting Tregs over effector T cells diminishes the expression of specific Treg markers including PD-1, ICOS, and LAG-3, which is associated with its suppressive function.

Further investigation, including an examination of the expression levels of other proteins involved in this process, is necessary to deepen our understanding. However, such identified hits show potential for modulating FoxP3. Moreover, they can serve as valuable tools for investigating the precise tumor immune evasion mechanisms by Tregs. By gaining insights into these mechanisms, we can expand our range of strategies for enhancing antitumor immunity.

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T-FINDER: A HIGHLY SENSITIVE, PAN-HLA PLATFORM FOR FUNCTIONAL T CELL RECEPTOR AND LIGAND DISCOVERY

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T-cell activation by antigen-presenting cells (APCs) defines both protective and pathological adaptive immune responses. T cells express clonally unique TCRs, and next-generation sequencing can provide cohorts of “TCRs of interest” based on phenotype and/or extent of clonal expansion¹, but no information regarding functional specificity. Mapping cognate ligands of disease-relevant TCRs remains a technologically challenging goal, with effective, unbiased, high-throughput methods to functionally identify both class II and class I HLA-presented antigens and their cognate TCRs currently underdeveloped. In silico approaches predicting the identity and HLA restriction of a TCR ligand, while offering promising throughput and efficiency, are limited by the quality of their training datasets, and generalize poorly to novel epitopes that do not share structural similarity to the training data. Major obstacles to deconvoluting TCR specificities include HLA diversity (more than 12,000 known alleles) and the ability to screen libraries of putative targets for class I- and class II-presented epitopes simultaneously. To address these bottlenecks, we designed a highly sensitive TCR deconvolution platform that avoids engineering the molecular TCR:target interaction, is agnostic to predicted epitopes and presenting alleles, operates for both class II and class I-presented peptides, and is independent from mass spectrometry, tetramer labeling, and other ligand/TCR prediction steps. Based on its ability to physiologically identify and intrinsically validate TCRs and their epitopes, we named this platform T-FINDER (T cell Functional Identification and (Neo)-antigen Discovery of Epitopes and Receptors).

Key features of T-FINDER include a highly sensitive (greater than 100-fold signal-to-noise ratio) and specific (absence of false positive activating interactions) TCR reporter cell line, which can be used to validate even low-affinity/avidity interactions, and a novel approach to epitope processing which overcomes previously unobserved challenges to class II HLA presentation of cDNA-encoded antigens. These tools enable the platform to be flexible across receptors (engineered TCRs, CARs, CD3 engagers, etc.), antigen-presenting cells (professional APCs, tumor cell lines, organoids, primary tissue), and ligands (peptides, genetic constructs encoding proteins or fragments up to 400 amino acids, biotherapeutics, CAR targets) at scale.

After establishing and benchmarking the performance of our platform, we performed functional ligand deconvolution screens across infectious disease, autoimmunity, and oncology to demonstrate the versatility and potency of the system. In addition to validating gliadin-reactive TCRs in celiac disease and identifying novel epitopes from rheumatoid arthritis patient-derived expanded TCRs, we rapidly cataloged more than 50 tumor vaccine peptide-reactive (and, notably, CD4-derived) TCRs in diffuse midline glioma. Analyzing the functional HLA restriction and validated epitope-specific TCR repertoire enabled a deeper analysis of the mechanisms potentially driving anti-tumor responses and providing a foundation for precision cellular therapies against solid tumors.

With several alternatives to TCR and epitope discovery currently available, but none satisfying our strict requirements for potency and flexibility, we have established a multicomponent system that can be adapted to serve many needs across T cell-based applications. Importantly, T-FINDER drives new biological insights at a pace that was previously not possible, enabling and improving the development of immune-specific diagnostics and therapeutics.

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MODULATION OF TUMOR ASSOCIATED MACROPHAGES BY ARGINASE-1 SPECIFIC T CELLS

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Myeloid cells such as tumor associated macrophages (TAMs) often comprise a dominant fraction of the tumor immune infiltrate and are known to be major contributors to the establishment and maintenance of the immunosuppressive TME. TAMs exert their suppressive function by expressing proteins such as arginase-1(Arg1) which acts as a metabolic immune regulator by reducing availability of L-arginine and thus reducing T cell functionality and proliferation. Previously, a novel approach to target Arg1-expressing cells was proposed, based on earlier observation that spontaneous CD4+ and CD8+ T-cell responses against Arg1 can be found in cancer patients and healthy individuals. In the present study we examined the potential of Arg1-specific T cells as immune modulators of the tumor microenvironment through targeting of tumor associated macrophages.

To examine the impact of Arg1-specific T cells on TAM, (i) an in vitro model was established where differentially conditioned human primary myeloid cells and myeloid cell lines (THP-1 and MonoMac1) were used as target cells, (ii) anti-tumor activity of Arg1 peptide vaccine was assessed in syngeneic mouse models, and (iii) functionality of T cells isolated from Arg1 peptide vaccinated mice were tested by co-culturing with TAMs. The phenotypic changes in the TAM immune suppressive function were assessed using flow cytometry and protein multiplex immunoassays.

We demonstrate that human and murine Arg1-specific CD4+ T cells specifically recognize Arg1-expressing TAMs by producing pro-inflammatory cytokines. In co-culture experiments TAMs were directly targeted by both ex vivo-isolated and in vitro-generated Arg1-specific T cells. This led to a reprogramming of the TAMs as detected by increased expression of CD80 and HLA-DR in human in vitro models and increased CD80 and CD86 and reduced CD206 expression in murine ex vivo models. The Arg1-specific T-cell immune attack on Arg1-expressing TAMs was dependent on HLA II presentation and led to the local secretion of the pro-inflammatory cytokines IFN γ , TNF α , IL-2, IL-6, and IL-8.

We demonstrate that Arg1-specific T cells modulate an immune-suppressive tumor microenvironment by promoting a pro-inflammatory response and the reprogramming of TAMs. Our study provides a rationale for vaccination against Arg1 in several cancer indications where Arg1 is known to play an important role. With this rationale, we are currently undertaking IND enabling studies to explore this approach in a clinical setting.

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GUT BACTERIA ASSOCIATED WITH COLORECTAL CANCER EXPAND CD4- CD8- T CELLS ENDOWED WITH ANTITUMOR PROPERTIES

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Colorectal cancer (CRC) is the second leading cause of cancer-related mortality in the world. The composition of the CRC microenvironment, and in particular, infiltration by different types of immune cells, the so-called immune contexture, has been recognized to critically impact on CRC clinical course and to outperform the prognostic significance of TNM staging. Tumor infiltration by T lymphocytes has been recognized to predict increased survival probability. However, lymphocyte antigenic specificity and mechanisms underlying their anti-tumor effects are still largely unknown. Our group has previously identified intra-tumoral gut commensal bacterial species associated with high tumor-infiltrating lymphocyte densities and prolonged patient survival. These findings urge the analysis of the immunostimulatory capacity of these bacteria and the investigation of mechanisms underlying the elicitation of their anti-tumor potential.

Human peripheral blood mononuclear cells (PBMCs) from healthy donors (HD) or CRC patients were CFSE-labelled and co-cultured ex vivo with cryopreserved preparations of previously identified bacteria. T cell phenotypes, activation, and proliferation, were assessed after 5 days by flow cytometry, based on the expression of subset-specific markers, activation markers, and dilution assays, respectively. Clones of bacteria-reactive T cells were generated by limiting dilution method upon sorting as CFSEintCD25high. Cytokine production from culture supernatants was assessed by multiplex assay kit. Cytotoxicity of T cell supernatants on CRC cell lines was determined by flow cytometry based on Annexin V and live/dead staining, upon a 24h culture. Single-cell suspensions of processed fresh tumor tissues and adjacent mucosa obtained from CRC patients were analyzed by large-scale flow cytometry, and some of the primary tumor samples were expanded as patient-derived xenografts in s.c. injected NRG mice.

All tested bacterial species induced activation, proliferation, and cytokine production in T cells from HD and patients to comparable extents. Notably, T cell expansion required the presence of antigen-presenting cells. Expanded T cells mostly consisted of TCRαβ CD4-CD8- (double-negative, DN) effector memory T cells, and in part of MAIT and TCR gamma-delta T cells. Following activation, DN T cells upregulated innate-like receptors DNAM-1 and NKG2D and released cytotoxic molecules, such as IFNγ, TNFα, Perforin, and Granzymes. Cytotoxic cell supernatants derived from DN T cell cultures induced cell death in CRC cell lines. Detailed immune characterization of primary tumor samples by unsupervised clustering revealed abundant tumor-infiltrating DN T cell populations, which are currently the focus of our investigation.

Gut bacteria associated with immune infiltration of CRC are capable to expand unconventional cytotoxic DN T cell population from peripheral blood. These cells may induce tumor cell death by secreting cytotoxic factors. The therapeutic potential of bacteria-reactive DN T cells in vivo is currently under investigation.

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RESISTANCE MECHANISMS IN ADVANCED NSCLC AFTER IMMUNE CHECKPOINT BLOCKADE

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Advanced non-small cell lung cancer (NSCLC) without a targetable driver mutation is commonly treated in the first line with immune checkpoint blockade (ICB) +/- chemotherapy, depending on PD-L1 expression. Unfortunately, while most patients benefit from ICI, overall survival rates remain low and recurrence rates high (MacManus & Hegi-Johnson, 2022). Primary resistance rates are estimated at 75-90%, while acquired resistance rates between 32% and 64% have been reported for NSCLC (Schoenfeld & Hellmann, 2020). Current research has primarily focused on the discovery of biomarkers for response, using pre-treatment patient datasets (Litchfield, et al., 2021) (Anagnostou, et al., 2020).

Mechanisms of tumor escape on ICB, however, have been postulated, but rarely assessed in larger post-ICB datasets. Because of the evident clinical need, we've focused our study on ICB-resistant NSCLC patients in order to characterize the type and prevalence of resistance mechanisms in this population, as well as the timing of occurrence of those mechanisms.

If we encounter patients that cannot be categorized by previously described resistance mechanisms, we aim to uncover alternative reasons for their failure to respond. We have already collected a cohort of 167 newly sequenced advanced NSCLC patient samples supplemented with published sequencing data for a total of almost 300 specimens from non-responding patients. Roughly half of these are post-treatment samples, with matched pre-post treatment material available for 31 patients. For these samples either WES, RNAseq, WGS or a combination thereof is available.

The first results of this study will be presented.

Retrospective collection of patient samples for RNA and whole-exome sequencing. The study is based entirely on bioinformatic analysis of these samples.

To be presented

To be presented

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AN EX VIVO MODEL FOR FUNCTIONAL IMMUNE RESPONSES WITHIN THE RENAL CELL CARCINOMA TUMOR MICROENVIRONMENT

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Tumors comprise a diversity of supporting cell types including mesenchymal-derived cells, stromal, endothelial cells and infiltrating immune cells in addition to neoplastic cells. Together these various cell types and their secreted components establish a complex milieu termed tumor microenvironment (TME). The TME composition impacts immunological treatment responses, and recent efforts are aimed at developing patient-derived models that recapitulate the complex immune TME and immune cell functionality. Such models would allow testing of immunotherapy treatments and their detailed functional and mechanistic evaluation in comparison to reconstituted 2D co-culture models comprising few cell types.

We established an ex vivo renal cell carcinoma model allowing the testing of early immunological responses. The model utilizes as starting material renal carcinoma tissue obtained from nephrectomy surgeries, which is further processed into tumor dissociates. The tumor dissociates are embedded in a supportive extracellular matrix for subsequent culturing. Under these conditions, both adaptive and innate immune cell contexture resembled the parental tumor. Furthermore, the model preserved immune cell viability at high level for 2 days ex vivo. We utilized the model to discern early immunological responses to approved and experimental IO-treatments and T-cell stimulation using CD3/CD28/CD2 activator as a positive control. Immune cell responses to anti-PD1 checkpoint inhibition, STING-pathway and macrophage activation was monitored utilizing cytokine profiling, and Nanostring analysis was performed to obtain differentially expressed genes and pathways. Additional immune cell profiling from the tumor, adjacent healthy tissue and peripheral blood was performed using flow cytometry.

We acquired sufficient tumor material from six patients to establish the model for functional drug testing. Nanostring analysis verified the presence of major tumor infiltrated immune cell types, including prominent T- and NK-cells, macrophages, neutrophils and dendritic cells, for each patient model. The baseline cytokine profile was characteristic of high levels of both pro-inflammatory cytokines (eg. IL-15) and chemokines (eg. CXCL10, IL-8, IL6 and CCL3) produced by both adaptive and innate immune cells. Additionally, high VEGF-levels were observed in line with high vasculature of the renal tumors. Patient samples showed robust T-cell activation with anti-CD3/CD28 ex vivo stimulation. Modest pro-inflammatory response to PD-1 blockade and macrophage activation was observed and PD-1 blockade was accompanied with a negative feedback response as observed with upregulation of checkpoint molecules and other T-cell suppressive mechanisms (eg. IDO1). Each patient sample responded strongly to STING agonist treatment which was noticed by increased release of various cytokines and chemokines (e.g TNF-alpha, GM-CSF and CCL3, CCL5) to the TME. Gene expression analysis with Nanostring further verified a prominent type-I interferon response. These functional responses were not significantly correlated to any studied baseline immunological feature.

We established and validated an ex vivo model testing for functional activation of intratumoral immune cells within a more physiological TME context. Our results are in line with known renal cell cancer immune biology. The results encourage to expand investigations utilizing patient-derived ex vivo models for resolving patient-specific response mechanisms.

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ARGINASE-1 VACCINE PROMOTES T CELL IMMUNITY AGAINST ARGINASE 1+ CELLS AND CONTROLS TUMOR GROWTH VIA IMMUNE MODULATION OF TUMOR MICROENVIRONMENT

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Arginase-1 (Arg1) is involved in several pathways that allow cancer cells to escape immune responses within the tumor microenvironment (TME). Although numerous strategies have targeted Arg1 in a clinical context, success has been limited. Harnessing the immune system to attack TME-related targets has recently shown promise as a potential treatment strategy. We previously demonstrated that immunization against Arg1 strengthens anti-tumor activity in mouse models (1). This research aims to build on these prior findings and elucidate the mechanisms by which the anti-tumor activity of the Arg1 vaccine functions.

The anti-tumor activity of the Arg1 peptide vaccine was evaluated in Arg1+ mouse models: MC38 and 4T1. The immune response prompted by the vaccine was confirmed via an IFN γ Elispot assay on splenocytes and tumor-isolated CD4 cells. Excised tumors were analyzed using immunohistochemistry, RNA sequencing, qPCR, and flow cytometry analyses.

Treatment with the Arg1 vaccine in two Arg1+ tumor models, MC38, and 4T1, led to the expansion of Arg1-specific T cells and a reduction of tumor growth. This was associated with a higher infiltration of CD4+ T cells in the TME and a decrease in Arg1+ expression in the lymph nodes of tumor-bearing mice from the 4T1 model. Moreover, F4/80 cells isolated from MC38 tumors showed a reduced Arg1 expression and an increased Fpr2 gene expression, a marker related to pro-inflammatory characteristics. The anti-tumor response to the ARG1 peptide treatment was enhanced when combined with anti-PD-1 monoclonal antibodies or other vaccines. Further analyses will investigate the cellular and molecular mechanisms at play in the Arg1 vaccine treatment.

Arg1 emerges as a promising target within the TME for immunotherapy. Targeting Arg1 through vaccination to bolster T cell immunity results in anti-tumor activity by skewing the balance from an immunosuppressive to a pro-inflammatory microenvironment. This presents a potential alternative to other strategies currently undergoing nonclinical and clinical trials. These results strengthen the clinical development of an Arg1 vaccine as a viable therapeutic strategy for several solid tumors.

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EXPLORING THE EFFICACY OF A NOVEL COMBINATORIAL CAR-T CELL-BASED SENOLYTIC APPROACH IN PROSTATE CANCER

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Prostate cancer ranks as the second most prevalent cancer among men worldwide and stands as the sixth leading cause of male mortality. Patients with metastatic prostate cancer, specifically those with disease progression following primary androgen ablation therapy, are considered refractory to hormonal therapy. Unfortunately, chemotherapy only provides limited survival benefits and mainly serves as palliative care in this context. Besides, prostate cancer is immunologically cold with a low tumor mutation burden, which translates to a low degree of anti-tumoral immune cell infiltration, mainly of NK and T cells. Numerous ongoing clinical trials investigating immune checkpoint blockade in prostate cancer have shown limited impact, highlighting the importance of identifying immune checkpoints that are specific to prostate cancer. This identification will play a crucial role in bolstering the effectiveness of immunotherapy for patients with prostate cancer.

By analysing available bulk-RNA and single cell sequencing datasets from prostate cancer patients, we found that BSKA2, a transmembrane protein expressed by epithelial cells, was upregulated in prostate cancer. BSKA2 is an adhesion protein that also displays immunomodulatory functions, in particular on T and NK cells. Interestingly, we found that one of its coupled receptors, BSKA-R, was upregulated in prostate cancer datasets, in particular by T and NK cells.

Additional analysis confirmed the upregulation at transcriptional and translational level of BSKA2 upon treatment of different human prostate cancer cell lines with chemotherapeutic drugs used in the clinic, such as Docetaxel and Palbociclib. Since the resistance to these treatments has also been associated to their capability of inducing senescence, we evaluated the correlation between senescence and BSKA2 upregulation. We observed that senescence induction further increased BSKA2 levels, both in vitro and in vivo. Indeed, the proteomic and bioinformatic analysis of PTEN-null prostate tumour from mouse models, identified BSKA among the upregulated transmembrane proteins expressed by senescent epithelial cells. Results from in vivo studies confirmed the induction of BSKA2 expression level upon senescence in PTEN-null mice treated with Docetaxel, and interestingly, in castration.

Based on the obtained data, we proceed with the design of CAR-T cells targeting BSKA2. These strategies were optimized by combining BSKA2 with a validated prostate cancer target, the prostate specific membrane antigen (PSMA), already used in prostate cancer diagnosis and therapy. We developed DualCAR T cells targeting PSMA and BSKA2 simultaneously, to increase selectivity and specificity. The more promising anti-PSMA and anti-BSKA2 scFvs sequences were selected from a scFvs phage display library and cloned into human T cells. In vitro and in vivo studies showed a higher activation and killing capability against human prostate cancer cell lines expressing both PSMA and BSKA2.

In conclusion, the study sheds light on the potential of BSKA2 as a viable target for immunotherapy in prostate cancer. By developing DualCAR T cells targeting BSKA2 in combination with PSMA, we demonstrated improved efficacy in killing cancer cells expressing both targets. This research offers a promising avenue for advancing immunotherapy approaches for patients with prostate cancer, especially those with limited treatment options due to disease progression following standard therapies.

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MODULATION OF NEUTROPHILS' METABOLISM IMPACTS THEIR FATE AND FUNCTION IN COLORECTAL CANCER PROGRESSION

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a) Introduction: tumor contexture has emerged as a major prognostic determinant and tumor infiltrating CD8+ T cells have been associated with a better prognosis in several solid tumors, including early-stage colorectal cancer (CRC). However, the tumor immune infiltrate is highly heterogeneous and understanding how the interplay between different immune cell compartments impacts on the clinical outcome is still in its infancy. We have recently described a novel CD8+ T effector memory population, which is characterized by high levels of Granzyme K (GZMK^{high} CD8+ TEM) and is correlated with CD15^{high} tumor infiltrating neutrophils. In addition, we have demonstrated that stromal cell-derived factor 1 (CXCL12/SDF-1) drives functional changes on neutrophils at the tumor site, promoting their retention and increasing the crosstalk with CD8+ T cells. Mechanistically, the interaction with neutrophils skewed CD8+ T cells towards a CD8+ TEM phenotype and to produce high levels of GZMK, which in turn decreases E-cadherin leading to poor prognosis.

b) Methods: here, we combine in vitro and in vivo approaches to investigate how neutrophil's fate and function are shaped by the tumor microenvironment in a novel perspective cohort of resectable CRC patients. We used multi-parametric flow cytometry, metabolic stainings, immunofluorescence on patient's tissues, bead-based multiplex ELISA and RNA sequencing on freshly isolated neutrophils to reveal the link between CD15^{high} neutrophil's state and worse prognosis.

c) Unpublished results: our new data indicate that CD15^{high} neutrophils have increase production of reacting oxygen species (ROS) and neutrophils extracellular traps (NETs) coupled with peculiar metabolic features. Pharmacologic modulation of neutrophil's metabolism impacts their fate and function, which determine their pro-tumorigenic behavior.

d) Conclusions: overall, our results highlight the importance of the tumor metabolic landscape in driving functional changes in immune along with tumor cells. In particular, targeting clinically-relevant metabolic pathways in neutrophils may represent an effective strategy to improve prognosis of high-risk CRC patients and, potentially, other types of gastro-intestinal tumors.

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HARNESSING MITOCHONDRIA TO EMPOWER CANCER IMMUNE SURVEILLANCE IN MULTIPLE MYELOMA

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Multiple myeloma (MM) is an incurable cancer of plasma cells, characterized by impaired innate and adaptive immunity, which poses a significant challenge for effective therapy and long-term disease control. Our recent findings highlight the crucial role of the major mitochondrial protease, ClpP, in MM growth through its involvement in maintaining mitochondrial homeostasis. Mitochondria, originating from bacteria, can release potent immunogenic signals into the cytosol upon damage. Both mouse and human ClpP null fibroblasts exhibit mitochondrial DNA release, followed by the activation of cGAS-STING and a type-I interferon (IFN) response. Given the emerging significance of STING in reshaping and stimulating anti-cancer immunity, we aimed to evaluate the therapeutic potential of targeting ClpP in MM cells to induce immune microenvironment activation through mitochondrial-dependent mechanisms.

We employed shRNA-mediated knockdown (KD) to manipulate ClpP expression in human MM cell lines. Subsequently, we assessed the activation of cGAS-STING, type-I IFN transcriptional response, and cytokine secretion. To evaluate the impact of ClpP manipulation on innate and adaptive immune cells, we optimized coculture platforms of MM cells with human dendritic and T cells, and performed flow cytometry analysis. To assess the in vivo effects of ClpP manipulation in MM cells on anti-tumor immunity, we injected ClpP-KD human myeloma cells into immune-compromised mice (NSG) and ClpP-knockdown murine myeloma cells into a syngeneic immunocompetent host (KaLwRij mouse model). We characterized the tumor onset, immune cell infiltration in the bone marrow and spleen, activation and exhaustion status of immune cells, and cytokine production profiles.

Through unbiased transcriptomics and proteomics analyses following ClpP ablation, we observed that human MM cells exhibited enrichment in signatures associated with immune activation, antigen presentation, and IFN signaling, similar to the changes observed in ClpP null fibroblasts. Western blot and RT-qPCR analysis confirmed the activation of cGAS-STING, evidenced by the phosphorylation of STING and TBK1, along with increased transcription of classical interferon-sensitive genes (ISGs). Furthermore, we verified the enhanced expression and exposure of HLA-I and HLA-II molecules, potentially contributing to increased antigen presentation.

In coculture experiments involving human dendritic cells (DCs) and supernatant derived from ClpP-KD or ClpP-mock MM cells, we demonstrated a significant increase in IFN β production in the former. Multiparametric flow cytometry staining revealed that conditioned medium from ClpP-KD MM cells rendered DCs more susceptible to activation and production of inflammatory cytokines. Indeed, these DCs upregulated CD86, CD80, and to a lesser extent HLA-DR (MHC-II), while showing elevated IL-1 β and IL-12 production. Additionally, DCs conditioned with ClpP-KD MM supernatant stimulated CD4 $^+$ T cells to produce more IFN γ .

In our in vivo experiments conducted in NSG mice challenged with human KMS26 MM cells, we observed a delayed tumor onset resulting from ClpP silencing, indicating a tumor-cell intrinsic effect. To assess the impact of ClpP-KD on anti-cancer immunity, we challenged immunocompetent mice with ClpP-KD or ClpP-mock 5TGM1 MM cells. ClpP-KD was associated with reduced tumor burden and lower bone marrow infiltration by Mo-MDSC. Additionally, the bone marrow of mice challenged with ClpP-KD 5TGM1 cells showed more CD3 $^+$ /CD4 $^+$ T cells and a reduced representation of exhausted CD8 $^+$ T cells.

Overall, our data define that ClpP ablation activates cGAS-STING in MM cells and induces a reshaping of the immune microenvironment towards a more effective anti-tumor immunity. Our

findings therefore establish modulation of ClpP as a novel immunogenic chemotherapy and pave the way for the exploration of mitochondria as targets to stimulate otherwise indolent anti-tumoral immunity against MM.

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ACTIVATING LYMPHOCYTE INFILTRATION IN TUMORS TO SPARK ANTI-TUMOR IMMUNITY

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An important factor limiting the development of anti-tumor immunity lies in the extent of tumor infiltration by immune cells, including tumor infiltrating lymphocytes (TILs) and dendritic cells, which is positively correlated to immune checkpoint blockade (ICB) response. Tertiary Lymphoid Structures (TLS) are found in chronic inflammations, autoimmune diseases, and cancer. They are characterized by a lymph-node (LN) like structure filled by B cell and T cell zones. TLS are associated with TILs and favorable outcomes in multiple cancers to the extent that they constitute a predictive biomarker for the responsiveness to ICB immunotherapy.

Here, we aim at inducing the formation of TLS within a breast cancer tumor to induce anti-tumor immunity. We have undergone to explore new methodologies to deliver TLS-inducing factors associated with the development of secondary lymphoid organs within the tumor microenvironment.

We observed that a TLS-inducing intervention slightly diminished the tumor growth compared to untreated mice. Furthermore, T and B cells as well as MECA-79+ vessels resembling High Endothelial Venules (HEV) of lymph nodes are increased in treated mice.

Altogether these data suggest that immunotherapeutic interventions purposed to induce TLS represent an attractive avenue to stimulate the infiltration of solid tumors by lymphocytes and stimulate anti-tumor immunity.

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IMMUNE CELL COMPOSITION AND FUNCTIONALITY IN MURINE IDH1WT/MUT GLIOBLASTOMA MICROENVIRONMENT AT SINGLE CELL AND SPATIAL RESOLUTION

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Glioblastoma (GBM) is the most prevalent primary malignant brain tumor in adults. Despite numerous conventional therapies, significant improvements in the survival outcomes of GBM patients have been elusive. IDH (Isocitrate Dehydrogenase) mutations have emerged as key players in glioma development and progression, particularly in lower-grade gliomas and secondary glioblastomas. However, the composition of the microenvironment in gliomas with distinct genetic backgrounds remains unresolved. This study aims to provide the most accurate and comprehensive insight to date into the immune microenvironment of murine glioma models with two distinct genetic backgrounds: wild type or harboring the IDH1 R132H mutation.

For this study, we utilized two murine glioma models: NRAS; shTP53-GFP; shATRX; wt/mutIDH1 and PDGFB; shTP53; shATRX; Ink4a; Arf^{-/-}; wt/mutIDH1. The mice were implanted with these models to investigate the differences in immune microenvironment composition, phenotype, and spatial localization. We employed a range of single-cell techniques, including Flow Cytometry, CITE-seq, and Visium Spatial Transcriptomics (10X Genomics). CITE-seq sequencing of CD45⁺ cells and Spatial Transcriptomics data were used to meticulously characterize and describe the proportions and phenotypes of 36 immune cell types, with a particular emphasis on their spatial localization within the tumor microenvironment. Additionally, Ligand-Receptor analysis was conducted to elucidate the differences in the intricate interplay between myeloid cells and lymphocytes under different conditions.

Our analysis revealed significant differences in the immune microenvironment between the IDH1wt/mut glioma models. In IDH1wt gliomas, there was a remarkable enrichment of T cells, particularly an abundant population of CD8⁺ exhausted T cell progenitors. However, their activity appeared to be dampened by myeloid cells expressing elevated levels of PDL1 and CD86, leading to heightened interactions with PD1 and CTLA4, as revealed by Ligand-Receptor analysis. Despite the increased levels of PDL1, the myeloid cells in IDH1wt gliomas exhibited a proinflammatory phenotype, characterized by elevated levels of Stat1, B7-1/2, and MHC2, as well as lower levels of Tgfb1 and Arg1. This favorable phenotype creates an ideal condition for immune checkpoint blockade immunotherapy.

The findings from this study hold great promise for the development of targeted immunotherapies for IDH1mt/wt patients, offering multiple potential therapeutic avenues. The meticulous characterization of the immune microenvironment in these distinct glioma models contributes valuable insights for future GBM studies. Importantly, this research underscores the significance of considering genetic background in the design of effective immunotherapeutic approaches. By unraveling the complexities of the immune-microenvironment interplay, we hope to pave the way for improved treatments and better outcomes for GBM patients in the future.

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PROCOAGULANT NEUTROPHILS PROMOTE RESISTANCE TO PROSTATE CANCER THERAPY

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Prostate cancer (PCa) is the second most frequent cancer diagnosis made in men and the fifth leading cause of death worldwide. Despite the advances in prostate cancer therapies targeting AR signaling, PCa patients invariably progress to metastatic castration-resistant prostate cancer (mCRPC).

We previously reported that the intratumor accumulation of CD11b⁺; Ly6G⁺; Ly6Clow myeloid cells (also known as PMN-MDSCs or immunosuppressive neutrophils) drives the onset of castration-resistance in both patients and mouse models by paracrine IL23 signals. Moreover, we demonstrated that inhibition of CXCR2 receptor can enhance the efficacy of both chemotherapy and androgen-deprivation therapy in prostate cancer.

By exploiting single-cell RNA sequencing, we further characterized immunosuppressive neutrophils in CRPC mouse models resistant to either enzalutamide or anti-IL23 treatment.

Our extensive analysis identified a novel subset of CD11b⁺Ly6G⁺ intratumor neutrophils that are a key extra-hepatic source of some coagulation factors. Within the TME, this procoagulant neutrophil subset enhances androgen-independent prostate tumor cell proliferation. Genetic and pharmacological inhibition of procoagulant neutrophils hinder tumor progression to castration resistance (CR) and synergizes with enzalutamide to prevent tumor growth.

These data were clinically validated by analyzing blood and tumor specimens from prostate cancer patients.

This study highlights a novel additional paracrine axis by which immunosuppressive neutrophils directly promote endocrine resistance in prostate cancer.

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TRANSLATION MACHINERY IS A THERAPEUTIC VULNERABILITY AND ITS TARGETING SUPPRESSES MDSCS RECRUITMENT AND POTENTIATES IMMUNOTHERAPY IN PROSTATE CANCER

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Prostate cancer is infiltrated by myeloid-derived suppressor cells (MDSCs), a diverse group of immature immune cells that can dampen the activation and function of T cells, a crucial component of the body's immune response against cancer. Accumulating evidence suggests that the frequency of MDSCs is positively correlated with tumor growth, indicating their role in promoting cancer progression.

The role of MDSCs in promoting prostate cancer progression is becoming increasingly recognized, and clinical trials targeting CXCR2 with compounds like AZD5069 are being evaluated for their potential to modulate MDSC recruitment and function. However, the complexity of the tumor microenvironment and the heterogeneity of immune responses in patients make prostate cancer treatment a challenging and ongoing area of research.

The research approach aims to identify and characterize new factors involved in recruiting myeloid-derived suppressor cells (MDSCs) in preclinical models of prostate cancer. The study combines gene expression profiling of MDSCs with genome-wide analysis of the translome in various genetically altered mouse models of prostate cancer, each representing different levels of disease aggressiveness: Ptenpc^{-/-}, Ptenpc^{-/-};TMPRSS2-ERGpc^{+/+}, Ptenpc^{-/-};CDCP1pc^{+/+}, Ptenpc^{-/-};TIMP1^{-/-} and Ptenpc^{-/-};Trp53pc^{-/-}.

The study design involves the genome-Wide Analysis of the Translatome, to identify which specific genes are actively translated in MDSCs, providing insights into the genes that are functionally relevant at the protein level. By integrating the gene expression and translome data, the researchers can identify candidate genes actively translated in prostate cancer. These candidate genes represent potential MDSC recruiting factors that could be involved in prostate cancer progression.

The research demonstrated that Pten-null prostate cancer cells drive a translational program that promotes the recruitment of MDSCs. This suggests that specific factors within Pten-null prostate cancer cells are crucial in attracting and recruiting MDSCs to the tumor microenvironment.

The researchers identified a set of genes constituting a translational signature associated with MDSCs recruitment. This signature includes Hgf, Spp1, and Bgn, which are likely involved in MDSC migration and suppression of T cell function. These factors seem crucial in creating an immunosuppressive environment within the tumor.

Mechanistically, the translation of the MDSCs recruiters (Hgf, Spp1, and Bgn) is controlled through the Akt/mTOR pathway and the MNK/eIF4E pathway.

By combining Pcd4 rescue and MNK1/2 inhibition, the researchers were able to downregulate the translation of the MDSCs recruiting factors (Hgf, Spp1, and Bgn) in Pten-null prostate cancer cells. In preclinical animal models (Ptenpc^{-/-};Trp53pc^{-/-} prostate cancer), treatment with the MNK1/2 inhibitor eFT508 and the Akt inhibitor ipatasertib resulted in significant reductions in MDSC infiltration and tumor growth.

Furthermore, the combination of the CXCR2 antagonist AZD5069 with eFT508 and ipatasertib showed enhanced effects, further reducing MDSC infiltration and tumor growth. This combination approach could potentially provide a more effective and comprehensive strategy for targeting MDSC recruitment and prostate cancer function.

The identified MDSCs recruiting factors (Hgf, Spp1, and Bgn) were highly expressed in human adenocarcinoma and castration-resistant prostate cancer (CRPC). Their expression positively correlated with phospho-eIF4e and the MDSCs marker CD33.

Overall, this research highlights the importance of translational regulation in driving the recruitment of MDSCs in Pten-null prostate cancer and identifies potential therapeutic targets for disrupting this process. The combination of CXCR2 inhibition with inhibitors of translational pathways may offer a promising strategy for improving the treatment of prostate cancer, particularly in aggressive and advanced stages.

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APOPTOTIC PRIMING PREDICTS ENHANCED PERSISTENCE AND ANTI-TUMOR CYTOTOXICITY OF CAR T CELLS OVEREXPRESSING BCL-XL

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Chimeric antigen receptor (CAR) T cell therapy has recently provided a promising treatment option for patients with hematologic malignancies; however, relapse remains a common hurdle in the setting of aggressive disease. A strong correlate identified in patients that relapse from CAR T cell therapy is poor persistence of their engineered T cells, placing a spotlight on the quality of the T cell product and how it can be improved. Programmed cell death plays a vital role in culling the natural T cell pool during infection and the role of apoptosis in CAR T cell therapy is only just beginning to be understood. Here, we sought to investigate the apoptotic priming, a measure of the likelihood of a cell to undergo apoptosis, of CAR T cells using BH3 profiling and explore whether overexpression of members of the Bcl-2 family of antiapoptotic proteins improves CAR T cell apoptotic priming and persistence.

Coexpression constructs were used to generate CD19-targeted CAR T cells expressing Bcl-2, a mutant form of Bcl-2 found in patients with CLL resistant to the BH3 mimetic venetoclax (Bcl-2 G101V), Mcl-1 and Bcl-xL. BH3 profiling was used to determine the overall apoptotic priming as well as any antiapoptotic dependencies of wild type and Bcl-2 family overexpression CAR T cells. CAR T cell killing of NALM6 cells (B-ALL) and persistence were investigated in coculture assays in vitro with and without BH3 mimetics and in vivo.

Coculture of CD19-targeted CAR T cells with NALM6 cells revealed a transient reduction in CAR T cell apoptotic priming by BH3 profiling upon receptor ligation. Reduced apoptotic priming following CAR ligation was further observed in CD19 CAR T cells overexpressing the antiapoptotic proteins Bcl-2, Bcl-2(G101V), and Bcl-xL. While expression of Bcl-2 and Bcl-2(G101V) did reduce the apoptotic priming of CD19-targeted CARs, CAR T cells overexpressing Bcl-xL were found to have the lowest overall apoptotic priming of all CARs analyzed. Interestingly, both Bcl-2(G101V) and Bcl-xL overexpressing CD19 CAR T cells exhibited enhanced tumor control in vivo and CARs overexpressing Bcl-xL exhibited significantly improved in vivo persistence, suggesting a relationship between apoptotic priming and CAR T cell function. Of note, BH3 profiling revealed a reduced dependence on the antiapoptotic protein Mcl-1 to resist cell death in CAR T cells overexpressing Bcl-xL. This finding was consistent with in vitro coculture assays where Bcl-xL overexpressing CAR T cells exhibited enhanced persistence and anti-tumor cytotoxicity in the presence of the Mcl-1 inhibitor AZD5991, demonstrating that BH3 profiling reveals apoptotic dependencies in CAR T cells that can be exploited with BH3 mimetics to further improve CAR T cell function.

In summary, we found that overexpression of antiapoptotic proteins, namely Bcl-xL, reduces the apoptotic priming of CAR T cells and that reduced apoptotic priming strongly correlates with improved CAR T cell persistence and anti-tumor cytotoxicity with and without BH3 mimetics. These findings suggest that investigating CAR T cell apoptotic priming by BH3 profiling may be useful when engineering novel CAR T cell products aimed at improving CAR T cell persistence and killing.

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STROMAL EXPRESSION OF THE ACTIN REGULATOR HMENA REGULATES TGF- β -RELATED MYOFIBROBLAST FUNCTIONALITY AND CONTRIBUTES TO THE IMMUNOSUPPRESSIVE FUNCTIONS OF CANCER-ASSOCIATED FIBROBLASTS IN NSCLC

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Cancer-associated fibroblast (CAF) functional heterogeneity plays a pivotal role in tumor progression and influences the clinical outcome of cancer treatments. The myofibroblastic CAF subset (myCAF) represents the major mediator of tumor progression, immunosuppression and resistance to immune checkpoint blockade (ICB), mainly by regulating ECM remodeling, cytokines secretion, and the interaction with tumor and immune cells. TGF- β signaling in CAFs leads to tumor resistance to immunotherapy by inducing an immunosuppressive TME. We have shown that the actin regulator hMENA/hMENA Δ v6 defines pro-tumoral CAF phenotype and orchestrates the dialogue between CAFs and tumor cells via the regulation of the immunosuppressive GAS6-AXL pathway in both PDAC and NSCLC (Melchionna et al. 2020). Stromal hMENA/hMENA Δ v6 expression is restricted to CAFs, and high levels of ENAH (hMENA gene) characterize FAP positive ECM-myCAF subtype with immunosuppressive functions and a role in immunotherapy resistance in breast cancer (Kieffer et al 2020). The role of hMENA/hMENA Δ v6-expressing CAF in immunotherapy response is also supported by our recent data showing that hMENA Δ v6 overexpressing CAFs are associated with poor response in ICB treated patients. Here, we gain new insights on the role of hMENA as a key player in the regulation of TGF- β 1-signaling and myCAF functionality.

Primary CAFs from NSCLC patients and normal fibroblasts were used for loss/gain of function experiments, respectively. The CAF-mediated paracrine effects were studied by co-culture assays of CAF/tumor cells. Multi-parametric flow cytometer analysis was used to study the CAF/immune cell cross-talk.

By interrogating the single-cell RNA sequencing data set, focused on fibroblasts populations (Lambrechts et al, 2018), we found that ENAH expression is strongly enriched in the fibroblast subset, with a strong EMT signal, an extensive repertoire of extracellular matrix proteins and TGF- β -associated genes. ENAH expression positively correlates with TGF- β 1/2 in NSCLC CAFs, but not in the matched non-malignant distal fibroblasts, as revealed by the analysis of Navab dataset. Experimentally, hMENA/hMENA Δ v6 isoform expression is increased in TGF- β 1-treated normal fibroblasts as well as in CAFs. The downregulation of hMENA/hMENA Δ v6 by siRNA inhibits the TGF- β 1-mediated ECM remodeling and production of immunosuppressive factors i.e IL-6 and CXCL12, while induces the expression of CXCL10, crucial in cytotoxic T cell recruitment. Noteworthy, the inhibition of autocrine TGF- β 1-signalling, while reducing the expression of hMENA in tumour cells, is not effective in regulating hMENA/hMENA Δ v6 in CAFs. Functional analysis of CAF/PBMC co-cultures shows that the depletion of hMENA/hMENA Δ v6 in CAFs decreases the proliferation of both CD4+ and CD8+ T cells, the percentage of Treg cells and PD-1 expression in CD8+ T cells, whereas increases the secretion of granzyme B in CD8+ T cells. Interestingly, hMENA/hMENA Δ v6 silencing inhibits PD-L1 expression in CAFs as well as the CAF-mediated paracrine induction of PD-L1 in tumor cells, by inhibiting CAF-mediated TGF- β 1/SMAD2 signaling activation, which is also sustained by hMENA expression in cancer cells. Furthermore, hMENA/hMENA Δ v6 silencing in CAFs also inhibits the CAF-conditioned medium-induced phosphorylation of STAT-1 in tumor cells, which is triggered by TGF- β 1 (but not TGF- β 2) treatment.

Further investigation is needed to delineate the role of stromal hMENA in the TGF- β signaling regulation.

In conclusion, we propose that hMENA/hMENADv6 regulate TGF- β 1-related myCAF functionality, play a significant role in TGF- β 1-induced up-regulation of PD-L1 in CAFs and in tumor cells, and contribute to CAF-mediated immunosuppressive roles. Our findings support the potential clinical relevance of identifying hMENA-related inhibitors to increase the efficacy of stromal-targeting therapy also in combination with ICB.

P481

STUDY OF EXOSOMES INTERNALIZATION BY ANTIGEN-PRESENTING CELLS

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Small extracellular vesicles (sEV) are gaining recognition as important mediators of intercellular communication, playing a crucial role in modulating immune responses. Both normal and tumor cells release sEV, but it is unclear whether they are selectively or non-selectively taken up by recipient cells. Recent studies have shown that sEV of endosomal origin (exosomes) are coated with high mannose glycans on their surface. The aim of this study was to investigate the mechanisms of exosomes internalization by antigen-presenting cells, specifically immature dendritic cells (iDCs) expressing the mannose receptor (MR) on their surface. The glycosylation profiles of both cells and exosomes can be altered in hypoxic conditions, such as those found in the tumor microenvironment. Tumor cells change their metabolism from oxidative phosphorylation to glycolysis, leading to changes in glycosylation patterns. We wanted to evaluate the specific uptake mediated by the MR of exosomes secreted under normoxic or hypoxic condition.

Fluorescent exosomes (Bodipy exosomes) were obtained by pulsing melanoma cells in normoxic or hypoxic conditions with BODIPY FL C16, a fluorescent palmitic acid analogue, isolated through differential ultracentrifugation, and quantified by Flow Cytometry (FC). iDCs were used to determine the specific uptake of Bodipy exosomes. The uptake of exosomes and their impact on DC and T-cells was evaluated by FC.

The dose and time-dependent kinetics of internalization of Bodipy exosomes was determined at 4°C and 37°C. Bodipy exosomes were specifically taken up by iDC but not by PBMC or other cell lines. Furthermore Bodipy exosomes were found to induce phenotypic changes in primary DC.

The results confirmed that exosomes are selectively internalized via the MR by iDCs prompting phenotypic changes in the DCs. These findings shed light on the immunomodulatory properties of exosomes in the innate immune system. However, further research is required to fully understand the underlying mechanisms and utilize the therapeutic potential of these findings.

P482

IMMUNOTHERAPY OF BRAIN METASTASIS APPLYING A RETROVIRAL (MMTV-P14) SIGNAL PEPTIDE

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Mouse Mammary Tumor Virus (MMTV) is a Beta-Retrovirus that causes mammary carcinoma and lymphoma in mice. An increasing body of evidence in recent years supports its involvement in over 30% of human sporadic breast cancers.

Previously, we have demonstrated that the signal peptide of the envelop precursor protein of MMTV (named p14) is a multi-functional 98 amino-acids long peptide. In addition to its signal peptide function, it localizes to nucleoli where it binds key proteins (B23 and RPL5) involved, among other activities, in Ribosome biogenesis and p53 stabilization. p14 is also expressed on the surface of cells that contain MMTV.

In addition, polyclonal anti-p14 antibodies were used in immune histochemistry analyses of human breast cancer samples supporting association of MMTV with the disease. p14 has been used for preventive vaccination towards murine tumors that contain the virus, demonstrating both antibody and T-cell mediated activities. Also, passive immunization with monoclonal anti-p14 antibodies impaired in vivo growth of murine lymphoma and mammary carcinoma that contain the virus.

Furthermore, we have developed an experimental model for CNS and Ocular metastasis by lymphoma whereby intraperitoneal inoculation of variant lymphoma cells (Rev-2-T-6 and HU-1) to young mice (day 7 post-natal) resulted in homing to the brain, spread within it along defined routes, including the optic nerve sheath and into the eyes. These cells harbor MMTV and express p14 on their surface. Taken together, this opens a window of opportunity to analyze a putative role for p14 in brain metastasis of MMTV-associated cancers.

Indeed, here we report that maternal vaccination with p14 impairs CNS and Ocular metastasis by the abovementioned murine lymphoma cells. These findings are of special interest as ≈20% of breast cancers metastasize to the brain.

P483

PECULIARITIES OF PHENOTYPIC TRAITS AND METABOLIC FEATURES OF B REGULATORY CELLS AND CD162+ TISSUE RESIDENT MEMORY T CELLS IN THE MICROENVIRONMENT OF NON-SMALL CELL LUNG CANCER

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The prognosis of non-small cell lung cancer (NSCLC) is greatly impacted by tumor infiltrating lymphocytes (TIL). This study explores the main phenotypic and metabolic features of distinct populations of T cells, expressing the P-selectin glycoprotein ligand-1 (PSGL-1, CD162) complex, and B cells expressing one of its connected ligands, V-domain immunoglobulin suppressor of T cell activation (VISTA).

Twenty-seven individuals with resectable non-small cell lung cancer (NSCLC) were enrolled in the study and the phenotype of tumor infiltrating B and T cells were analyzed with two flow cytometry panels (18 and 21 markers for B and T cells, respectively), paying special attention to recently-discovered immune checkpoints such as CD226, CD96, CD162, and VISTA. A 45-parameter mass cytometry panel was used to obtain the metabolic profile of these cells (single-cell metabolic regulome profiling, scMEP by CyTOF). NicheNet and NSCLC spatial transcriptomics datasets (CosMX, Nanostring) were used to map the ligand-receptor interactions and their effect on interacting cells.

The analysis of CD4+ and CD8+ tissue resident memory cells indicated that 11% of CD4+ and 19% of CD8+ displayed varying levels of CD162, and had metabolic profiles that ranged from high metabolic activation to poor glycolytic capacity. A further study of B cells revealed a tumor increased numbers of a type of B regulatory cell that expressed VISTA and secreted IL-10; these cells were characterized by enhanced activity of metabolic pathways including amino acid, glycolysis, pentose, fatty acid oxidation, and the tricarboxylic acid cycle. Information obtained through computer modeling of interactions between T and B cells identified CD162-VISTA as a probable receptor-ligand pair in non-small cell lung cancer samples. Spatial analysis of data from NSCLC cancers demonstrated that T and B cells were often in close proximity within tertiary lymphoid structures.

CD162-VISTA axis could point out a novel functional interaction between B and T cells in the TME of NSCLC patients. Ongoing experiments are investigating mechanisms of this interaction that could be associated to local immune modulation and to possible therapeutic interventions.

P484

UNTANGLING MYELOID DIVERSITY IN PEDIATRIC HIGH GRADE GLIOMAS – MICROGLIA EMERGE AS DRIVERS OF TUMOR PROGRESSION AND ARE TRANSCRIPTIONALLY SHAPED BY TUMOR SPECIFIC HISTONE MUTATIONS

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Significant progress has been made molecularly defining pediatric high-grade gliomas (pHGG), including diffuse midline gliomas (DMG), yet little progress has been made with respect to delineating the inflammatory microenvironment.

We utilize molecularly defined human samples, immunocompetent genetic mouse models (RCAS-Tva), multi-parameter flow cytometry, and single cell RNA sequencing on over 100,000 cells to study how tumor location and histone genetic driver mutations influence the tumor microenvironment (TME).

We report the novel observation that human DMGs have a significant enrichment of Elane+ neutrophils compared to their hemispheric counterparts. We validate this utilizing the RCAS-Tva mouse model, which histologically and genetically recapitulates human pHGGs. Preventing neutrophil infiltration via genetic ablation of Cxcl1 did not prolong survival, suggesting neutrophils are not primary drivers of disease malignancy in pHGG/DMG. We then demonstrate each distinct pHGG/DMG entity confers unique transcriptional identities. H3.3WT DMGs and H3.3K27M DMGs cluster together and have high expression of inflammatory genes such as Ptpcr, Trem2, Lag3, and Cd274 while H3.3WT and H3.3G34R hemispheric tumors, and H3.1K27M DMGs cluster together with low expression of these genes. H3.3K27M DMGs were found to have transcriptional signatures enriched for astrocytes, an astrocyte-like tumor signature, and EMT signature genes, while all subtypes contained oligodendrocyte progenitor cell-like signatures. We report the identification of several myeloid cell and microglia sub-populations acquiring unique inflammatory transcriptional signatures that are dependent on the histone H3.1K27M or H3.3K27M mutation and tumor location (cortical hemisphere vs brainstem). Specifically, H3.3K27M DMGs have significantly more proliferating microglia, downregulated interferon signaling pathways, and downregulated antigen presentation compared to H3 wild type or H3.1K27M DMGs. Further, we identify high expression of chemokines and chemokine receptors including CCR1 and CCR5 in microglia. Identification of rare tumor infiltrating T cell subsets was also done, including stem-like CD8 T cells, lineage negative CD4+ T cells, NKT cells, and others. Genetic knockout of Ccl8/Ccl12 was done to prevent chemokine signaling and tumor associated macrophage (TAM) infiltration in these tumors. A significant extension of survival in H3.1 and H3.3K27M DMGs was observed, which was also met with an increase in CD4+ and CD8+ T-cells and decreased neutrophil infiltration. CD4+ T-cell depletion and anti-PD1 therapy was performed to further study the role of lymphocyte infiltration in Ccl8/Ccl12 DKO mice. No survival benefits were observed with anti-PD1 therapy, while anti-CD4 treatments significantly extended survival in WT but not CCl8/12 DKO tumor-bearing mice. CCR1/CCR5 inhibitors were also utilized to abrogate TAM infiltration, resulting in decreased microglia infiltration and significant survival extension comparable to standard of care radiation therapy.

Together, this work establishes the role histone mutations play in shaping the tumor immune microenvironment and provides credence for targeting TAMs as a novel therapeutic option. Further, these studies provide a foundation for developing or improving immunotherapies designed at specific subgroups of pHGG and DMGs, such as CAR-T-cell, oncolytic viral therapy, and checkpoint blockade.

P485

TRANSLATION MACHINERY IS A THERAPEUTIC VULNERABILITY AND ITS TARGETING SUPPRESSES MDSCS RECRUITMENT AND POTENTIATES IMMUNOTHERAPY IN PROSTATE CANCER

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Prostate cancer is infiltrated by myeloid-derived suppressor cells (MDSCs), a diverse group of immature immune cells that can dampen the activation and function of T cells, a crucial component of the body's immune response against cancer. Accumulating evidence suggests that the frequency of MDSCs is positively correlated with tumor growth, indicating their role in promoting cancer progression.

The role of MDSCs in promoting prostate cancer progression is becoming increasingly recognized, and clinical trials targeting CXCR2 with compounds like AZD5069 are being evaluated for their potential to modulate MDSC recruitment and function. However, the complexity of the tumor microenvironment and the heterogeneity of immune responses in patients make prostate cancer treatment a challenging and ongoing area of research.

The research approach aims to identify and characterize new factors involved in recruiting myeloid-derived suppressor cells (MDSCs) in preclinical models of prostate cancer. The study combines gene expression profiling of MDSCs with genome-wide analysis of the translome in various genetically altered mouse models of prostate cancer, each representing different levels of disease aggressiveness: *Ptenpc^{-/-}*, *Ptenpc^{-/-};TMPRSS2-ERGpc^{+/+}*, *Ptenpc^{-/-};CDCP1pc^{+/+}*, *Ptenpc^{-/-};TIMP1^{-/-}* and *Ptenpc^{-/-};Trp53pc^{-/-}*.

The study design involves the genome-Wide Analysis of the Translatome, to identify which specific genes are actively translated in MDSCs, providing insights into the genes that are functionally relevant at the protein level. By integrating the gene expression and translome data, the researchers can identify candidate genes actively translated in prostate cancer. These candidate genes represent potential MDSC recruiting factors that could be involved in prostate cancer progression.

The research demonstrated that *Pten*-null prostate cancer cells drive a translational program that promotes the recruitment of MDSCs. This suggests that specific factors within *Pten*-null prostate cancer cells are crucial in attracting and recruiting MDSCs to the tumor microenvironment.

The researchers identified a set of genes constituting a translational signature associated with MDSCs recruitment. This signature includes *Hgf*, *Spp1*, and *Bgn*, which are likely involved in MDSC migration and suppression of T cell function. These factors seem crucial in creating an immunosuppressive environment within the tumor.

Mechanistically, the translation of the MDSCs recruiters (*Hgf*, *Spp1*, and *Bgn*) is controlled through the Akt/mTOR pathway and the MNK/eIF4E pathway.

By combining *Pdcd4* rescue and MNK1/2 inhibition, the researchers were able to downregulate the translation of the MDSCs recruiting factors (*Hgf*, *Spp1*, and *Bgn*) in *Pten*-null prostate cancer cells. In preclinical animal models (*Ptenpc^{-/-};Trp53pc^{-/-}* prostate cancer), treatment with the MNK1/2 inhibitor eFT508 and the Akt inhibitor ipatasertib resulted in significant reductions in MDSC infiltration and tumor growth.

Furthermore, the combination of the CXCR2 antagonist AZD5069 with eFT508 and ipatasertib showed enhanced effects, further reducing MDSC infiltration and tumor growth. This combination approach could potentially provide a more effective and comprehensive strategy for targeting MDSC recruitment and prostate cancer function.

The identified MDSCs recruiting factors (Hgf, Spp1, and Bgn) were highly expressed in human adenocarcinoma and castration-resistant prostate cancer (CRPC). Their expression positively correlated with phospho-eIF4e and the MDSCs marker CD33.

Overall, this research highlights the importance of translational regulation in driving the recruitment of MDSCs in Pten-null prostate cancer and identifies potential therapeutic targets for disrupting this process. The combination of CXCR2 inhibition with inhibitors of translational pathways may offer a promising strategy for improving the treatment of prostate cancer, particularly in aggressive and advanced stages.

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DESIGN, CHARACTERIZATION AND PRECLINICAL VALIDATION OF A COMBINATORIAL CAR-BASED IMMUNOTHERAPY AGAINST COLORECTAL CANCER WITH HER2 AMPLIFICATION

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In the last decade, many studies highlighted HER2 amplification and overexpression as an effective pharmacological target for metastatic colorectal cancer (mCRC) treatment. However, two major unmet clinical needs remain: (i) primary resistance to HER2/EGFR-targeted therapy, and (ii) limited duration of the response, due to acquisition of resistance-promoting mutations. As a consequence, a sizeable fraction of HER2-amplified CRCs needs alternative therapies. In this perspective, a promising strategy is CAR-based adoptive immunotherapy, provided that the known “on-target off-tumour” toxicity of HER2-CAR effectors is kept at bay.

We considered a combinatorial strategy to selectively kill only CRC cells co-expressing high levels of HER2 and carcinoembryonic antigen (CEA), an antigen whose expression is restricted to the digestive tract and increased in cancer. For combinatorial targeting of the two antigens, we employed an artificial regulatory network based on the synthetic Notch (synNotch) signal transduction system. The first component is a synNotch receptor in which the extracellular domain is an anti-HER2 scFv and the intracellular domain contains the GAL4VP64 artificial transcription factor. Engagement of the anti-HER2 domain by target cells drives GAL4VP64 cleavage and translocation to the nucleus, where it drives expression of the second component, an anti-CEA CAR under a GAL4-driven promoter. The CEA CAR is therefore expressed only when the HER2-synNotch is engaged. In this way, only cells co-expressing both HER2 and CEA at high levels are killed.

Lentiviral vectors encoding the two components were transduced in the NK-92 natural killer cell line. Repeated sorting and cloning led to selection of the optimally responsive clone 5F. In vitro, the 5F clone displayed selective cytotoxicity against HER2^{amp}/CEA⁺ CRC cells, with minimal activity against HER2^{amp}/CEA⁻ cells, or against HER2⁻/CEA⁺ cells. Additional assays on 3D organoids highlighted better recruitment and infiltration by the 5F clone respect to NK-92 WT cells, only in HER2^{amp} models. In vivo, the clone 5F significantly impaired tumor growth in two different HER2^{amp} CRC models. Considering the known limits of CAR-killer therapy against solid tumours, i.e. insufficient penetration and killing, we considered whether the above approach could be directed against minimal residual disease under HER2/EGFR treatment, when the tumour burden is minimal. Indeed, drug-tolerant persister cells become quiescent and survive until treatment is suspended, or resistance is acquired. We therefore verified whether, under prolonged in vitro treatment with HER2/EGFR therapy, persister cells are still targetable by the 5F clone. Flow cytometry analysis showed that both HER2 and CEA proteins remain high in persister cells. Indeed, the 5F clone displayed remarkable in vitro efficacy against persister cells surviving under HER2/EGFR therapy, also at low effector/target ratio.

The observed selective efficacy both in vitro and in vivo of the HER2-synNotch/CEA-CAR system opens a perspective for possible clinical applications in cases of HER2^{amp} CRC displaying primary or secondary resistance to HER2/EGFR blockade. Further evolutions of the HER2-synNotch/CEA-CAR system, incorporating additional synNotch-driven elements to improve effector efficacy, will also be presented.

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ROLE OF HMENA-MEDIATED AUTOPHAGY IN THE DIALOGUE AMONG CAFs AND CANCER CELLS IN NSCLC

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Autophagy, a self-catabolic system, is an evolutionary conserved and tightly controlled process, involved in the degradation and recycling of cellular components to maintain cellular homeostasis. Emerging evidence indicates that autophagy machinery is involved in non-degradative processes including unconventional secretion mechanism. In cancer, autophagy is context-dependent and can promote or suppress tumor development and progression. Actin cytoskeleton dynamics participate in the early events of autophagic flux by acting on the autophagosome biogenesis, with the role in cancer progression still poorly understood.

The actin cytoskeleton regulatory protein hMENA, along with its isoforms hMENA11a epithelial associated and the mesenchymal hMENA Δ v6, participates to progression of non-small-cell lung cancer (NSCLC). The expression of hMENA Δ v6 in tumor cells, has been associated with mesenchymal phenotype and cancer progression by regulating crucial pathways including TGF- β , AXL and β 1 integrin/Fibronectin. hMENA/hMENA Δ v6 are key players of Epithelial Mesenchymal Transition (EMT), characterize pro-tumor cancer associated fibroblasts (CAFs) and are involved in tumor/CAF cross-talk and resistance to immunotherapy.

Aim of this study is to understand how hMENA and its splicing isoforms participate in the regulation of autophagy in reciprocal interaction between CAFs and cancer cells, to define new targetable pathways in NSCLC.

Employing NSCLC patient derived CAFs we found that the depletion of hMENA/hMENA Δ v6 isoforms counteracts basal autophagy as demonstrated by the accumulation of LC3-II and the reduction of p62 levels, leading to the YAP stabilization.

The increased YAP expression level observed in the hMENA/hMENA Δ v6 silenced cells is the result of a stabilization of the protein by the Aurora A kinase which promotes the stability of YAP in the inhibited autophagy condition. We posit that the different cytoskeleton tension generated in hMENA/hMENA Δ v6 silenced CAFs may trigger a mechanical stimulus for induction of YAP expression that regulates autophagosome trafficking. Recent studies show that there are many interconnections between degradative autophagy and secretion of extracellular vesicles (EVs). The actin polymerization generates branched actin networks on the vesicle precursor surface to drive either the fusion of mature autophagosomes with lysosomes or with the plasma membrane to secrete the cargo proteins. This form of autophagy-based unconventional secretion is involved in the export of a variety of cellular cargos and cytokines such as IL-1 β , IL-18, HMGB1, IL-6 and TGF- β 1. To demonstrate the pro-tumor role of hMENA-related secretory autophagy in the cross-talk between CAFs and NSCLC cells, we analysed the conditioned media derived from hMENA/hMENA Δ v6 silenced CAFs in order to evaluate and quantify changes of secreted cytokines. We demonstrated that CAFs conditioned media promotes lung cancer cell proliferation in a CAF autophagy-dependent manner and that the hMENA/hMENA Δ v6 depletion as well as inhibition of chloroquine-mediated autophagy mitigates the effect of CAFs on NSCLC cell proliferation. Reciprocally, the silencing of hMENA/hMENA Δ v6 in lung cancer cells counteracts basal autophagy through the activation of mTORC2/AKT axis.

We are exploring the role of signatures including ENAH (hMENA gene) and autophagy genes, i.e. Atg9A, BECN1, MTOR, RICTOR, that are positively correlated in TCGA NSCLC patient database, in immune checkpoint blockade treated NSCLC patient datasets where we have found that hMENA isoforms are related with therapy response.

Collectively, these data support a novel function for the actin cytoskeleton regulator hMENA and its isoforms in the autophagy mediated cross-talk between tumor cells and CAFs, paving the way for novel combinatorial therapies to enhance response to immunotherapy.

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REPROGRAMMING IMMUNOSUPPRESSIVE TUMOR-ASSOCIATED MACROPHAGES POTENTIATES STANDARD-OF-CARE THERAPY IN MELANOMA

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Cutaneous melanoma is a highly aggressive cancer capable of distant and lethal metastatic spread. Breakthroughs in treatment have come from understanding oncogenic signaling and cancer immunobiology. Targeted therapies successfully block MAPK signaling in BRAFV600e mutant melanoma with remarkably high clinical responses followed, by rapid relapse, whereas checkpoint inhibitors activating the immune response induce long-lasting responses, albeit only in a subset of patients. These limitations have driven interest in understanding innate and acquired resistance mechanisms.

Using a refined, immunocompetent genetically-engineered mouse model of BRAF-driven melanoma (iBIP2), which phenocopies an appreciable subset of patients suffering from the human disease in its development, histopathology, and transitory response to therapy, we focused on the TME, seeking to elucidate resistance mechanisms by performing *in vivo*, *in vitro* and *ex vivo* analyses.

Using a refined, immunocompetent genetically-engineered mouse model of BRAF-driven melanoma (iBIP2), which phenocopies an appreciable subset of patients suffering from the human disease in its development, histopathology, and transitory response to therapy, we focused on the TME, seeking to elucidate resistance mechanisms by performing *in vivo*, *in vitro* and *ex vivo* analyses. Our investigations revealed that tumor-associated macrophages (TAMs) are involved in tumor development and therapeutic resistance mechanisms following a standard-of-care therapy (inhibition of MAPK signaling; MAPK-i). We characterized this myeloid population and showed that TAMs are a significant component of the TME, predominantly polarized toward a pro-tumoral "M2-like" phenotype and produce numerous immunosuppressive factors leading to potent immunosuppressive capabilities. We identified three distinctive TAM-reprogramming agents, a PI3Ky inhibitor (Eganelisib), a CD11b agonist (GB1275), and a tricyclic antidepressant (Imipramine). Each can convert immunosuppressive into pro-inflammatory tumor-antagonizing macrophages, leading to improved survival and responsiveness to targeted therapies (MAPKi). Interestingly, each proved to be inefficient as monotherapy, and only remodeled TAMs in combination with MAPK-i, emphasizing the crosstalk between cancer and stromal cells and the need to target different cell compartments to disrupt immunosuppressive TME. We observed that TAM reprogramming facilitated tumor infiltration by activated cytotoxic CD8+ T cells mediating tumor destruction and anti-tumor immunity. We then performed integrative transcriptomic and spatial analyses to illustrate, at the single-cell level, the complex interplay occurring in the TME and further validated our results in human melanoma.

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Our work highlights the central role played by immunosuppressive macrophages in melanoma resistance to therapy and demonstrates that pharmacologic reprogramming of macrophages represents a therapeutic modality with the potential to elicit more effective anti-tumor immune responses against this devastating disease.

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THROMBOSPONDINS 1 AND 4 UNDERGO COORDINATED TRANSPORT TO THE MULTICORE CYTOTOXIC GRANULES TO REGULATE SUPRAMOLECULAR ATTACK PARTICLES (SMAPS) BIOGENESIS AND FUNCTION IN CTL-MEDIATED CYTOTOXICITY

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Supramolecular attack particles (SMAPs) are autonomous killing entities released by cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells to kill virally infected or cancerous targets. SMAPs consist of a core of cytotoxic proteins surrounded by a non-membranous glycoproteic shell, of which thrombospondins (TSPs) are prominent components. A better understanding of how SMAPs are assembled within the CTL will set the basis for the development of SMAPs as a new strategy to overcome major limitations associated with engineered T cell-based cancer immunotherapy. As a first step towards this goal, we have started to dissect the trafficking pathways responsible for SMAP biogenesis in human CTLs.

For this study, we optimized a protocol for in vitro generation of CTLs from CD8+ T cells purified from healthy donors. First, we monitored expression of endogenous TSP-1 and TSP-4 during CTL differentiation both at the mRNA and protein levels by quantitative reverse transcription PCR and immunoblotting, respectively. Second, we analysed the intracellular localization of fluorescently tagged TSP-1 and TSP-4 under steady state conditions and following activation on a surface mimicking target cell membrane as well as their SMAP-associated release at the cytotoxic synapse using a combination of imaging techniques. Third, we tested the requirement of TSP-1 and TSP-4 for the formation of SMAPs and their specific contribution both to CTL- and SMAP-mediated killing of target cells by performing functional cytotoxicity assays on CTLs knocked out for TSP-1 or TSP-4 expression by CRISPR/Cas9 gene editing.

We demonstrated that the SMAP shell components TSP-1 and TSP-4 show opposite expression trends during the differentiation of CD8+ T cells to cytotoxic effectors, with TSP-1 dropping from initially high levels and TSP-4 increasing from initially low levels. Additionally, we found that TSP-1 and TSP-4 likely share a common pathway for transport and localization to multicore granules, a newly identified class of lytic granules that store SMAPs under steady state conditions, accumulate at the immune synapse formed by CTLs on activating surfaces, and eventually fuse with the plasma membrane to release SMAPs following stimulation. Finally, preliminary data show that both TSP-1 and TSP-4 are required for the killing activity of SMAPs.

Collectively, our results demonstrate that TSP-1 and TSP-4 coordinately regulate SMAP biogenesis and function. This not only furthers our knowledge of the mechanisms underpinning CTL-mediated killing but also potentially paves the way to new, effective and safe cell-free immunotherapeutic strategies through engineering the glycoprotein shell of SMAPs with specificity for tumor antigens.

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PERIVASCULAR MACROPHAGES COOPERATE TO FORM MULTICELLULAR CCR5-DEPENDENT NESTS THAT INFLUENCE THE IMMUNE LANDSCAPE AND RESPONSE TO CHEMOTHERAPY IN CANCER

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Tumour associated macrophages (TAMs) are an abundant infiltrating immune cell in solid tumours that have been associated with poor clinical outcomes. TAMs are a heterogeneous population and develop refined effector function in response to cues in the tumour microenvironment (TME). TAMs have been implicated in facilitating a variety of pro-tumoral pathways including resistance to anti-cancer therapeutics, immune suppression, angiogenesis, and metastasis. However, anti-tumoral TAM subsets also arise and co-exist within the TME. Recent data points to a developmental specialisation of these cells towards polarisation extremes. Identifying the most specialised pro-tumoral TAM subsets and understanding their functionality provides therapeutic opportunities for targeting their activity, while preserving the anti-tumoral subsets in the TME. We have recently characterised one such highly polarised TAM subset which has pro-angiogenic and immune-suppressive functions and is identified by the expression of the lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1) and its spatial location proximal to blood vasculature. Interestingly, LYVE-1+ TAMs are heterogeneously distributed along the endothelium where they form multicellular 'nest' arrangements in a spontaneous murine model of mammary adenocarcinoma (MMTV-PyMT), a cellular arrangement that can also be observed in human breast cancer. In the current study we investigated the development of LYVE-1+ TAMs and their nest formation.

To investigate their role in the TME, we utilised a combination of genetically modified murine models, photo-labelling studies, advanced immunofluorescence imaging, flow cytometry and RNA sequencing approaches to study these cells.

We demonstrated that LYVE-1+ TAMs are collaborative and form their nest arrangements in a CCR5-dependent manner. We demonstrate that the phenotypic identity of LYVE-1+ TAMs are derived from an IL-6 signal, a cytokine that is expressed by the adjacent endothelium, and genetic knockout of IL-6 prevents the formation of this subset in vivo. We demonstrate in vitro that IL-6 directly induces key markers associated with the phenotypic identity of this population including LYVE-1 and high expression of CD206 and the immunosuppressive enzyme heme oxygenase-1 (HO-1). Furthermore, IL-6 induces CCR5 expression which connects a communication axis between the TAM subset through their co-expression of the ligands CCL3/4 allowing these cells to form multicellular nest structures in the perivascular niche. We go on to demonstrate that HO-1 represents a key effector molecule for this subset. Genetic inactivation of HO-1 in LYVE-1+ TAMs resulted in an improved CD8+ T-cell infiltration to the TME and, in doing so, improved the immune-dependent effects elicited by chemotherapy, providing prolonged tumour control in MMTV-PyMT mice. Interestingly, in the absence of CCR5 this effect was abrogated suggesting that the nest formation by these macrophages, and their collaborative function, may represent an active biological unit that plays a 'gatekeeper' role in the perivascular niche.

This study highlights an unappreciated collaboration between a TAM subset and uncovers a spatially driven therapeutic resistance mechanism of these cells in cancer which can be therapeutically targeted.

P492

REPROGRAMMING TUMOR ASSOCIATED MACROPHAGES IN IN VITRO AND IN VIVO MODELS OF MESOTHELIOMA

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Chronic inflammation and the presence of an unfavorable inflammatory tumor microenvironment (TME) can promote tumor development. A typical example is malignant mesothelioma (MM), an aggressive and incurable tumor arising from the exposure to asbestos fibers. High Mobility Group Box 1 protein (HMGB1), a non-histone DNA chaperone that also works as a signal of danger, contributes to MM onset and progression

We recently discovered that CXCR4 activation, so far associated only with tumor progression and metastasis, also flags tumor cells to immune recognition.

Both CXCL12, the natural ligand of CXCR4, and BoxA, a fragment of HMGB1 that shows anti-inflammatory activity, promote immunogenic surrender (IGS) (Mezzapelle et al. 2021). Macrophages play a central role in IGS and are crucial for the mode of action of BoxA, thus we want to investigate their role more deeply.

Single cell RNA sequencing was used to study at single cell level human mesothelioma biopsies. 3-dimensional (3D) system of murine mesothelioma cells (AB1 cells) was used to study tumor spheroid growth. Spheroid growth was measured by using an inverted microscope. M0 macrophages were obtained from cells isolated from murine bone marrow using M-CSF. In vivo experiment was performed as described in Mezzapelle et al., EMBO Mol Med 2021. To deplete macrophages we used anti CSF1-R antibody (clone AFS98), 250 ug/injection.

Single cell RNA sequencing of human MM biopsies showed that macrophages (MOs) are the main infiltrating immune cells in MM. By using a 3D spheroid system of MM (murine cells) co-cultured with bone marrow derived macrophages (BMDMs) we observed that MOs that are initially M0, polarized to a pro-tumoral M2 phenotype MOs when in contact with MM spheroids. Moreover, M2 MOs fostered spheroid growth while M1 MOs reduced it, as expected. However, when we treated MOs with an antibody against CSF1 receptor (CSF1-R), MOs switched from an M2 to an M1 polarization state. This was true also when BMDMs were exposed to conditioned medium (CM) collected from MM spheroids; the CM induced a polarization to an M2 phenotype, nevertheless by adding an anti CSF1-R antibody we observed a shift to an M1 phenotype.

We also tested the effect of BoxA on this co-culture system; we found that BoxA reduced the growth of MM spheroids cultured with M0 (non activated) MOs, but even more significantly when cultured with M2 MOs.

Next, we tested the impact of MOs depletion in vivo in a syngeneic mouse model of MM using an anti-CSF1R antibody. We were successful in depleting a large proportion of MOs (tested by IHC and flow cytometry). Tumor growth and survival analyses indicate that MOs in fact support MM growth, and that their depletion extends the survival of mice. Treating macrophage-depleted mice with BoxA does not afford any further advantage, both in terms of tumor growth or survival. This result is in accordance with our hypothesis that macrophages are needed for the mode of action of BoxA, and we were initially very excited. However, we realized that since macrophage depletion already confers some advantage in MM mice, proving or disproving an additional advantage of BoxA would have required a much larger number of mice.

To conclude, we found that MOs are essential in supporting MM growth and that their depletion could be therapeutic. In addition, the treatment with anti CSF1R antibody skews TAMs polarization towards M1, could be a promising approach to induce an immune-permissive TME in MM, and this approach

could synergize with BoxA activity. This would make MM and other inflammatory tumors more suitable for cancer immunotherapies.

P493

THE ROLE OF QUIESCENCE IN ACUTE MYELOID LEUKAEMIA GROWTH

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Acute myeloid leukemia (AML) is the most common leukemia in adults and its prognosis is usually poor. The major responsible for therapy failure and leukemia relapse is the genomic and biological heterogeneity of the tumor. At biological level, AML is hierarchically organized with leukemia stem cells (LSCs) at the apex. LSCs are a rare cell sub-population with self-renewal capacity, responsible for leukemia initiation and maintenance. Standard AML therapies have limited effects on LSCs, mainly due to their quiescent status. Preliminary data obtained in our group have shown that different leukemia-initiating oncogenes (NPMc+, PML-RAR α and MLL-AF9) share the property of enforcing quiescence in pre-leukemic hematopoietic stem cells (HSCs), and that this is critical for the progression and maintenance of the leukemia clone. Underlying molecular mechanisms, however, are unknown.

To identify quiescence-related genes indispensable for AML growth, we performed an in vivo shRNA screening. Three of the identified hits were subsequently chosen to analyze the effect of their silencing on AML growth in vivo: control and interfered blasts were intravenously transplanted into sublethally irradiated C57BL/6J recipient mice. Then, to evaluate whether the silencing of these genes affected the cell cycle status of growing AML, an in vivo time course analysis was performed by sacrificing transplanted mice at different time points post transplantation for downstream FACS analyses (cell cycle -Ki67 and DAPI and apoptosis -cleaved caspase3). To investigate underlying molecular mechanisms, we performed a single-cell RNAseq (scRNAseq) analysis of both interfered and control blasts, which was followed by blasts transplantation into immunocompromised mice and into mice that were systematically depleted from macrophages.

Among the identified hits, Socs2, Stat1 and Sytl4 silencing prevented AML outgrowth in vivo. Notably, Socs2 and Stat1 interference increased proliferation, prevented accumulation of quiescence and induced apoptosis only in vivo, suggesting that their effects are largely dependent on the in vivo context.

ScRNAseq analysis of Socs2-interfered blasts showed marked down-regulation of genes characterizing the dormant status of quiescent HSCs, suggesting that loss of quiescence in Socs2-interfered blasts is linked to the loss of their regenerative potential. Since dormancy and self-renewal potential in HSCs are antagonized by prolonged stress signals, we evaluated the activation of the integrated stress response (ISR). Strikingly, Socs2-interfered cells showed aberrant activation of ATF4, UPR and autophagic transcriptional programs. Since elevated levels of UPR may act as danger signals and favor immune-mediated clearance in vivo, we analyzed immune-related pathways. Consistently, Socs2-silenced blasts showed a dramatic down-regulation of specific immune check-point molecules (ICMs), including CD24a, galectin 9 and VISTA, which are involved in the regulation of B, T and NK cells and macrophages.

Based on these findings, we hypothesized that Socs2 regulates the resolution of stressful signals that accumulate in hyper-proliferating AML blasts by allowing cells to enter quiescence and activate pathways of ISR resolution, including upregulation of ICMs. If cells fail to enter quiescence (e.g. Socs2 interference), they maintain ISR activation and downregulate ICMs, thus triggering immune-mediated cell death. Consistently, the anti-leukemic effect of Socs2-silencing was partially rescued upon propagation into immunocompromised mice. As well, macrophage depletion prolonged disease latency of Socs2-interfered blasts in immunocompetent mice.

These findings provide preliminary evidence of the existence, in AML blasts, of an adaptive response to hyperproliferation that involves ISR activation, induction of quiescence and immune evasion. Targeting this adaptive response, as by Socs2 interference, may activate potent mechanisms of AML immune clearance.

P494

THErapy-INDUCED SENESCENT CELLS UPREGULATE ANTIGEN PROCESSING AND PRESENTATION MACHINERY AND ELICIT ANTI-TUMOR IMMUNITY IN ACUTE MYELOID LEUKEMIA

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Acute myeloid leukemia (AML) is an aggressive hematological malignancy currently treated with high-dose chemotherapy. Nevertheless, treatment failure is still frequent, thus underscoring the need to better investigate the molecular events occurring early after chemotherapy. Therapy-induced senescence (TIS) is a stress-triggered fail-safe mechanism that may elicit immune-mediated responses. However, its functional role in AML eradication and immune surveillance by the adaptive immune system remains ill-defined.

Our work aims to functionally interrogate the crosstalk between senescent leukemic blasts and the immune system. The final long-term goal is to unveil mechanistic insights into the factors dictating AML response to conventional chemotherapy and immunotherapy.

We exploited ex-vivo cultures of primary AML samples or cell lines undergoing senescence upon different treatments. Multiparametric flow cytometry analysis integrated with quantitative imaging will be used to measure senescent markers and human leukocyte antigen (HLA) expression in a cohort of primary AML samples at diagnosis and upon ex-vivo therapy. The mixed lymphocyte reaction assay was performed to evaluate T cell activation in response to senescent blasts.

By combining transcriptional and cell-based evaluation of senescence markers in AML patient samples, we identified two groups of patients based on their ability to accumulate SA- β -GAL upon ex vivo chemotherapy and defined them as Senescence High or Senescence Low samples. Importantly, only in Senescence High patients, we found upregulation of Human Leukocyte Antigens (HLA) class I and II molecules and their regulators, both at RNA-seq and protein level, revealing a link between TIS establishment and increased blast immunogenicity. Consistently, senescent AML samples activated autologous CD4⁺ and CD8⁺ T cells, leading to improved recognition of leukemic blasts.

Mechanistically, we found that in senescent competent blasts the upregulation of HLA molecules was due to Polycomb Repressor Complex 2 (PRC2) and Histone methyltransferases 1 and 2 (EHMT 1/2) mediated derepression of interferon and immune-related genes.

Indeed, we reported downregulation of Polycomb Repressor Complex 2 (PRC2) core subunits in Sen High AML patients that was paralleled by enrichment of gene lists of PRC2 targets and genes marked by H3K27me3 histone modification.

In line with this, driven by the hypothesis that the senescence competency of patients and the consequently increased immunogenicity may be driven by epigenetic modalities, we identified epigenetic drugs inhibiting PRC2 (i.e., EZH2 inhibitor) and EHMT 1/2 (i.e., GLP/G9a inhibitors) which are able to revert the immunological mechanisms at the basis of senescence low patients, leading to the reactivation of immune cells against the tumour.

Lastly, we found enhanced T cell activation for both T cell subsets when combining TIS and immune checkpoint blockade (ICB) therapy, both ex-vivo and in PDX models, indicating that senescence competence upon chemotherapy may be exploited to stratify AML patients that would benefit from this treatment.

Overall, our findings uncover a novel link between senescence induction and leukemia immune recognition by T cells via upregulated components of the antigen presentation machinery, elucidating the basis for conceptually novel senescence-based targeted immunotherapeutic regimens for AML patients.

P495

A FINE-TUNED BALANCE BETWEEN CD28 EXPRESSION AND CO-STIMULATOR/INHIBITOR MOLECULES IN THE PERIPHERY AND TUMOR SITE CONTROLS CD8+ T-CELL FUNCTIONALITY AND INFLUENCES THE RESPONSE TO INHIBITORY CHECKPOINT BLOCKADE IN PATIENTS WITH ADVANCED NSCLC

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Immune checkpoint blockade (ICB) has significantly prolonged survival of NSCLC patients. However, only a minor percentage of the patients have a durable response. The challenge is to understand the complex mechanisms of resistance, recently partially highlighted thanks to the advent of single-cell RNA (scRNA-Seq) technology. An enormous variability in terms of phenotypic and (dys)functional states among the T-cell subsets, either circulating or infiltrating the tumor site has emerged, fine-tuned by the complex network between T-cell differentiation state and local cues. T-cell exhaustion is recognized as an adaptation in terms of functional skills rather than a terminal condition of T-cell inactivation, with a dynamic progression from a long-lived "pre-exhausted stem-like progenitor", likely more responsive to immunological re-invigoration by ICB, to a "terminally exhausted" state. On the other hand, the interplay between co-stimulatory and inhibitory molecules dictate T-cell functionality, and CD28 co-stimulatory molecule has been described as the primary target downstream of PD1 mediated inhibitory signaling.

Considering the relevance of the relationship between periphery and tumor site, herein we have in depth explored CD8+ T-cell subsets characterized by the presence/absence of PD1 and CD28, by integrated multiparametric and targeted multi-omic scRNA-seq analyses. To gain a baseline scenario of the composition, functional states, and transcriptomic signatures predictive of prognosis, we analyzed a cohort of treatment-naïve NSCLC patients in periphery, adjacent non-tumor tissue and tumor site with the aim to identify a T-cell subset sharing features of functionality in periphery and in tumor tissue.

We observed an improved PD1+CD28+ T-cell polyfunctionality with the transition from periphery to the tumor site, while PD1+CD28- T cells decreased their effector function, along with amplified PD1 levels and reduced Ag-experienced marker CD11a. Of note, the absence of TIGIT, TIM3 and CTLA4, either alone or in combination, was associated with higher polyfunctionality in CD28- T cells, while the CD28+ T-cell subset displayed higher efficacy in the absence of TIGIT, TIM-3 and LAG-3. ScRNA-Seq analysis revealed that PD1+CD28- and PD1+CD28+ T-cell subsets cluster into 10 different groups characterized by distinctive transcriptional profiles and heterogeneously represented within the different districts. Then, we found a CD28-associated gradual phenotypic change of CD103+PD1+ CD8+ T cells from the periphery, where are mainly CD28+ and capable to self-upregulating CD103 through an autocrine TGF β production, to the tumor where TRM are mostly enriched of a CD28- phenotype. Furthermore, we found that highly CXCL13-producing TRM lack CD28, while CXCR5-expressing T cells are mainly CD28+, suggesting a crucial role of CD28 in establishing this axis. Notably, when we interrogated the TCGA dataset of patients with lung adenocarcinoma (LUAD) and metastatic NSCLC treated with atezolizumab, we found signatures of

heterogeneous TRM and "pre-exhausted" long-lived effector memory CD8⁺ T cells associated with improved response to immune checkpoint blockade (ICB) only in the presence of high intratumoral CD28.

Our findings identify gene expression signatures able to stratify survival of lung adenocarcinoma (ADC) patients and predict ICB response in advanced NSCLC. CD28 is advocated as a key determinant in CD8⁺ T cells with different functional states in the signatures identified, in both periphery and tumor site, thus likely providing feasible biomarkers of ICB response to be monitored in peripheral blood.

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DNA-LOADED TARGETED NANOPARTICLES AS AN INNOVATIVE PLATFORM FOR THE LOCAL PRODUCTION OF THERAPEUTICAL PROTEINS IN TUMOR MICROENVIRONMENTS

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Cancer immunotherapy is an emerging field based on the stimulation and thus the improvement of the immune system's response through different ways including therapeutic proteins (e.g., therapeutic monoclonal antibodies or cytokines) injected directly into the patient. Side effects and the limited reach of the target site are some of the key contributors to the variable response rate. Approaches to turning cells of the body into 'in situ bioreactors' has been a long-term goal of the gene therapy area. Polymeric nanoparticles (NPs) coated with a targeting mechanism and loaded with protein-coding plasmid DNA can represent a versatile nano platform for the selective production of therapeutic proteins directly in tumor microenvironments.

The current study focuses on B cell malignancies and aimed to develop a biodegradable transfection system represented by an antiCD19-targeted nanocarrier encapsulating a therapeutic antibody-coding plasmid ensuring its local production.

PLGA-PVA NPs functionalized with a recombinant antiCD19 antibody fragment as a targeting agent and loaded with a protein-coding DNA plasmid have been developed and morphologically characterized. NPs were incubated with plasma/calcium or with serum to investigate the interference with coagulation and complement activity, respectively. Direct cell cytotoxicity was also achieved. NPs uptake and consequent transfection studies were performed, both in the CD19+ cell line (Bjab, Burkitt Lymphoma cell line) and CD19- cell lines (RPMI 8226, Plasmacytoma cell line, and Jurkat, Acute T leukemia cell line) by flow cytometry and fluorescence microscopy. Bjab cells were also used to set up in vivo models of B-cell malignancies in zebrafish larvae in which NPs were injected into the bloodstream.

The production yield, the integrity of the protein, and thus the therapeutic potential were established by combining Complement Dependent Cytotoxicity (CDC) and Antibody Dependent Cell Cytotoxicity (ADCC) tests.

The manufacturing process for the protein-coding DNA-loaded targeted PLGA- PVA NPs produces nanocarriers with a round shape with a diameter of 250nm, a negative charge, and a safe toxicological profile, avoiding direct lysis of red blood cells, clotting formation, complement system activation, and cell cytotoxicity. NPs were able to bind and be internalized efficiently in CD19+ cells but not in CD19- ones. In zebrafish models, NPs were safe and easily diffused in tissues through the bloodstream. Surface modifications favor the targeting of B-cells both in vitro and in vivo. The protein expression confirmed the integrity of the encapsulated DNA and then its nuclear import. This results in the production of a structurally intact protein, able to maintain binding specificity and the activity of killing to target and eliminate malignant cells.

This study demonstrated that DNA can be encapsulated into a biodegradable NPs delivery system, which specifically and safely releases the payload inside cells and can be formulated as a relevant and innovative device suitable for gene therapy. In conclusion, this formulation will be used to investigate the therapeutic efficiency in preclinical in vivo models of B-cell malignancies but can also be easily modified to address different targets for the production of tailored therapeutic proteins.

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FUNCTIONAL MAPPING OF AHR-DRIVEN TUMOR MICROENVIRONMENT CHANGES IN HIGH-RISK BREAST CANCER BY SPATIAL TECHNOLOGIES

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Single-cell and spatial mapping of tumor, immune and stromal cells within the tissue microenvironment have revolutionized our understanding of cancer, including breast cancer. Although advances in tumour microenvironment composition have greatly expanded our knowledge on how cancer originates and progresses, the translation of this knowledge into better patient treatments and care has been complex. One of the major limitations has been the difficulty to identify key vulnerabilities within the heterogeneous tumour ecosystem and to translate these changes into actionable biomarkers. AHR is a ligand-dependent transcription factor whose expression correlates with critical changes in the tumor microenvironment thus affecting the response to several anti-cancer strategies, including immunotherapy. Besides its role in xenobiotic detoxification, AHR is now recognized to be an essential gatekeeper that integrates dietary, environmental, microbial, and endogenous ligand signals to modulate immune cell homeostasis and inflammation. For these reasons, targeting AhR is considered an attractive strategy “to kill two birds with one stone”, as it will both dampen tumor immune escape and intrinsic survival signals, at once.

Here we describe the generation of a novel mouse model, to track the expression and regulation of AHR in tissues. To dig into AHR-dependent modulation of the tumor microenvironment, we have also established an experimental platform combining a custom, barcoded, CRISPR/Cas9 screen with advanced spatial imaging.

Preliminary transplantation studies show that AHR expression increases in macrophages invading BRCA1/p53 deficient mouse mammary tumors. These initial results confirm the opportunity to use this mouse model to track AHR levels within the tumor microenvironment. We will combine this genetic model with the CRISPR/Cas9 screen to dissect functional links between cancer-promoting pathways and AHR activities in the tumor microenvironment.

Our study combines advanced genetic technologies with state-of-art imaging systems for a functional analysis of breast tumor microenvironment. It is our goal to translate our imaging findings into digital pathology tools, for better informed decisions in cancer patient care.

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DISSECTION OF MELANOMA-MICROENVIRONMENT INTERACTIONS DURING TUMOR PROGRESSION

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Despite recent advances in melanoma management, metastases appearance remains life-threatening for patients, dramatically impacting on their prognosis and overall survival. It was shown that tumor-surrounding cells, such as stromal components and immune infiltrate have a key role in driving metastasis formation. The development of reliable and adequate preclinical tools to mimic the tumor microenvironment is therefore urgently required. Moreover, the identification of melanoma metastasis drivers is crucial to design novel effective therapeutic approaches.

To investigate the mechanism behind melanoma/microenvironmental interaction, achieving translationally relevant insights into melanoma spreading and metastasis, we developed an innovative and highly reproducible fully human in vitro pre-clinical model of patient-derived melanoma cells, fibroblasts and endothelial cells. The system better recapitulates patients than the previous 2D or 3D models used in the field, maintaining melanoma cell heterogeneity while resembling tissue structures and organization. In our organotypic cultures (OCs), TME components foster melanoma invasion of collagen matrix in vitro and metastatic potential in vivo. We proved an upregulation of phenotype-switch and extracellular matrix remodeling pathways during co-cultures growth. Indeed, the presence of fibroblasts and endothelium in the OCs significantly modifies the melanoma transcriptome, secretome and surface molecule expression, skewing melanoma cells toward a more aggressive phenotype.

Our findings advocate for a prominent role of the L1 adhesion molecule (L1CAM) in mediating melanoma relationship with the surrounding stroma and the consequent switch toward an invasive state. Notably, L1 cell adhesion molecule (L1CAM) expression results highly increase in melanoma cells interacting with fibroblasts and/or endothelial cells. Moreover, L1CAM overexpression on melanoma prompts melanoma aggressiveness in vitro and in vivo. At the same time, in vitro L1CAM silencing abrogates melanoma invasiveness, while its depletion strikingly abolishes melanoma metastatic burden and highly prolongs mice overall survival.

Plasminogen Activator Inhibitor 1 (PAI1 – SERPINE1) is among the secreted factors mostly altered in co-cultures of melanoma, fibroblasts and endothelium and its production is strictly dictated by melanoma/stroma cross-talk. Interestingly, the increase in L1CAM levels sensitizes melanoma to anti-PAI-1 agent cytotoxic effects. Even if additional studies are required to determine the reciprocal role of the two proteins, our results suggest the existence of an L1CAM/PAI1 axis, possibly exploitable as a novel vulnerability of highly aggressive cancer cells.

During the past decade, immunotherapy has changed the way melanoma is treated, significantly improving patients' survival. Nevertheless, the overall fraction of patients benefiting from this therapy is still low, due to innate and acquired therapy resistance. TME plays a crucial role in the acquisition of immune-resistance mechanisms by tumor cells. Thus, we produced an organotypic culture in which the immune system could be, even if partially, represented. Preliminary data suggest that we are able to maintain vital healthy donor PBMCs in co-culture with melanoma cells up to 7 days. Insertion of immune-system components will further improve the complexity and reliability of the model, allowing the test of both targeted- and immune-therapy.

The proposed project will shed new light on the mechanisms behind the relationship between malignant melanoma and the surrounding ecosystem. In particular, we expect that our model will deepen the knowledge of the tumor-stroma relationship in the metastatic context, finally leading to the identification of new druggable vulnerabilities and markers of melanoma evolution. The pharmacological targeting of the identified metastasis drivers could have profound implications for

cancer patients management.

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DEVELOPMENT OF NOVEL THERAPEUTIC COMBINATIONS IN DRUG RESISTANT BRAF-MUTANT MELANOMAS

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Metastatic melanoma is the most aggressive and lethal type of skin cancer. The introduction of systemic combination strategies targeted to specific mutations (BRAF and MEK inhibitors) and immune checkpoints (anti-PD1, anti-CTLA4) dramatically improved patients' overall survival rates. Nevertheless, a large fraction of BRAF-mutant patients develops primary or secondary resistances. To either prevent or overcome resistance in BRAF-mutant melanoma patients, numerous therapeutic approaches were tested, many of them showing unbearable toxicities, mainly due to overlapping adverse events of the targeting agents, or no efficacious results. New combinations are now being tested, exploiting triple-agent combinations (targeted therapy with immunotherapy), but with unsatisfactory results. Thus, the identification of novel vulnerabilities for drug resistant BRAF-mutant melanoma patients is still an unmet clinical need.

We performed an shRNA-based in vivo genetic screen with a library that targets 195 actionable genes (FDAome library), where each gene is linked to specific FDA-approved available drugs that can be repurposed for melanoma treatment. A375 parental cells and A375 cells resistant to dabrafenib and trametinib (A375-DT) were used as a model to uncover new dependencies.

Our FDAome screen unveiled 37 genes which are essential to the in vivo growth of the DT-resistant cells. We validated two candidates, namely CREB-binding protein (CREBBP) and Prolyl 4-Hydroxylase Subunit Beta (P4HB), whose inhibition reduces tumor growth in vivo and increases overall survival of A375-DT but not of A375 parental cells, suggesting a putative point of intervention in resistant tumors. Moreover, CREBBP and P4HB silencing in vitro reduces the migratory capacity of A375-DT but not of A375 cells, recapitulating the in vivo results. We further validated the in vitro results in another cell line, namely SKMEL28-DT and in resistant patient-derived primary cultures. We then tested if CREBBP (CCS1477) and P4HB (PACMA31) inhibitors are effective as monotherapies. Both compounds were able to reduce cell viability in vitro and tumor growth in vivo of A375-DT cells.

It was previously shown that CREBBP and P4HB targeting re-sensitize tumor cells to the conventional therapy in many cancer types but not in melanoma. We will therefore investigate if CCS1477 and PACMA31 in combination with dabrafenib and trametinib are able to re-sensitize cells to the standard-of-care therapy. In parallel, we will exploit patient-derived primary cultures resistant to dabrafenib and trametinib to improve the translational potential of our findings.

The newly identified cytotoxic agents will be also combined with immunotherapy, to reach a more durable response. To this end, we are now generating a syngeneic mouse BRAF-mutant melanoma model resistant to dabrafenib and trametinib, using D4M-3A cells.

To conclude, these approaches and models will allow us to develop new combinatorial treatments for relapsing melanoma patients by drug repurposing.

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DROP-OUT FUNCTIONAL SCREEN UNVEILS MYD88 AS A DRIVER GENE OF MELANOMA METASTASIS

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Despite the significant improvement in the overall survival rates, metastatic melanoma prognosis remains poor and still represent an unmet clinical challenge. More predictive preclinical models are needed to deepen our comprehension of the leading mechanisms of cell dissemination and identify novel driver genes.

Focusing on NRAS-mutant metastatic melanoma patient-derived xenografts (PDXs), we set up a relevant model of spontaneous metastasis in vivo unveiling a remarkable polyclonality of lesions composition. Then, we proved that drop-out functional screens are feasible in these systems and identified numerous metastasis-specific driver genes, describing high functional heterogeneity among individual metastases.

In particular, we described for the first time the role of Myeloid Differentiation Primary Response Protein (MyD88) in driving melanoma metastasis formation to both regional and distant sites, and prolonging mice overall survival. MyD88 silencing did not impact on the primary tumor growth rate, but induced a profound transcriptional reprogramming at this stage, involving metabolic functions, cytoskeletal reorganization and epithelial-to-mesenchymal transition (EMT), that leads to reduced invasive properties of melanoma cells. Furthermore, pharmacological targeting of MyD88 reproduced analogous effects halting cell spreading properties, suggesting that its therapeutic exploitation may represent an innovative treatment approach and will be therefore further developed. Moreover, we started dissecting the downstream cascade mediating MyD88 mechanism of action. MyD88 knock-down reduced the activation of many signaling pathways, namely NF- κ B, MAPK and STAT3. In particular, pharmacological inhibition of STAT3 reduced cell invasive capacities in melanoma PDX 3D models, phenocopying MyD88 genetic silencing, suggesting a role for STAT3 in MyD88 signaling.

We then exploited an experimental model of metastasis, injecting shMyD88 cells directly in the bloodstream (intravenously) and showing a dramatic reduction of organs colonization potential. Indeed, upon MyD88 knock-down we assessed a decreased expression of integrins and adhesion molecules mediating tumor-endothelium attachment. We moreover demonstrated that conditioned medium derived from melanoma MyD88-silenced cells impairs angiogenic potential of endothelial cells in vitro. Further investigations are ongoing to confirm the role of MyD88 in the angiogenesis and adhesion to endothelial cells during the extravasation phase of the dissemination process.

We also analyzed a putative activation mechanism for MyD88. The administration of several environmental stimuli promoted cell plasticity, inducing the upregulation of EMT/metastasis genes. This modulation is abolished upon MyD88 silencing, indicating that MyD88 is contributing to cell plasticity. Using single-cell multiplexing staining approach on PDX tumor samples, we defined two populations based on MyD88 expression, namely MyD88^{high} and MyD88^{low}. We proved the MyD88^{high} population is characterized by increased expression of MyD88 downstream elements (phospho-IRAK1/4 and phospho-ERK1/2) and the known invasive markers AXL and PDGFR- β , unveiling this MyD88^{high} subpopulation may be endowed with improved metastasis-initiating capacities.

We proved a prominent role of MyD88 in the dissemination dynamics of melanoma progression, thus making it a promising target to arrest cell dissemination.

P501

SPATIAL TRANSCRIPTOMIC ANALYSIS OF A PATIENT WITH NON-SMALL CELL LUNG CANCER (NSCLC) WITH PATHOLOGICAL COMPLETE RESPONSE (PCR) AFTER NEOADJUVANT CHEMOIMMUNOTHERAPY

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In the past few years, immune checkpoint inhibitors (ICIs) alone or in combination with chemotherapy (ChT) dramatically changed the treatment scenario of advanced NSCLC, with a not negligible fraction of patients deriving long-term survival benefit. On this base, there is a strong rationale to test ICI with or without ChT even in the neoadjuvant setting, where a definitive cure is the goal of treatment. Available data demonstrated that neoadjuvant chemoimmunotherapy enhanced surgical outcomes by increasing the rate of pCR. However, the picture of molecular mechanisms underlying pCR are poorly characterized and little is known about the role of tumor immune microenvironment (TIME) and immune cell localization. In addition, standardized immune-related criteria for pCR assessment are lacking.

Here, we leveraged recent advances in spatial transcriptomics by 10X Visium technology and quantitative digital pathology coupled with machine learning to decipher the molecular and clinical landscape of pCR following neoadjuvant chemoimmunotherapy in a patient with NSCLC.

Case Presentation: A 49-year-old female was admitted to our Institution after the resection of a single brain metastasis. She was a heavy smoker without significant comorbidities. The histological and molecular evaluation revealed an adenocarcinoma of the lung, PD-L1 40%; ALK not rearranged, EGFR wild type, ROS1 not rearranged. Radiological assessment with an 18-FDG/PET and a contrast-enhanced CT scan evidenced the primary lesion in her right upper lobe and the patient was staged as cT2bcN0pM1a according to the 8th TNM classification. After a multidisciplinary discussion, a neoadjuvant treatment including platinum-based chemotherapy and the anti PD-1 pembrolizumab followed by surgery, was planned. After completing three cycles of treatment, tumor assessment showed a partial response according to RECIST criteria and the patient underwent lobectomy.

Pathological evaluation of the resected lung specimen was negative for malignant cells and revealed the presence of several immune features associated to response to immunotherapy, including Tertiary Lymphoid Structure (TLS), proliferative fibrosis and cholesterol clefts.

Spatially resolved transcriptomics of paired pre-treatment surgically removed brain metastasis and post-treatment resected primary lung cancer showed that under the immunological pressure of treatment, the TIME of this exceptional responder was enriched in B cells and plasma cells producing IgG and IgA, supporting the pivotal role of B cells in modulating the TIME and favouring response to cancer immunotherapy. Furthermore, spatial transcriptomic revealed the presence of a cluster of specific cancer associated fibroblasts (CAFs), randomly distributed in the core regions whereas ECM remodelling CAFs are in the peritumoral area. Conversely, the analysis of the brain metastasis showed paucity of adaptive immune cells and relevant presence of innate immune cells. Interestingly, we identified an area with localized neutrophils.

These findings highlight how spatial transcriptomics may help in identifying immune-related parameters guiding precise immunotherapeutic treatment.

* Equal contribution

P502

TYPE III INTERFERONS DRIVE THYMIC DC1 ACTIVATION TO PROMOTE CENTRAL TOLERANCE

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The world incidence of autoimmune diseases has been on the rise in the last decades and is a growing concern as a side effect of immunotherapy for cancer. The major process protecting the organisms against the development of autoimmune diseases is immune tolerance to self. The thymic medulla is essential for the establishment of immune tolerance and accommodates several specific subtypes of antigen-presenting cells (APCs), which produce and present self-antigens to developing T cells. These APCs consist of medullary thymic epithelial cells (mTECs), B cells, and several populations of dendritic cells (DCs). Recently it was discovered that the thymic DC compartment contains an activated population of CCR7+ DC (aDCs) that resembles those present in inflamed or tumor tissues. Although the general role of DCs in promoting thymic tolerance is extensively studied, the direct function of those aDCs and the molecules that are responsible for their activation in the thymus are completely unknown.

Transgenic mouse models with defective interferon signaling were used to study the function of Type III interferons in the thymus. The data were acquired using Single-cell RNA sequencing, lineage tracing experiments and multi-parametric flow cytometry analysis.

Our data suggests that aDCs in the thymus are of dual origin differentiating either from XCR1+ DC1 (aDC1) or SIRP α + DC2 (aDC2). The analysis of the transcriptomic data also revealed that thymic DCs in general and XCR1+ DC1 in particular are enriched for the expression of interferon stimulated genes (ISGs). By analysis of several mouse models with defected interferon signaling we found that activation of thymic aDC1 was specifically abrogated in IFNLR deficient strain, suggesting the dependency of those cells on type III interferon signaling. Furthermore, we observed that type III interferons are in the thymus specifically produced by a small population of mTECs.

Together, our data provide evidence that type III interferons produced by mTECs are essential for activation of XCR1+ DC1 to become CCR7+ aDC1 that possess enhanced antigen processing and presentation and are thus crucial for the proper selection of T cells.

P503

THE ROLE OF HMENA IN CAF-DERIVED EXTRACELLULAR MATRIX REMODELLING AND IMMUNE CELL FUNCTIONALITY IN NON-SMALL CELL LUNG CANCER

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Mechanical aberrations in tumour, such as increased stiffness, fluid flow and solid stress significantly impact innate and adaptive anti-tumour immune response in solid cancer, including Non-Small Cell Lung Cancer (NSCLC). Recently, the contribution of Extracellular Matrix (ECM) remodelling in the modulation of immune cell functionality and response to therapies is emerging, yet a deep understanding of the dynamic and multi-directional interconnectivity between cellular and non-cellular components of the NSCLC Tumor Immune Microenvironment (TIME) is still lacking. Cancer Associated Fibroblasts (CAFs) and in particular the ECM-myCAF subset, are the main players of the altered ECM deposition and assembly, thus shaping the mechanical and biochemical landscape of tumour stroma. Our laboratory has identified hMENA, an actin regulatory protein and its splicing isoforms, hMENA11a and hMENA Δ v6, as a central node in epithelial mesenchymal plasticity with specific isoforms differentially involved in critical pathways affecting cancer progression, β 1 integrin-ECM axis, immune suppression, Tertiary Lymphoid Structures (TLS) presence and localization, with an impact on the clinical outcome and response to immune checkpoint inhibitors of NSCLC patients. Recently, a novel role of hMENA in gene expression rewiring has been described linking the adhesome to the nuclear envelope. In this study, we aimed to elucidate how the expression of hMENA in CAFs impacts ECM remodelling, and in turn the anti-tumor immune response.

We leveraged confocal and Brillouin microscopy to evaluate the topography and to measure the viscoelastic properties of ECM produced by NSCLC patient-derived CAFs silenced or not for hMENA/hMENA Δ v6 (sihMENA(t)).

Confocal staining of decellularized ECM (dECM) deposited by sihMENA(t) CAFs revealed less organized fibers compared to dECM obtained from siCTR CAFs which are characterized by anisotropic features, i.e. more aligned tracks. The morphological features revealed by confocal microscopy will be further extended by exploiting second harmonic generation signals of two-photon illumination, which provides a label free effective way to study the structural composition of ECM fibers, and are reflected in the results of Brillouin microscopy, an innovative optical elastography method based on inelastic light scattering process. Comparing the Brillouin spectra acquired on dECM of siCTR and sihMENA(t) CAFs, we found that the Brillouin peak remains at the same frequency while decreasing its width. This variation highlights differences in term of acoustic attenuation, quantity linked to the viscous response and to the mechanical heterogeneity of the material. When we assessed the impact of the diverse viscoelastic and topographical features of the hMENA-related matrisome on the immune cell functionality by multiparametric flow cytometry, we found that immune cells isolated from healthy donors' peripheral blood (PBMC) cultured on the dECMs obtained from sihMENA(t) CAFs showed different phenotype and functionality. Specifically, we observed a decreased frequency of the regulatory CD19⁺CD24^{high}CD38^{high} B cells in PBMC cultured on dECM of sihMENA(t) CAFs, a decreased frequency of PD-1⁺CD19⁺ along with increased frequency of memory CD27⁺CD19⁺ B cells in sihMENA(t)-dECM compared to siCTR-dECM. Furthermore, we found increased monocyte to macrophage differentiation and a polarization toward a M1-like phenotype in sihMENA(t)-dECM.

These findings suggest that hMENA regulates immunosuppressive functions via regulation of ECM architecture and bio-physical properties, likely acting as a critical determinant of anti-tumor immunity. If confirmed on a larger number of samples, by shedding light on the complex interplay between physics and immunity in the context of ECM modulation, these findings may pave the way for innovative therapeutic interventions combined with immunotherapy in NSCLC.
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* Equal contribution

P504

RECRUITING INNATE IMMUNE CELLS UNIVERSALLY: DEVELOPMENT OF A NOVEL UNIVERSAL INNATE CELL ENGAGER FOR CANCER-IMMUNOTHERAPY

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Novel antibody-engineering approaches have enabled the development of targeted innate cell engagers which redirect innate immune cells to eliminate cancer or suppressive immune cells. These antibodies function through binding to surface antigens on target cells and engaging activating receptors on innate immune cells such as natural killer cells and macrophages. Although early innate cell engagers have shown promise in controlling various cancer types, several major challenges remain in the clinics, including high tumor heterogeneity, low tumor immunogenicity, and reduced efficacy in solid tumors. Here, we present a universal innate cell engager as part of a novel adaptor-based antibody platform. Our universal innate cell engager utilizes a two-step approach combining the power of an adaptor and an effector antibody. First, an Fc-silenced adaptor antibody comprising P329G LALA mutations is used to target the desired antigen in the tumor microenvironment. Then, as an effector antibody, a universal innate cell engager directed against the P329G mutation on the adaptor antibody is used. Innate immune cells are recruited by the universal innate cell engager through its active Fc, leading to antibody-dependent cellular cytotoxicity against the target cells. To maximize innate cell activation, the Fc portion of the universal innate cell engager can be glycoengineered for enhanced FcγRIII affinity.

We conducted reporter cell assays using Jurkat ADCC cells transfected with FcγRIII as effector cells and several cancer cell lines as target cells in the presence of universal innate cell engager to test whether it can lead to FcγRIII engagement. Moreover, we performed ex vivo ADCC assays using allogenic NK cells isolated from healthy donors as innate immune cells and several different cancer cell lines as targets cells to test the cytotoxic potential of universal innate cell engager.

In vitro assays showed that our universal innate cell engager is inducing innate immune cell activation and innate immune cell-mediated target cell killing when combined with different antigen-targeted adaptor antibodies, while no effect was observed in the presence of the non-targeting adaptors used as controls.

These results provide preliminary in vitro evidence that the universal innate cell engager can be used as an efficacious cancer treatment. Ultimately, this approach may enable off-the-shelf personalization via combination of universal effector cell engagers and selected adaptor antibodies specific to the patient's tumor profile.

P505

GRB2, A NOVEL BIOMARKER FOR CANCER CELLS GENOMIC INSTABILITY, PARPi SENSITIVITY AND THE ASSOCIATED INNATE IMMUNE RESPONSE OUTCOME.

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Growth factor receptor-bound protein 2 (GRB2) is a cytoplasmic adaptor for tyrosine kinase signaling and a nuclear adaptor for homology-directed-DNA repair. Here we find nuclear GRB2 protects DNA at stalled replication forks from MRE11-mediated degradation in the BRCA2 replication fork protection axis. Mechanistically, GRB2 binds and inhibits RAD51 ATPase activity to stabilize RAD51 on stalled replication forks. In GRB2-depleted cells, PARP inhibitor (PARPi) treatment releases DNA fragments from stalled forks into the cytoplasm that activate the cGAS–STING pathway to trigger pro-inflammatory cytokine production. Moreover in a syngeneic mouse metastatic ovarian cancer model, GRB2 depletion in the context of PARPi treatment reduced tumor burden and enabled high survival consistent with immune suppression of cancer growth. Collective findings unveil GRB2 function and mechanism for fork protection in the BRCA2-RAD51-MRE11 axis and suggest GRB2 as a potential therapeutic target and an enabling predictive biomarker for patient selection for PARPi and immunotherapy combination.

GRB2 is an important intracellular adaptor protein that consists of a central SH2 domain flanked by two SH3 domains and is essential for cell proliferation. Classically its SH3 domains direct complex formation with proline-rich regions of other proteins, and its SH2 domain binds tyrosine phosphorylated sequences⁶⁻¹⁰. In the cytoplasm GRB2 acts in initial steps of receptor tyrosine kinase (RTK) signaling to the Ras-MAPK cascade. In the nucleus GRB2 adaptor moonlights in initial steps for efficient homology-directed-DNA of DNA double strand breaks (DSBs). Given these functions, we reasoned that GRB2 could potentially act more generally in the DNA damage response (DDR) including its DNA replication stress responses and the activation of the innate immune response by loss of genome stability during replication. In particular, tumor cells with oncogenic replication stress might select for GRB2 activities associated with proliferation and the DDR. Yet GRB2 nuclear activities and mechanisms as well as possible connections to cancer are largely unappreciated.

Various

Here we tested and defined GRB2 interactions and activities with DNA replication fork proteins in vitro, in cells, and in vivo. We find that GRB2-depleted cells strikingly mirrored DNA replication fork protection characteristics of the BRCA2 deficient phenotype. In response to replication stress from hydroxyurea (HU) and PARP inhibitor (PARPi), GRB2 stabilized RAD51 on single-stranded DNA (ssDNA) at stalled replication forks to reduce cytoplasmic DNA accumulation and cGAS/STING activation. Furthermore, BRCA2 knockdown (KD) in a GRB2-KO background showed no added fork degradation defects consistent with an epistatic relationship. During RF stress, GRB2 depletion promoted cGAS/STING activation that translated into the production of inflammatory cytokines and recruitment of cytotoxic T-cells. In a GRB2-depleted syngeneic ovarian cancer mouse model, PARPi treatment led to enhanced targeted destruction of tumor cells by the host immune system compared to PARPi alone. These findings align GRB2 with BRCA2 for replication fork protection and PARPi responses that include the therapeutic activation of innate immunity.

Here we discovered that GRB2 acts in response to HU and PARPi by stabilizing RAD51 on DNA to reduce both cytoplasmic DNA accumulation and cGAS/STING activation. Thus, in the context of RF stress, GRB2 depletion promotes cGAS/STING activation that translated into the production of inflammatory cytokines and recruitment of cytotoxic T-cells. Moreover, in the immune competent

mouse model, PARPi treatment of cancer cells with low-GRB2 leads to an enhanced targeted destruction tumor cells by the host immune system compared to PARPi alone. This combination of GRB2 depletion and PARPi treatment enabled longer survival and less cancer-associated complications.

P506

ANTI-GPC1 IGM ACTIVATING THE COMPLEMENT SYSTEM IS AN EFFECTIVE TREATMENT IN A PANCREATIC DUCTAL ADENOCARCINOMA MOUSE MODEL

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Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive malignancies with a very low survival rate at 5 years. Chemotherapy agents causes only a modest enhancement in the survival and is usually associated to toxic effects. The proteoglycan GPC1 showed a cell surface localization in PDAC tissues but limited or absent expression in the most of adult tissues and in chronic pancreatitis. Exploiting this promising tumor-associated antigen, an anti-GPC1 monoclonal IgM was produced with the aim to induce a strong complement activation on PDAC cells, a consequent modification of tumor microenvironment associated with a direct antibody-based immunotherapeutic effect.

Several monoclonal antibodies were produced in collaboration with Takis S.R.L. using hybridoma technology; to specifically address epitopes of GPC1 very close to cell membrane, animals were immunized with a vector encoding for 46 amino acids. The most promising mAbs were produced, characterized, and tested in vitro for the ability to induce Antibody-Dependent Cellular Cytotoxicity (ADCC), Complement-Dependent Cytotoxicity (CDC), and apoptosis. Then, a xenograft model of the pathology was set up and employed to evaluate the biodistribution of the molecules and its therapeutic efficacy.

The antibody was characterized both in vitro and in vivo. Biodistribution studies, using near-infrared optical imaging technology, confirmed the capacity of anti-GPC1 IgM to selectively bind its target in a localized PDAC model developed in Nude mice, with a pick after 4 days; this antibody was mainly eliminated by the liver. Moreover, a single injection of anti-GPC1 IgM induced a strong activation of the classical pathway of the mouse complement system; as expected, C1q, C3 and C9 deposits were documented in tumor microenvironment by immunofluorescence analysis, causing extended necrotic areas as well as the recruitment of CD14+ macrophages and CD56+ NK cells. Repeated injection of anti-GPC1 IgM (twice a week) controlled tumor growth in all tumor-bearing mice. All saline-treated animals died in 19 days after the first treatment while 60% of anti-GPC1 IgM treated mice survived at the end of the study (50 days after the first treatment).

Collectively, these findings showed the capacity of anti-GPC1 IgM to strongly activate the complement system on PDAC cells, causing cancer cell death, modifying tumor microenvironment and finally increasing mice survival.

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DELINEATING THE EPIGENETIC LANDSCAPE OF T CELL EXHAUSTION IN LUNG CANCER USING MULTIMODAL SINGLE-CELL ANALYSIS

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Adoptive cell therapy using autologous tumor infiltrating lymphocytes (TIL) is a promising avenue for treatment of lung cancer. Despite preliminary results demonstrating its effectiveness, autologous TIL therapy suffers from several limitations including progressive reduction in cytotoxic ability of T cells. One of the major challenges is that T cells become exhausted upon persistent exposure to antigens in tumor. Exhausted T cells display attenuated cytotoxic function and overexpression of inhibitory receptors which are critical factors for the therapeutic potential of T cells. While cellular properties that characterize exhaustion states of T cells in various biological contexts have been described, the molecular mechanisms underlying T cell exhaustion and heterogeneity within exhausted T cells in lung cancer remain unclear. To address this challenge, we investigated the transcriptional and epigenetic landscape of T cells in lung cancer using multimodal genomic sequencing data.

We conducted a computational analysis of single cell multiome sequencing data that contains both chromatin accessibility and gene expression in the same cells of TILs from six non-small cell lung cancer (NSCLC) patient samples. The TILs had been expanded with IL-2 for two weeks prior to sequencing. To identify cell types that are present in the lymphocytes, we performed unsupervised clustering of the gene expression data. We then applied CellSpace, a novel computational algorithm developed by our group that learns a low-dimensional embedding of chromatin accessibility data based on both the accessibility and the DNA sequence of genomic loci. By combining the cell type labels determined from gene expression and the CellSpace embedding of chromatin accessibility data, we identified subpopulations of TILs that represent epigenetically distinct cell states within T cell exhaustion and conducted downstream analyses to interrogate these states.

We identified multiple cell types including CD8+ T cells, CD4+ T cells, and NK cells based on gene expression from the TIL multiome data. Application of CellSpace to the accessibility data generated a low-dimensional embedding of cells from the six patient samples. Using this embedding, we identified epigenetically heterogeneous subpopulations of exhausted T cells including progenitor exhausted T cells, NK-like exhausted T cells, and exhausted T cells that are represented by several marker genes such as TCF7, GZMB, TOX, and CTLA4. Furthermore, the integration of data from multiple patients using CellSpace resulted in a higher number of genomic loci that are highly accessible in exhausted T cells compared to analyzing each patient data separately. Lastly, we inferred regulatory interactions in TILs by combining the gene expression and chromatin accessibility data and identified co-regulatory modules in exhausted T cells.

In conclusion, our multiomic analysis using novel computational methods uncovered distinct states of T cell exhaustion in NSCLC. Integration of multimodal assay from multiple patients elucidated the molecular landscape of TIL subpopulations in NSCLC and the regulatory logic behind their heterogeneity.

P508

ITERATIVE STIMULATION OF T CELLS IMBUES RESISTANCE TO EXHAUSTION

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CD8 T cells are critical components of anti-tumor immunity through their release of cytokines and direct killing of malignant cells. Unfortunately, cancer can develop in tissues with low T cell surveillance and tumors diminish T cell responses via chronic stimulation-induced exhaustion. The infiltration of tumors and continued functionality of T cells are two major hurdles for successful immunotherapeutic approaches.

Using a heterologous prime-boost immunization strategy and adoptive transfers over the last 10 years, we generated an abundant murine memory T cell population that have been exposed to greater than 50 viral infections. These cells, termed iteratively stimulated T cells (ISTCs), maintain proliferative capacity and cytokine production throughout each infection. We hypothesized that this continued functionality may provide an advantage when exposed to chronic antigen. We subjected ISTCs to rapid, iterative viral infections for chronic antigen stimulation and evaluated survival, functionality, and transcriptional responses of these cells.

We find that ISTCs survive and function longer than other populations of T cells when exposed to chronic antigen stimulation. ISTCs continue to proliferate and produce cytokines in response to antigen for twice as long as their naïve counterparts. Interestingly, despite maintaining function, ISTCs have high expression of exhaustion markers including PD-1, LAG3, and TIM3 as well as the exhaustion-associated transcription factor TOX. Moreover, we performed single-cell RNA-Sequencing analysis to evaluate the transcriptional profiles between ISTCs and other populations of T cells undergoing chronic antigen stimulation. We find differential transcriptional programs between ISTCs and other populations of T cells that may underlie the functional differences observed.

These results suggest that ISTCs maintain functional capacity under chronic antigen stimulation and may have molecular adaptations to subvert canonical exhaustion programs. Investigating the adaptations of ISTCs provides a unique opportunity to discover cellular mechanisms for maintaining T cell function in the context of cancer. Future directions will investigate the infiltration and functionality of ISTCs in a melanoma model to evaluate the role of these adaptations in maintaining anti-tumor T cell responses.

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INEFFICIENT CROSS-PRIMING BY IRF4-DEPENDENT MIGRATORY DENDRITIC CELLS IS REQUIRED FOR THE ACTIVATION OF TUMOR-INFILTRATING TISSUE RESIDENT CD8+ T LYMPHOCYTES WITHIN TUMOR DRAINING LYMPH NODES

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Tumor infiltration by CD8+ T cells endowed with a tissue-resident phenotype (TRM) is generally associated to favorable outcomes in human solid tumors. These cells are characterized by expression of CD103 and CD69 together with multiple exhaustion markers. CD8+ T cells are typically primed in the tumor-draining lymph nodes by dendritic cells (DCs) cross-presenting tumor-antigens, thereby shaping adaptive immune responses. XCR1+ DCs play a major role in CD8 activation overall. However, little is known on : i) the extent of the overlap between dysfunctional features and the residency program within CD8+ T lymphocytes, ii) at which stage of T cell activation and in which location (tumor-draining lymph node vs tumor) CD8+ T cells acquire the transcriptional program underpinning tissue residency and iii) which DCs subsets are needed for TRM specification.

In this study, we delineate the activation of lung-associated CD103+CD69+ TRMs in the KP (KrasG12D, p53-/-) model of lung adenocarcinoma and identify DC requirement.

Here we show that the residency and exhaustion programs are distinct but partially overlapping in tumor-infiltrating CD8+ lymphocytes. Using an unbiased single-cell RNA-sequencing approach, we found that activated CD8+ T cells in tumor-draining lymph nodes comprise a rare population of CXCR6+CD103+ cells endowed with tissue-residency features. Both XCR1 and IRF4-dependent migratory have uptake tumor materials and cross presentation tumor-associated antigens by MHCI although IRF4-dependent DCs do it much less efficiently. TRM-like cells are generated in the tumor-draining lymph node in a process dependent on both XCR1 and IRF4-dependent migratory DCs. Mechanistically, we found that IRF4-dependent DCs are less efficient at driving T cell proliferation but they maintain CD103 expression during T cell activation, thereby enabling TRM specification.

Altogether, these findings highlight the fine tuning of T cell fate and memory formation by multiple DCs subsets within draining lymph nodes. These findings have important implication for the design of immunotherapeutic strategies purposed to activate long-lived T lymphocytes persisting within tumor beds.

P510

GENERATION OF IGA PRODUCING B CELLS IN TERTIARY LYMPHOID STRUCTURES OF LUNG ADENOCARCINOMA

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Tumour infiltrating immune cells (TILs) often represent host immune reaction and in many solid tumours high number of TILs is associated with favourable clinical outcome. In recent years, it became evident that particular, still undefined, tumour microenvironments are able to promote the generation of germinal center-like structures, the so called tertiary lymphoid structures (TLS), rich in B cells.

In order to characterize B-cells present in Lung Adenocarcinoma we have isolated TILs from tumour and adjacent non-tumoral tissues, as well as from peripheral blood lymphocytes from two patients whose primary tumor tissue revealed that CD20+ B cells were not scattered as occurs for CD3+ T cells, but rather found in aggregates surrounded by CD3+ T lymphocytes, a feature of TLS. We performed a transcriptomic analysis using BD Rhapsody and Illumina next generation sequencing platform from TILs and PBMC.

To identify B-lymphocytes, we firstly screened all cells, based on differently expressed genes and proteins present on our Abseq preparation. We have recognized B cells by the up regulation of genes such as MS4A1 (CD20), IGKC (Ig kappa light chain), IGHM/D/A/G, CD74, Pax5, CD24, CD40, FCRLA (Fc receptor like A for gamma) among others and by the presence of the Abseq positivity for CD19, CXCR5 and HLA-DR. We then focused on the analysis of tumour resident B cells and found that tumour resident B cells could be divided in three clusters corresponding to three independent signatures. One, of naïve B cells characterized by high expression of IgM, IgD, CD79A and IL-4R but still presenting TCL1A, PCDH2, and the transcription factors FOXO1 and FOXP1 associated to a more immature B cell status. Thus, these naïve B cells are likely intimately related to recent bone marrow B cell migrants. Another cluster with features of activated B cells showed high expression of transcripts related to survival, such as Bcl2A1 and MYC, and CD40, CD83, ICAM, IL-21R and MHC class II molecules all involved in B-T interaction required for T-dependent Ig class-switching. In fact, this cluster showed a reduction on IgM/IgD expression and starts to present transcripts of IgG1 heavy chain. Finally, the "effector" B cell cluster that expressed mostly IgA1 and 2 transcripts, has lost all the other Ig subclasses. The cluster showed high expression of AICDA suggesting that B cells still have the ability of performing Ig hypermutation in the context of GC reaction. We also found that in this cluster B cells started to express syndecan-4 and Blimp-1 with reduction of Pax-5, in agreement with a possible transition CD27pos switched memory B cells into fully developed IgA plasma cells.

These results strongly suggest that in the lung adenocarcinoma tissues analysed, active TLS were formed and that these structures were able to generate IgA activated/plasma cells from early immature B cells recruited from the peripheral blood into tumour site. The functional significance of IgA in the clinical follow up of patients also in the context of tumour mutational profile is under evaluation in a larger cohort of patients.

P511

CHARACTERIZATION OF THE EPIGENOMIC PROFILE SHAPING HUMAN CD4 REGULATORY T CELL IDENTITY IN THE TUMOR MICROENVIRONMENT

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Tumor infiltrating regulatory T (tiTregs) cells can suppress specific anti-tumor immune responses coordinated by effector CD4+ T lymphocytes thus promoting tumor growth. We previously demonstrated that tiTreg cells display a unique transcriptional signature with respect to normal adjacent tissue (NAT) Treg cells (De Simone et al. Immunity 2016), while their epigenetic landscape is still largely uncharacterized. Among regulatory elements, enhancers are key to coordinate gene expression programs in a tissue-specific manner, we therefore focused on enhancer rewiring as the epigenomic process that orchestrates the response of tiTregs to the microenvironmental cues within the tumor.

We performed integrated multi-omics (ChIPseq and ATAC-seq) on Tregs isolated from blood, tumor and NAT of colo-rectal cancer (CRC) patients and we drew a comprehensive map of active enhancers and key transcription factors (TFs) reconstructing the crucial nodes of regulatory networks that are activated by tiTregs.

Through the integration of epigenomic and transcriptomic data we identified active enhancers specific for tiTreg signature genes and showed that the epigenomic changes between NAT and tiTregs reflect their differences at gene expression level. Then, using in silico footprinting, we systematically determined the TF binding dynamics across Treg cell states highlighting key trans regulators that preferentially act upon the enhancerome of tiTregs.

We are now evaluating the relevance of candidate TFs identified as potential key regulators for the acquisition of the tiTreg cell state by both functional modulation and targeted spatial profiling technologies able to capture both RNA and protein information (1,000-plex and 64-plex respectively) at sub-cellular resolution. This analysis, integrated with CRC single-cell RNAseq datasets, will provide a comprehensive molecular characterization of the niches surrounding Treg cells enriched for the specific gene signatures whose expression is coordinated by the TFs under investigation. We envisage spatial transcriptomics as the layer where all our findings can be mapped back on tissues, allowing to assess to what extent tiTreg phenotype is affected by the spatial organization of the tumor microenvironment.

we expect that our studies will contribute to unveil key regulatory nodes underlying the acquisition of tiTreg hyperactivated phenotype thus providing actionable targets for novel therapeutic approaches.

P512

DIRECT ACTIVATION OF STAT5 ENHANCES CD8+ T CELL SURVIVAL AND ANTI-TUMOR IMMUNITY

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Cancer can evade T cell mediated destruction by establishing a suppressive tumor microenvironment (TME) that deprives T cells of pro-survival cytokines, such as IL-2. These cytokines bind to their cognate receptor to cause the JAK-STAT signaling required for T cell survival and effector functionality. Attempts to harness cytokines therapeutically have resulted in the direct administration of IL-2 to cancer patients. However, only a subset of patients responds to this treatment, and the therapeutic potential of IL-2 has been limited by its poor pharmacological properties and potential for serious adverse events. Because cytokine-receptor binding causes Janus kinases (JAKs) to phosphorylate and activate STAT proteins, we hypothesized that the direct activation of STAT proteins in a cytokine-independent manner could restore the signals needed by T cells to mount an effective and sustained attack against cancer. We reasoned that the direct recruitment of tyrosine kinase activity to a STAT protein could be used to enforce its activation and thereby rewire T cells to persist in a hostile TME that is depleted of pro-survival cytokines. Because the cytokine IL-2 activates STAT5, we developed a STAT5 activator and evaluated its potential to enhance T cell therapies for cancer treatment.

To evaluate whether direct activation of STAT5 can enhance T cell survival and functionality, we developed a bifunctional peptide that contains a kinase binding site and a STAT binding site. The STAT binding site was tailored to recruit specific STAT proteins. Specifically, a STAT5 binding site derived from the IL-2 receptor was incorporated into the bifunctional peptide to develop a STAT5 activator. The specificity of the STAT5 activator was determined by immunoblotting to assess the profile of phosphorylated STAT proteins. Viral transduction was used to incorporate the STAT5 activator into primary CD8+ T cells isolated from C57BL/6 mice. We evaluated whether cytokine-independent activation of STAT5 enabled CD8+ T cells to persist and retain their function *ex vivo* under suppressive culture conditions where the pro-survival cytokine IL-2 was absent. *Ex vivo* analyses were complemented by syngeneic mouse tumor models (EL4-OVA and B16-OVA) to determine whether direct activation of STAT5 enhanced the persistence and function of adoptively transferred tumor-specific OT-I T cells *in vivo*.

We determined that the direct activation of STAT5 enabled CD8+ T cells to remain viable over several days under suppressive culture conditions lacking the pro-survival cytokine IL-2. Despite the absence of IL-2, CD8+ T cells modified with a STAT5 activator retained their functional capacity to become activated by antigen, produce IFN γ upon restimulation, and kill cancer cells *ex vivo*. We therefore incorporated a STAT5 activator into tumor-specific T cells and adoptively transferred them into tumor-bearing mice. OT-I CD8+ T cells modified with a STAT5 activator caused potent tumor regression and reduced tumor outgrowth in the EL4-OVA tumor model. We also observed that incorporation of a STAT5 activator enhanced CD8+ T cell persistence *in vivo* using the suppressive B16-OVA model. We determined that the reduction in B16-OVA tumor outgrowth could be attributed to a marked increase in the abundance of tumor-specific T cells within the tumor, blood, and tumor-draining lymph node.

These data demonstrate that CD8+ T cell survival and cytotoxic capacity can be maintained in cytokine-depleted environments by the direct activation of STAT5. Our findings reveal that T cells can be improved to resist the miscommunication that cancer causes by targeting essential intracellular signaling pathways needed for a sustained anti-tumor response. We envision that a STAT5 activator can be used to enhance T cell therapies for cancer treatment.

P513

DEFINING A CROSS-PRIMED ANTI-TUMOR T CELL SIGNATURE TO GUIDE IMMUNOTHERAPY DEVELOPMENT

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Immunotherapies such as checkpoint blockade have revolutionized cancer therapy, but responses are seen only in a subset of patients. Though tumor-intrinsic factors such as tumor mutational burden (TMB) or IFN γ “inflamed” signature partially predict sensitivity to checkpoint blockade, these correlations are limited—most patients with “inflamed” tumors or high TMB still fail to respond to therapy. A critical step for efficacy of T cell-mediated immunotherapies, including checkpoint blockade, is Antigen Presenting Cell (APC), e.g. dendritic cell (DC), cross-presentation of tumor antigen (Ag) to CD8 $^{+}$ T cells. Cross-presentation in vivo requires Batf3-expressing type 1 dendritic cells (cDC1). However, these DC have additional functions, including secretion of T cell-recruiting chemokines, that also drive tumor-reactive T cell (TRT) responses. Despite the critical role of cDC1 cross-priming of CD8 $^{+}$ T cells for effective therapy, there is no established method for measuring T cell cross-priming (via APC MHC-I) or direct-priming (via tumoral MHC-I) in vivo. Consequently, there is a critical need for methods to directly measure CD8 $^{+}$ T cell cross-priming, both to identify novel therapeutic targets to enhance cross-priming and to understand mechanisms of therapy resistance.

We hypothesized that cross- and direct-primed CD8 $^{+}$ T cells would harbor distinct signatures mirroring their differential antitumor efficacy. To this end, we developed novel mouse models of cross- and direct-priming antitumor T cells using GFP-expressing A20 lymphoma with or without H2-Kd expression (direct-prime capable or incapable), and transfer of GFP-specific, H2-Kd-restricted CD8 $^{+}$ T cells into syngeneic (cross-prime capable) and allogeneic (cross-prime incapable) RAG $^{-/-}$ mice. We sorted tumor-infiltrating, Ag-specific and nonspecific (passenger) CD8 $^{+}$ T cells and performed bulk RNA sequencing. We then performed differential gene expression analysis to identify cross-primed and direct-primed antitumor CD8 $^{+}$ T cell signatures.

We identified a set of 49 genes uniquely upregulated in cross-primed antitumor T cells and a set of 23 genes uniquely upregulated in direct-primed antitumor T cells. The cross-primed gene signature included genes involved in T cell activation, tumor recruitment, effector differentiation, and tissue residency, including *Ccr2*, *Cxcr3*, *Gzmb*, and *Cxcr6*, and checkpoints including *Ctla4*. Direct-prime signature genes included activation markers such as *Cd109* and costimulatory receptors including *Cd9*.

Thus, cross-primed antitumor T cells express unique signature genes. Future studies could target checkpoints or costimulators that are uniquely present on cross-primed T cells to enhance their function, or those on direct-primed T cells to prevent T cell exhaustion. Further work could also utilize this cross-primed T cell signature to monitor immunotherapy responses.

P514

STEAROYL-COENZYME A DESATURASE 1 PROMOTES REGULATORY T CELLS EXPANSION IN HEPATOCELLULAR CARCINOMA

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Tumor infiltrating regulatory T cells have evolved metabolic adaptations to sustain their fitness in the tumor microenvironment. It is well known that Treg frequency increases at the tumor site in both experimental models and cancer patients and accumulating evidence indicates that both lipid and mitochondrial metabolism play a critical role in tumor-Treg expansion and suppressive functions. Here we investigated the role of stearoyl-coenzyme A desaturase 1 (SCD1), a monounsaturated fatty acid (MUFA) synthesizing enzyme, in the expansion of tumor-infiltrating Tregs.

The expression of SCD1 was assessed through intracellular flow cytometry in human hepatocellular carcinoma (HCC)-infiltrating CD4 T cells. The SCD1 inhibitor A939572 (hereafter A93) was used in vitro, in human and murine T cell cultures, and in vivo, in a mouse tumor model. Fatty acids, cardiolipin, and mitochondrial mass were quantified through liquid chromatography-high resolution mass spectrometry, fluorometric assay, and flow cytometry, respectively.

In human HCC, SCD1 was upregulated in tumor-infiltrating Tregs, as compared to both tumor-infiltrating conventional T cells and circulating Tregs. Of note, SCD1 was co-expressed with the activation marker OX40 and the proliferation marker Ki67, suggesting a link with Treg expansion at the tumor site. Upon polyclonal stimulation in vitro of human or murine CD4 T cells, SCD1 was strongly upregulated in both Tregs and Tconvs, and treatment with SCD1 inhibitor A93 inhibited proliferation in both cell types. Specifically, SCD1 inhibition reduced Treg suppressive function, decreased the content of several MUFA (such as oleic acid) and of cardiolipins (which are enriched in MUFA), and reduced the expression of Foxp3. The in vivo treatment of tumor-bearing mice with A93 significantly reduced tumor growth, and this was accompanied by a reduced expression of Foxp3 and OX40 in tumor-infiltrating (but not splenic) Tregs. Concomitantly, tumor-infiltrating CD8 T cells displayed a stronger effector function.

In summary, these results demonstrate that SCD1 inhibition may exert anti-tumor functions also through destabilizing Tregs, and that SCD1-dependent MUFA synthesis is involved in Treg stability and suppressive function.

P515

SUV39H1 INACTIVATION REPROGRAMS CAR T CELL FATE TOWARDS MEMORY PERSISTENCE ENHANCING ANTI-TUMOR EFFICACY

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One of the main clinical challenges for adoptive T cell therapy with chimeric antigen receptors (CAR) is the limited persistence and expansion of infused products. We hypothesized that the lifespan of CAR T cells could be extended through epigenetic reprogramming via inactivation of SUV39H1, a histone methyltransferase that promotes effector CD8+ T cell differentiation during acute infection by silencing stemness and memory genes (Pace et al. 2018).

CRISPR/Cas9 mediated targeting of SUV39H1 promoted improved anti-tumor control in different preclinical models.

In an orthotopic lung tumor model, SUV39H1 deficient CAR T cells better reject established tumors, displaying stronger persistence and offering long-term protection against tumor rechallenges. Single-cell transcriptomic (scRNA-seq) and epigenetic analyses (scATAC-seq) from tumor infiltrating SUV39H1-deficient CAR T cells reveal increased chromatin accessibility and expression of stem/memory genes. We find an enrichment of CD4+ stem-like populations with characteristics of circulating versus resident phenotype, increased self-renewal and multipotency. SUV39H1-deficient CAR T cells show enhanced peripheral memory expansion and a stem-like transcriptomic signature that correlates with clinical response in diverse immunotherapy settings.

Altogether, our findings highlight the promising potential of epigenetic editing for adoptive cell therapy.

P516

CONSERVED TRANSCRIPTIONAL CONNECTIVITY OF REGULATORY T CELLS IN THE TUMOR MICROENVIRONMENT INFORMS NOVEL COMBINATION CANCER THERAPY STRATEGIES

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While regulatory T (Treg) cells are traditionally viewed as professional suppressors of antigen presenting and effector T cells in both autoimmunity and cancer, recent findings of distinct Treg functions in tissue maintenance suggest that their regulatory purview extends to a wider range of cells and is broader than previously assumed.

To elucidate tumoral Treg cell “connectivity” to diverse tumor-supporting accessory cell types, we explored immediate early changes in their single cell transcriptomes upon punctual Treg depletion in experimental lung cancer and injury-induced inflammation.

Factor analysis revealed shared Treg cell-dependent gene programs, foremost, prominent upregulation of VEGF and CCR2 signaling-related genes upon Treg deprivation in either setting, as well as in Treg-poor vs Treg-rich human lung adenocarcinomas. Spatial transcriptomics revealed distinct localization of VEGF related inflammatory gene programs to the tumor core after Treg depletion and found a lack of immune response in tumor regions marked by Sox9 expression. Accordingly, punctual Treg depletion combined with short-term VEGF blockade showed markedly improved control of PD-1 blockade-resistant lung adenocarcinoma progression in mice compared to the corresponding monotherapies.

This highlights a promising factor-based querying approach to elucidating novel rational combination treatments of solid organ cancers.

P517

ONCOLYTIC VIROTHERAPY AS AN "IN SITU" VACCINATION STRATEGY FOR NON-SMALL CELL LUNG CANCER TREATMENT

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Non-small cell lung cancer (NSCLC) is a major cause of cancer-related deaths. The development of resistance to molecular-targeted therapies and the heterogeneity of responses to immune checkpoint inhibitors pose challenges in improving NSCLC patient's outcomes. Oncolytic viruses (OV) emerge as a promising immunotherapy approach thanks to their ability to selectively replicate and kill cancer cells triggering an anti-tumor immune response. Currently, the only approved OV for clinical use is the herpes simplex virus (Talimogene laherparepvec), which has shown moderate response rate in advanced unresectable melanoma. Preclinical studies highlight bovine herpes virus (BoHV)4 as a candidate for oncolytic virotherapy since it is safe and no pre-existing neutralizing antibodies against it have been found in humans, ensuring the overcoming of resistance mechanisms associated with the use of other OV.

The oncolytic activity of the BoHV4 was tested by using NSCLC cell lines and exploiting NSCLC mouse models. The efficiency of BoHV4 infection of murine (KL-ROS) and human (A549) NSCLC cells was tested monitoring the fluorescence intensity of the cells incubated with a BoHV4 coding for the green fluorescent protein. The in vitro cytotoxicity of BoHV-4 was assessed by enumerating living cells and evaluating their viability (MTT assay) after the infection. The amount of ATP released in the supernatant of BoHV4 infected NSCLC cells was evaluated through ELISA. The in vivo anti-tumor effectiveness was tested by monitoring the growth of subcutaneous KL-ROS tumors injected three times at four days interval with 1x10⁷ unit forming plaques (PFU) of native and heat inactivated BoHV4. By exploiting transgenic K-RasG12D mice bearing multiple autochthonous NSCLC lesions, the feasibility of intratracheal (i.t.) instillation of BoHV4 was studied in vivo and ex vivo. Briefly, the luminescence of the chest area and of the explanted lungs of K-RasG12D mice receiving i.t. instillation of a recombinant BoHV4 coding for the luciferase (Luc) gene was quantified through optical imaging.

We demonstrated that BoHV4 infects and kill murine and human NSCLC cell lines and that in the supernatant of BoHV4 infected as compared to control cells a significantly higher amount of ATP is present, suggesting the induction of an immunogenic cell death. The safety and the anti-tumor potential of the BoHV4 administration was proved by exploiting a preclinical NSCLC model. A complete tumor regression was observed in 43% of intratumorally injected established KL-ROS tumors growing into immunocompetent mice. Interestingly, 100% of cured mice rejected a second challenge with a lethal dose of KL-ROS cells, suggesting the establishment of an immune memory. Indeed, the anti-tumor effect was associated with the stimulation of both a humoral and cellular immune response and with the induction of an effective immune cell infiltration in the tumors. In vivo quantitative analyses of the chest zone of K-RasG12D mice i.t. instilled with BoHV4 Luc showed that a higher luminescent signal was present in these mice as compared to those treated with heat-inactivated BoHV4 Luc. The ex vivo analysis of different organs confirms that the virus does not spread systemically, showing a tropism for tumor lesions.

Despite further investigations will be needed to evaluate the efficacy of i.t. BoHV4 instillation in halting the progression of autochthonous NSCLC in the K-RasG12D mice and to better define the underlying mechanisms of the anti-tumor efficacy, here we have demonstrated for the first time that BoHV4 has both a direct and an immune-mediated anti-tumor activity, providing a proof of concept

of its possible use also as an in situ vaccine. The safety of the i.t. instillations of BoHV-4 and its tropism for lung tumors hold promise for improving NSCLC patient's outcomes and open the possibility to extend this strategy to counteract lung metastases derived from tumors of different histotypes.

P518

NEORANKER™, A NEW AI-BASED TOOL FOR THE SELECTION OF IMMUNOGENIC CLONAL TUMOUR NEOANTIGENS FOR PERSONALIZED THERAPIES

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Personalized tumour neoantigen therapies have garnered significant interest as a promising approach to combat cancer. However, one of the primary obstacles encountered by researchers is the effective prioritization of neoantigen targets capable of eliciting sustained therapeutic responses. Screening of memory responses to non-synonymous mutations in tumor-infiltrating T cells showed that only 1-2% of these alternations are immunogenic illustrating the importance of accurate neoantigen prioritization. Furthermore, only a subset of all neoantigens capable of eliciting T-cell activation have a relevant role in vivo due to ongoing tumour immune evasion mechanisms. Overcoming these challenges is of paramount importance to achieve durable clinical responses in personalized tumour neoantigen therapies.

The correlation between clonal neoantigens and immunotherapeutic response is well-established across various indications, emphasizing the pivotal role of clonal neoantigens in facilitating T cell recognition of tumors. Achilles Therapeutics aims to deliver precision immunotherapies by specifically targeting clonal neoantigens. These clonal targets are identified through the Achilles Clonality Engine methodology within our state-of-the-art PELEUS™ bioinformatics platform. Utilizing a Bayesian approach incorporating various genomic properties, PELEUS™ accurately assesses the probability of each potential neoantigen being clonal, thereby aiding in the precise identification and prioritization of promising therapeutic targets.

To enhance the selection of immunogenic mutations beyond clonality, we have established a robust methodology for identifying tumor-derived memory T cell responses to clonal neoantigens. Leveraging data generated by screening up to 200 neoantigens per patient, providing a total dataset of approximately 10,000 neoantigens for T cell reactivity in expanded tumor-infiltrating lymphocytes, we developed and validated an AI-based tool, neoRanker™, for predicting neoantigen immunogenicity. This cutting-edge algorithm utilizes a limited set of genomic, transcriptomic, and proteomic features to rank candidate clonal mutations by their predicted capability to elicit CD8+ or CD4+ T cell responses. Using neoRanker™ across our patient cohort, we were able to rank, on average, over 70% of reactive neoantigens in patients top 30 neoantigen candidates. neoRanker™ demonstrated superior performance compared to well-known tools like BigMHC, Prime and the Gartner NMER model, as measured by the area under the receiver operator characteristic curve.

We believe this technology holds broad applicability for optimising target selection across personalized neoantigen vaccines and TCR-based cell therapies.

P519

USE OF NONENGINEERED MULTIANTIGEN SPECIFIC TISSUE-RESIDENT MEMORY T CELLS FOR ADOPTIVE CELLULAR THERAPY IN GASTROINTESTINAL PATIENTS WITH LIVER METASTASES

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UMR1098 RIGHT Interactions Greffon- Hôte-Tumeur/Ingénierie Cellulaire et Génique, INSERM, EFS BFC, University Hospital of Besançon ~ Besançon ~ France

One of the major challenges for adoptive cell transfer (ACT) is the ability of T cells to infiltrate the tumor and to be able to persist for the long term. Tissue-resident memory (TRM) T cells are endowed with increased functionality and cytotoxicity compared to non-TRM cells. Due to their efficacy and long-term persistence, TRM cells are gaining the researcher's attention being considered one of the most promising therapeutic strategies for solid tumors combined with checkpoint inhibitors. Liver metastases displayed a decreased T cell infiltration status and therefore, decreased tumor responses and long-lasting remissions achieved by immune checkpoint inhibitors. In this therapeutically challenging disease, the use of TRM cells could be a useful approach to overcoming this barrier and turn liver metastases into immune-infiltrated tumor lesions.

Our team developed a reproducible, well-validated protocol for in vitro generation of multiantigen-specific TRM cells from patient's peripheral blood mononuclear cells (PBMCs). Monocyte-derived dendritic cells were loaded with CD4 and CD8 peptide mix and co-cultured with autologous PBMCs in the presence of TRM cells polarizing cytokine cocktail (IL-15, TGF- β).

This antigen-specific TRM cells (ASTRM) generation protocol was tested on a cohort of patients presenting gastrointestinal cancers (cholangiocarcinoma, hepatocellular carcinoma, metastatic colorectal cancer, and stomach cancer) and compared to our specific T cell generation protocol without the use of TRM cell polarizing cytokine cocktail.

For the 15 tested patients, a CD4 and CD8 TRM phenotype was observed (CD69+CD103+) and (CD69+CD103-). These two TRM subsets highly expressed tissue homing biomarkers (CXCR6+CD101+CD49a+). Importantly, using the classical protocol, with no TRM polarizing cytokines, no TRM phenotype was observed which validated our ASTRM generation protocol. All generated TRM cells highly expressed activation biomarkers and highly expressed PD1 which is a hallmark of TRM cells and not associated with T cell exhaustion. However, no expression of LAG-3 nor TIGIT was observed. Seven of the tested patients (45%) presented TERT-specific CD4 TRM cells. NY-ESO1 and TERT-specific CD8 TRM cells were also observed. Remarkably, these in vitro generated TRM cells were highly functional compared to antigen-specific non-TRM cells. Over 50% of these generated CD4 and CD8 TRM cells were polyfunctional as they co-produced IFN γ , TNF α , and IL-2. Interestingly, these TRM cells were also highly cytotoxic as they were able to enact cancer cell lines eradication, mirrored by high expression of Annexin/7AAD.

Recently, our team successfully validated multiantigen-specific TRM cells production from PBCMs of patients with liver metastasis using G-Rex® technology.

In light of these results, our team will be soon conducting a phase Ib proof-of-concept study "MERIT trial" that will allow the investigation for the first time of a novel therapeutic strategy using nonengineered multiantigen-specific TRM cells combined with anti-PD-1 to treat patients with liver metastases.

P520

BLOCKADE OF PD-L1 AND B7-H3 CHECKPOINT INHIBITOR LIGANDS IN GLIOBLASTOMA

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Glioblastoma is an aggressive primary brain tumor with a short patient survival and is often associated with highly immunosuppressive tumor microenvironment. Gamma-delta ($\gamma\delta$) T cells are innate immunity effector lymphocytes with known prominent reactivity against glioblastoma. Plasmacytoid dendritic cells (pDCs) play crucial roles in anti-viral and anti-tumor immune responses. However, both pDCs and $\gamma\delta$ T cells have been shown to display also pro-tumor effects in some cancers. Recently, we determined tumor-infiltrated pDCs and $\gamma\delta$ T cell subsets in primary glioblastoma patients yet the potential mechanisms contributing to patient early relapse are still unknown.

Here we investigated the interactions between pDCs and $\gamma\delta$ T cells and glioblastoma tumor cells, which were previously phenotyped for the immune checkpoint inhibitor ligands B7-H1 and B7-H3 on pDCs and tumour cells by multicolor flow cytometry. Purified pDCs and glioblastoma cells were cultured in presence of PD-L1 inhibitor (atezolizumab, avelumab and durvalumab) or B7-H3 inhibitor (omburtamab) and subsequently used in killing assays with magnetically sorted $\gamma\delta$ T cells.

We showed that pDCs i) could be largely expanded with autologous glioblastoma whole tumor-lysates, ii) modulate $\gamma\delta$ T cell cytotoxic activity against glioblastoma cell lines and primary tumor cells and iii) are directly involved in B7-H1- and B7-H3- mediated suppression of tumor control.

Our study highlights the multiple cell interactions within the glioblastoma tumor that could be exploited in novel immunotherapeutic interventions to improve patient survival.

P521

CIRCULATING NEOANTIGEN-SPECIFIC CD8 T-CELL RESPONSES: A BIOMARKER OF RESPONSE TO IMMUNE CHECKPOINT BLOCKADE IN LUNG AND BLADDER CANCERS

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Tumor-specific T cells play a central role in tumor control, yet the attainment of their full tumor rejection potential is hindered by regulatory mechanisms, including expression of inhibitory immune checkpoints. Although immunotherapy by immune checkpoint blockade (ICB) has transformed cancer care, only a proportion of patients (~30%) experience clinical benefit. Ancillary studies to clinical trials evaluating ICB have revealed an association between high tumor mutational burden (TMB) and treatment efficacy, suggesting that unleashing T cells targeting neoepitopes encoded by somatic mutations could underlie ICB-mediated tumor control or regression. Nevertheless, TMB is not consistently related to clinical response to ICB, potentially because most studies have addressed the influence of TMB by in silico prediction without systematic analysis of actual neoantigen-specific T cells. We hypothesized that the presence of actual tumor antigen-specific T-cell responses to neoantigens is predictive of response to ICB, rather than the mere presence of a high TMB.

Here, using whole exome sequencing (WES) and whole transcriptome sequencing (WTS) in a cohort of 40 non-small cell lung cancer and bladder cancer patients receiving anti-PD-1/PD-L1 (programmed cell death protein 1/Programmed death-ligand 1), we show that a high TMB and neoantigenic load are not predictive of responses to ICB, supporting our hypothesis. For each patient, we evaluated the antigenome, defined as the largest set of tumor antigens expressed in his or her tumor. We focused on potential neoantigens, which are private to patients, as well as patient-shared tumor antigens, such as Cancer Testis Antigens (CTAs). We developed a workflow allowing for the assessment of circulating CD8 T-cell responses to more than 400 prioritized neoantigens, and for the assessment of circulating CD4 T-cell responses to neoantigens from the 50 best mutations, in ICB responder and non-responder patients.

Remarkably, for the first 21 patients tested, we showed that circulating CD8 T-cell responses to predicted neoantigens were of higher magnitude and breadth in clinical responder compared to non-responder patients.

Thus, clinical responses to ICB provide an opportunity to study T-cell responses and the tumor antigens for which they are specific, allowing to identify those that have the potential to mediate tumor rejection. T-cell responses to the antigenome (neoantigens and CTAs), their evolution along therapy and their relation to tumors molecular signatures could provide insights into the role of pre-existing adaptive immunity in responsiveness to ICB.

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P522

TOWARDS CAR-T CELL THERAPY AGAINST PDAC LIVER METASTASIS

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PDAC is among the most lethal tumors, typically diagnosed at late stages with a median survival of 4-6 months and a 5-year survival rate of 10%. It is mostly resistant to chemotherapy and radiotherapy because of a prominent desmoplastic reaction and immunosuppressive microenvironments, frequently causing liver metastases. Among most promising alternatives, therapies that exploit immune-based mechanisms are increasingly being considered. Among these, adoptive T cell therapy with tumor-specific T cells provides the unique possibility to replenish patients immune system with highly specific cytotoxic effectors in principle able to reject tumor cells, and also patrol against disease recurrence. Attempts against PDAC have so far failed. We hypothesize that combinatorial strategies would overcome current obstacles.

To advance adoptive T cell therapy against PDAC we developed a preclinical mouse model. We adopted the intra-portal seeding of primary and metastatic murine PDAC cells expressing the human tumor-associated CEA antigen (K8484 and 5M7101) and traced metastasis occurrence and growth by longitudinal Magnetic Resonance Imaging. Histologically, K8484 and 5M7101 liver metastasis closely reproduced features of human ones. We then adopted a CEA-specific Chimeric Antigen Receptor, and non-myeloablative Total Body Irradiation (TBI) to precondition the mice for efficacious T cell engraftment. We found that CAR-T cells rejected PDAC liver micrometastasis, allowing overall long-term survival and protecting mice against secondary tumors. However, CAR-T cells failed to cure mice carrying PDAC liver macrometastasis. Also in the case of cyclophosphamide driven preconditioning or intra-liver delivery, CAR T cells lacked anti-tumor activity. To understand the possible reasons for suboptimal therapeutic efficacy, we performed multiplex immunofluorescence and spatially analyzed the tumor and its associated microenvironment. We found that although clinical responses could not be found, histological responses were clearly identifiable in mice treated by the combination of cyclophosphamide and anti-CEA CAR T cells, compared to mice treated with the combination of cyclophosphamide and control untransduced T cells. The combined cyclophosphamide/CEA-CAR-T cells proved able to derange tumor margins, and cause a remodeling of the peritumoral microenvironment. M1 macrophages (F4/80⁺CD206⁻) and monocytes (F4/80⁻Ly6G⁻Ly6C⁺) immune cells were all enriched. In addition, both CD8 and CD4 T cells proved able to migrate within the lesions and accumulated within the inner metastatic core. However, these cells most likely lacked effector activity, given the absence of sign of clinical response. We speculate

that within the inner core of macrometastasis, CAR-T cells are functionally inactivated by the interaction with the immunosuppressive milieu. Next approaches to promote local inflammation will be adopted to unleash inner-core infiltrating T cell effector functions. Thus, together our study provides evidence that CAR-T cell therapy can effectively cure PDAC micrometastases, and also organize tranches against macrometastasis. Data however indicate that CAR-T cells would need allies to overcome the no man land and take over the inner core of the lesions.

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P524

IMPROVEMENT OF LYMPHOMA THERAPY BY SALINOMYCIN-INDUCED INHIBITION OF CMYC AND FOXO TRANSCRIPTION FACTORS.

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We discovered that salinomycin, previously shown to eradicate breast cancer stem cells, is also able to strongly upregulate the surface levels of CD20 antigen on B-cell-derived tumor cells, including a variety of Burkitt's lymphoma and diffuse large B-cell lymphoma (DLBCL) cell lines as well as patients-derived primary malignant cells. We, therefore, explored the hypothesis that salinomycin can be particularly beneficial when combined with anti-CD20 monoclonal antibodies for the improvement of the B-cell non-Hodgkin lymphoma therapy.

We employed the therapeutic anti-CD20 antibody, rituximab, in both complement- and NK cell-dependent assays in vitro and in Burkitt's lymphoma xenograft mouse model.

We found that the efficacy of rituximab was significantly increased by salinomycin, in assays in vitro and in vivo. Importantly, full CD20 upregulation was induced by salinomycin at concentrations as low as 250 nM, ensuring the reduction in the potential systemic toxicity of salinomycin. Additionally, we discovered that other members of the same class of antibiotics, known as cation carriers, namely monensin, narasin, and nigericin exhibit similar CD20-upregulating abilities. This effect was clearly dependent on the transcriptional regulation of the MS4A1 gene, encoding CD20, via inhibition of FOXO1 and cMYC transcription factors. Importantly, salinomycin derivatives unable to upregulate CD20 antigen were also unable to downregulate the level of cMYC, underlining the importance of the cMYC-CD20 axis for the therapy of lymphoma.

In light of our discoveries, it seems reasonable to consider the cation carriers as promising drug candidates, used in combination with therapeutic anti-CD20 antibodies for future treatment of B-cell non-Hodgkin lymphoma patients.

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P525

HARNESSING NANOMECHANICAL STROMA REMODULATION TO OVERCOME RESISTANCE TO IMMUNOTHERAPY

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In recent years the combination of immunotherapy with other therapies has proven successful in enhancing immune activation and tumor suppression in solid cancer. In particular, new subtherapeutic radiation protocols (low-dose) have shown effective in restoring response to a variety of immunotherapies, including immune checkpoint inhibition (ICI) and cell therapy [Barsoumian HB, Ramapriyan R, Younes AI, et al, 2020; Patel RR, He K, Barsoumian HB, et al, 2021; He K, Hong DS, Ke D, et al, 2023]. As the use of low-dose radiation (LD-XRT) gains popularity in the clinics, it becomes necessary to identify new clinically translatable methods to predict response and optimize treatment. Underlying the LD-mediated restoration of response to immunotherapy, is both an immune-activation enhancement and a physical stroma remodulation. In this work we have leveraged previous evidence that nanomechanical alterations of the tumor microenvironment are viable predictors of response [Plodinec M, Loparic M, Monnier CA, et al, 2012], to identify a clinically translatable signature of stroma-remodulation and enhanced immune response upon combination of ICI and LD-XRT in mice.

Our preclinical studies focus on a mouse model of lung adenocarcinoma resistant to anti-PD1 treatment, developed within MD Anderson cancer center from the 344SQ cell line established in 129Sv/Ev mice. Mice were treated with both anti-PD1 and anti-CTLA4 antibodies, and irradiated at low dose (2 x 1Gy) at the start of ICI therapy. In order to evaluate the early prediction value of tumor nanomechanics, mice were biopsied at different time points, early during treatment and at survival. Survival and tumor growth were monitored for the various groups. Fresh biopsies were measured within 2h from collection via ARTIDIS platform following the same protocol as previously validated in clinical studies in breast cancer (Basel Nano Study). The ARTIDIS platform is an innovative clinically implemented technology that combines atomic force microscopy for tissue mechanical measurements with artificial intelligence proprietary algorithms to provide accurate and reliable characterization of the nanomechanical properties of measured tissue. To complement mechanical properties, histopathology, multiplex immunofluorescence and Nanostring analyses characterization of stroma remodulation and immune infiltration was also integrated within ARTIDIS assessment of samples.

Our study led to the clear identification of a unique signature of radiation – induced restoration of response to ICI in lung cancer. This signature was identified in the nanomechanical parameter space, which includes physical parameters such as nanometric stiffness, plasticity, adhesion, and dissipation. In this analysis we achieved almost perfect stratification of responders vs non responders, with 90% sensitivity, 99.1% specificity and 96% AUC. Moreover, we identified the nanomechanical signature of immune cell infiltration and activation post radiation.

In this work we have investigated the nanomechanical signature of response to ICI combined with LD-RT. We have identified biomechanical drivers of immune infiltration, and how they can be used to optimize cancer diagnosis, orientate therapy choice, and support patient follow-up in combination radiation/immunotherapies. For the first time we demonstrated a clear nanomechanical signature of LD-RT-mediated response to both immune checkpoint inhibitors in lung cancer as assessed by ARTDIS. Our findings open the way to using ARTIDIS nanomechanical signature to predict and optimize response to combination radiation and immunotherapies.

P526

EVALUATING COMPLETE AND MAJOR PATHOLOGIC RESPONSE AS SURROGATE ENDPOINTS IN NEOADJUVANT IMMUNOTHERAPY CLINICAL TRIALS FOR EARLY-STAGE NON-SMALL CELL LUNG CANCER: A SYSTEMATIC REVIEW AND META-ANALYSIS.

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The use of neoadjuvant immunotherapy alone or in combination has rapidly evolved in the last 5 years and has been able to show complete and major pathologic responses in a substantial percentage of patients. There is significant debate as to whether pathologic response (pCR and MPR) can be considered a surrogate endpoint. We therefore performed a systematic review and meta-analysis to evaluate the surrogacy of pCR and MPR for event free survival (EFS) in neoadjuvant clinical trials for early-stage NSCLC.

We performed a systematic literature search on PubMed and reviewed abstracts of the most relevant international conferences until June 2023. Relevant information relative to response rates, Odds Ratios (ORs) of response, 2-years EFS rates, and Hazards Ratios (HRs) were retrieved from all eligible clinical trials and associations were analyzed. To evaluate patient level surrogacy, the association between pathologic response and 1- and 2-year EFS was evaluated. Publications showing EFS rates by achievement of pCR and MPR, a forest plot with a random effect model was also produced. For trial level surrogacy, ORs for pCR and MPR and HRs for EFS were retrieved and analyzed using a linear regression model weighted by sample size. R² and linear regression slope were used, respectively, to estimate the proportion of variation in EFS effect explained by pCR effect and the magnitude of change in EFS effect as a function of the magnitude of change in pCR effect. R²s with 95% CI were calculated by bootstrapping approach.

Five RCTs and ten single-arm trials were identified for a total of 2343 patients. At patient level, the R² of pCR and MPR with 2-years EFS were 0.79 (0.44-0.99) and 0.85 (0.57-0.99), respectively, while OR of 2-year EFS rates according to response achievement was 0.12 (0.05-0.30) and 0.04 (0.02-0.08), respectively. At trial level, R² for association of OR for response and HR for EFS was 0.64 for both analyses but with 95% CI ranging from 0.01 to 1.00.

We were able to show a strong correlation between pCR, MPR and EFS, and there is some suggestion of trial level surrogacy. However, the data are highly unstable and need confirmation. More effort should be made to reduce factors that might negatively affect surrogacy (e.g., heterogeneity in pathological evaluation, surgery, and staging harmonization) in order to advance pCR and MPR as a primary measure to predict EFS.

P527

EVALUATING APPROACHES FOR IMMUNE INDUCTION ACROSS COLD TUMOR TYPES - THE ONTARIO INSTITUTE FOR CANCER RESEARCH WINDOW OF OPPORTUNITY (WOO) CLINICAL TRIAL NETWORK

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Immunotherapy has shown remarkable progress in treating certain types of cancer known as "immune hot" tumors. However, many patients with "immune cold" tumors experience limited or no benefit from these therapies. To improve outcomes for such patients, it is essential to induce a tumor immune response, transforming "cold" tumors into "hot" ones, before administering immune-targeted therapies. This process of "immune induction" represents a novel but logical extension of immunotherapy. Identifying optimal approaches for immune induction is crucial, and the challenge lies in quickly evaluating multiple methods across various tumor types to optimize future combination therapies. Different approaches may be needed based on the mechanisms driving immune suppression in "cold" tumors.

Window of opportunity (WOO) trials offer a rapid and effective means of assessing the response of cancer to new treatment approaches. In these trials, treatment-naïve patients are exposed to one or several doses of a drug immediately after diagnosis but before the standard treatment (surgery or neoadjuvant therapy). WOO trials facilitate a comprehensive understanding of the mechanisms of action of new therapies and the variability in clinical responses after just a few weeks of preoperative therapy. In 2021, the Ontario Institute for Cancer Research (OICR) in Ontario, Canada, established a WOO Clinical Trials Network. The Network brings together clinicians, scientists, patient partners and industry with the goal of creating an integrated platform of clinical trials, testing the same agents across multiple tumor types or different agents in the same tumor type and stage.

The OICR WOO Network has made significant progress since its inception, supporting 12 trials targeting immune pathways in several cancer types, including breast, bladder, lung, pancreas, hepatobiliary, and head and neck cancers. To date, over 200 patients have been enrolled in these trials, with three randomized trials already completed. Each trial collects tumour and blood samples, with over 8000 biospecimens collected from the first three trials. The Network focuses on evaluating novel agents with a known safety profile, either alone or in combinations, that target pathways responsible for triggering antigen release and presentation, T-cell priming and activation, and tumor-infiltrating lymphocyte (TIL) recruitment and function, although the model is applicable across all targeted therapeutics. The approach emphasizes comprehensive molecular screening and deep, multi-omic biomarker analysis for each trial, with all data integrated into a database comprising genomic, transcriptomic, and proteomic profiles, accessible to qualified investigators. Standardized standard operating procedures for trial conduct, ethics and regulatory submission, sample collection, and core translational assays are implemented across all trials, enabling cross-trial meta-analyses. Randomized trials allow for development of robust predictive biomarkers that can then be tested and validated in future neo-adjuvant studies. Importantly, the Network includes a unique patient partner program focused on engaging patients in trial design, delivery, and dissemination of results. The OICR WOO network is also partnering with multiple groups to expand the value of the network internationally.

The OICR WOO Network represents a promising initiative in the field of immunotherapy, aiming to develop effective immune inductive therapies for "immune cold" tumors. By leveraging the rapid and comprehensive WOO trial approach, the Network brings together a collaborative effort from multiple stakeholders to explore novel immunotherapy agents across multiple tumor types in a focused,

efficient manner. The integration of advanced biomarker analysis further enhances the Network's potential to identify effective immunotherapy combinations and optimize host immune activation, ultimately improving the activity of immune oncology agents.

P528

SINGLE-CELL CYTOTOXICITY ANALYSIS IDENTIFIES SERIAL KILLER TRANSGENIC TCR-T CELLS MANUFACTURED USING NON-VIRAL S/MAR DNA NANOVECTORS

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T-cell therapies using transgenic T-cell receptors (TCRs) are a promising approach for cancer treatment. The aim of our work is to overcome key challenges with TCR-T cell therapies and at the same time generate effective therapies featuring high potency T cells using a novel DNA NanoVector platform which offers a safe and efficient method for generating genetically modified T cells.

Unlike integrating systems, nS/MAR DNA vectors remain episomal, eliminating the risk of insertional mutagenesis and reducing manufacturing time and costs. In this study, we refined the vector features, focusing on the promoter. The potency of generated TCR-T cells is assessed through single-cell cytotoxicity analysis, with a focus on identifying the presence and frequency of serial killer cells which can be defined as those which can kill three or more target cells. We use this as an indicator of our ability to enhance T-cell fitness by simultaneously conferring high potency with low exhaustion to the immune cells. Single-cell potency analysis is carried out with the novel VivaCyte® platform which enables multi-parametric and high throughput analysis of cell cytotoxicity and phenotype of individual immune cells. The platform is based on a microfluidic technology that can generate thousands of miniaturized co-cultures of immune and target cells. By defining a novel Potency Score we can compare the potency of cell therapy products, at the single-cell level. This score allows us to evaluate the effect of donor heterogeneity and assess the impact of vector composition on therapeutic potency.

We refined the S/MAR DNA platform to generate transgenic TCR T-cells targeting the melanoma antigen MART1. HLA-A2+ human healthy donors were used to produce TCR-T cells and the impact of the strong EF1 α or weak hTCR promoters in driving TCR expression and the functionality of the TCR-transgenic T cells was assessed. In a bulk-killing assay against the target MART-1 cell line the EF1 α promoter displayed the highest level of cytokine release, indicating a potential enhancement in the efficacy of T cells.

To characterise the potency of the products generated, we quantified the ability of single TCR-T cells to kill one or more target cells during a co-culture in the VivaCyte® system, allowing us to characterize each product generated at the single-cell level, determining the fraction of TCR-T cells with serial killing activity.

Interestingly, the frequency of serial killer cells increased after TCR transfection, ranging from 17 - 34% for EF1 α and 15 - 32% for the hTCR promoter. Using single-cell data, we then computed a Potency Score to compare the potency of the different batches calculating the weighted average of the number of killing events made by single effector cells and represents the average number of target cells that each effector cell kills in a specific amount of time. The Potency Score is highly variable depending on the donor. Notably, while non-transfected cells show a 24-hour score that can be lower or higher than 1, the TCR-T cells always show a Potency Score higher than 1, meaning that, on average, each TCR-T cell kills more than one target cell post-transfection.

In this work, we characterized the potency of T cells genetically engineered using a novel nano (nS/MAR) DNA Vector platform using a commonly used assay and the innovative VivaCyte® system. Single-cell analysis reveals that the (nS/MAR) DNA Vector can increase the frequency of serial killer cells to up to 34% of the immune cell population. These cells are expected to play a crucial role in the anti-tumor activity, exerting their cytotoxic function multiple times before their exhaustion. Further studies will aim at refining the Potency Score by correlating the single-cell data with conventional phenotypic analyses utilized to characterize cell exhaustion, for an even more comprehensive characterization of our engineered TCR-T cells.

P529

ANTIGEN-INDEPENDENT CAR DOWNREGULATION IS FOUND DURING IL-7/IL-15 T CELL EXPANSION AND MITIGATED BY SODIUM PROPIONATE

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The genetic retargeting of T cells with Chimeric Antigen Receptors (CAR) specific for a predefined antigen has delivered new treatment options for cancer patients. Although unprecedented clinical outcomes have been achieved against B-cell malignancies, >30% of patients who initially achieved complete remission encountered relapses 1-year after CAR T cells infusion. In addition, in case of solid tumors, efficacy has so far been only sparsely observed. Abundance and heterogeneity of tumor antigen expression, along with immunosuppressive tumor microenvironments limit CAR-T cell efficacy and persistence. The density of CAR at the T-cell surface also represents a key variable in controlling the cytolytic outcome.

We adopted an anti-human-CEA CD28/CD3 ζ second generation murine CAR. Murine T cells were transduced with CAR encoding retroviral vectors and expanded with mIL-7 and mIL-15 cytokines. Flowcytometry and confocal microscopy were used to follow CAR expression and subcellular localization at different times. RT-PCR was used to follow mRNA levels. Cocultures of CAR-T cells and hCEA/GFP-expressing PDAC cells were adopted to assess CAR T cell responses and intracellular staining and ELISA were used to measure cytokine expression.

We describe a mechanism of antigen-independent CAR downregulation over the course of in vitro IL-7/IL-15 CAR T cells expansion. Shortly after transduction surface CAR levels were gradually lost. This was found in spite significant intracellular accumulation of CAR molecules, and the persistence of GFP+ transduced cells.

Surface CAR loss was only partially due a decrease in mRNA levels, independent of the specificity, and rather linked to the activation status of the cells. Accordingly, surface CAR expression remained stable in transduced immortalized fibroblasts. How CAR trafficking is regulated in T cells remains largely unknown and might involve epigenetic silencing and protein ubiquitination and degradation. Given recent reports highlighting that short chain fatty acids can modulate TCR stability, we investigated the possibility that their implementation over the course of T cell expansion might rescue CAR internalization. CD3/CD28 activated primary T cells were transduced and then cultured in IL-7 and IL-15 with or without Sodium Butyrate or Sodium Propionate. As control T cells were also engineered with a large T antigen specific TCR. We found that sodium propionate best preserved CAR levels and favour CAR+ T cell expansion. Propionate-treated CAR T cells expressed the CAR at higher density and produced higher levels of TNF α and IFN γ and proved more efficacious in elimination hCEA+ tumor cells in coculture settings. Whether Sodium Propionate CAR T cell products have superior therapeutic effects for the treatment of subcutaneous PDAC metastasis is currently being investigated.

In summary, our data unveil an antigen-independent mechanism controlling CAR levels over T cell manufacturing. As down-modulation could limit CAR-T cell responsiveness and persistence in vivo, a further understanding of CAR fine tuning might be needed for optimal CAR-T cell exploitation.

P530

CD103+ NATURAL KILLER CELLS DISTINCTIVELY INFILTRATE THE TUMORAL EPITHELIUM OF HUMAN LUNG CANCER

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Natural Killer (NK) cells represent a promising target for cancer immunotherapies. Indeed, recent studies have shown that the anti-tumor function of NK cells may be not only restricted to cytotoxicity but can be associated to the recruitment and maturation of cross-presenting DCs. However, only limited progress has been made in deciphering complexity of the NK cell compartment in human cancers. In the present study, we aimed to investigate NK cell subsets specifically infiltrating lung cancer with the aim of providing a deeper understanding of their spatial distribution and functional specialization, with particular interest in their interaction with tumor cells.

We analyzed tumor-infiltrating NK cells in a cohort of 53 Non-Small Cell Lung Cancer (NSCLC) patients, specifically 36 non-squamous carcinomas (NON-SQK) and 17 squamous cell carcinomas (SQK). Tumor ranged from stage Ia to IIb. NK cells derived from tumor specimens were analyzed by multicolor flow cytometry for the expression of markers related to circulation, tissue-residency and functional activation. Phenotypic analyses were paired with assessment of the localization of subsets of NK cells within matched tumor samples, as well as correlation on patients' relapse-free survival (RFS) and overall survival (OS). Additional experiments were performed to investigate cytotoxicity and cytokine/chemokine production upon different types of stimulation. Finally, the potential of circulating blood NK cells to infiltrate tumor mass and engender tumor-resident NK cell subsets was assessed further by using a 3D spheroid model of NSCLC.

We showed that a specific subset of NK cells is able to display an intraepithelial tumor localization in NSCLC (NON-SQK=36 NK cell/mm²; SQK=7 NK cell/mm²). This is remarkable since, based on previous findings, NK cells had only been reported to localize in the stroma surrounding tumoral epithelium, thus hardly in direct contact with cancer cells. This tumor epithelium-infiltrating NK cell subset homogeneously express CD103/ α E integrin and display other markers of activation and tissue residency. We also found that CD103+ NK cells specifically accumulate in tumor tissues, but not in adjacent normal lung tissues (tumor vs. adj lung = 33% vs. 5% of total NK cells), regardless of the histotype and the clinical stage. Interestingly, higher frequency of CD103+ NK cells among total TILs appears associated to shorter relapse-free survival (HR=2.27; p= 0.132). In line, they showed dysregulation in classical NK cell functions even if maintaining high level of chemokine production. Nevertheless, culture with IL-15 was able to rescue both their cytokine secretion and degranulation properties against tumor cells.

Finally, by using a 3D spheroid model of NSCLC, we observed that only circulating CD56bright NK cells, but not the CD56dim counterpart, could efficiently adhere to and infiltrate tumor masses. Interestingly, circulating CD56bright NK cells, which were able to infiltrate in vitro tumor spheroids, acquired CD103 and other features that we observed in NK cells present in the epithelial area of patient's tumor samples. These data suggest that CD103+ NK cell subset localized in tumor epithelium might derive from circulating CD56bright NK cells upon conditioning by tumor microenvironment.

Overall, these findings emphasize a unique role of CD103+ tissue-resident NK cells in localizing within epithelial areas of lung tumors, thus supporting the rationale for developing strategies targeting endogenous NK cells to boost antitumor immunity.

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RORG BRIDGES CANCER-DRIVEN LIPID DYSMETABOLISM AND MYELOID IMMUNOSUPPRESSION

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Growing evidence highlights that lipid and cholesterol metabolism dysregulations contribute to tumor progression by altering inflammatory, immune and clinical responses of cancer patients. However, the mechanisms underlying the cholesterol-induced alterations are still elusive. Our group has recently reported that retinoic acid-related orphan receptor RORC1/RORg induction by myeloid growth factors (e.g., GM-, M-CSF) largely drives emergency myelopoiesis in tumor bearers. Of relevance, while hypercholesterolemia can modulate myelopoiesis, cholesterol and its derivatives may act as activator of RORg. Hence, we investigated the role of cholesterol-RORg interaction in cancer myelopoiesis

To clarify the relevance of cholesterol in cancer development, we experimentally increased cholesterol availability in mice bearing MN/MCA1 fibrosarcoma, K1735-M2 melanoma or Kras/p53-driven (KP) lung cancer with a hypercholesterolemic (HCD) or normal (NCD) diet feeding. Conversely, cholesterol levels were reduced administering a cholesterol-lowering anti-PCSK9 monoclonal antibody (mAb). RORg hematopoietic- (bone marrow cell transfer) or myeloid-specific (Rorc flox/flox Lyz2-Cre mice) genetic deficiency, or its pharmacological inhibition (SR2211) were used in HCD-fed tumor-bearing mice to dissect the cholesterol-RORg interaction. Blood cholesterol levels were screened during disease progression. Flow cytometry analysis of immune cell subsets, mainly myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs), bone marrow myeloid progenitors (CMP/GMP), as well as CD4 and CD8 T cells, were performed. Anti-PD1 mAb was administered in HCD-fed tumor-bearing mice

Of relevance, we observed a tumor-dependent increase of circulating cholesterol levels in both NCD and HCD feeding. Higher cholesterol levels supported tumor growth and metastasis formation. Flow cytometry analysis showed an HCD-dependent increase of monocytic (M-) MDSCs and TAMs, with a prominent pro-tumoral M2 polarization (TNF α low MHC-II low CD206high PD-L1high). Coherently, a dysfunctional phenotype of CD4 and CD8 T cells (PD-1high IFN γ low) was observed in HCD. Of relevance, anti-PCSK9 mAb administration in either fibrosarcoma-bearing or KP mice dampened cancer progression in both NCD and HCD conditions. This effect was paralleled by a reduced TAM frequency, which acquired an antitumor M1 activation state. Notably, in agreement with our previous report, HCD diet in RORg-proficient mice increased commitment of bone marrow common myeloid progenitors (CMP) to granulocyte-macrophage progenitors (GMP), which was prevented by anti-PCSK9.

Flow cytometry analysis showed that hypercholesterolemia increased RORg expression in mono/macrophage cells from different tissues (tumor, blood, lung, spleen), as well as in CMP/GMP myeloid progenitors. Accordingly, we underlined a positive correlation between cholesterol levels and frequency of circulating M-MDSC RORg⁺ in NSCLC patients (stage III-IV).

Notably, transplantation of Rorc^{-/-} bone marrow cells in lethally irradiated wt mice or myeloid-specific Rorc flox/flox Lyz2-Cre mice showed a drastic reduction of fibrosarcoma progression, regardless the diet type, while cholesterol levels were not affected. Consistently, the immunosuppressive profile of TAMs and M-MDSCs exacerbated by HCD was lost in RORg deficiency. Of note, while HCD increased the cholesterol-load in both TAMs and in CMP/GMP progenitors regardless of RORg expression, RORg deletion restored an M1 phenotype of TAMs and strongly reduced the CMP-to-GMP differentiation promoted by cholesterol. In agreement, the RORg

pharmacological inhibitor SR2211 drastically reduced tumor progression and immunosuppressive myelopoiesis in hypercholesterolemic fibrosarcoma- or melanoma-bearing mice, and improved the antitumor efficacy of anti-PD-1 immunotherapy

Overall, we identify ROR γ as a novel sensor of lipid disorders in cancer bearers, bridging hypercholesterolemia and pro-tumor myelopoiesis

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NANOMECHANICAL SIGNATURE OF COMBINED RADIOTHERAPY WITH ADOPTIVE CELL THERAPY FOR ENHANCED CAR-T CELL INFILTRATION INTO SOLID TUMORS: A KEY TO ANTITUMOR RESPONSE AND THERAPEUTIC SUCCESS

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The management of solid cancers, such as lung, gastric, and pancreatic cancers, faces challenges due to the persistence of therapy resistance and recurrence despite initial success. Researchers have combined cell therapy with other treatments to enhance treatment effectiveness, leading to improved T-cell activation and infiltration, resulting in better responses. However, the issue of varying responses and resistance remains a concern, as current clinicopathological markers used for prognosis and prediction often fail to predict immunotherapy outcomes accurately.

Recent studies have shed light on the significant role of mechanical properties in cancer cells and their microenvironment in cancer progression and metastasis. The tumor microenvironment's cellular and extracellular matrix components and associated molecular factors actively influence the tumor's response to therapy (Leight, Drain, et al., 2017). These findings highlight the importance of considering the mechanical aspects within the tumor microenvironment for a more comprehensive understanding of cancer treatment and response.

Notably, alterations in the mechanical characteristics of cancer cells at the nanoscale level show great potential as markers for assessing cancer aggressiveness (Plodinec, Loparic, et al., 2012). This offers exciting possibilities for optimizing cancer diagnosis, guiding therapy decisions, and supporting patient follow-up. Additionally, recent evidence has demonstrated that radiation therapy has immunomodulatory effects, which could help overcome resistance (Cushman TR, et al., 2018).

This study aimed to investigate the combined effects of multiple doses of low-dose radiotherapy (LD-RT) and T-cell therapy using an NSG mouse model implanted with gastric carcinoma (GSU) cells. We administered low doses of radiation therapy (RT) in conjunction with cell therapy and closely monitored survival and tumor growth. Tumor biopsies were collected for detailed analysis.

To precisely characterize cancer tissue nanomechanics, we employed cutting-edge atomic force microscopy with proprietary AI algorithms on the ARTIDIS platform, which allowed us to extract a multiparametric nanomechanical signature. Histopathology and multiplex immunofluorescence analyses also provided insights into stroma remodeling and immune infiltration processes.

In this study, we identified a specific nanomechanical signature indicating an enhanced response to cell therapy induced by radiation. The dataset revealed T-cell infiltration post-radiation with impressive precision, achieving 92% sensitivity, 100% specificity, and 95% accuracy. Importantly, these findings remained consistent across different T-cell infusion doses and low and high radiation levels.

This novel nanomechanical signature conclusively demonstrates the LD-RT-mediated response to cell therapy in NSG mouse models, offering the potential use of the ARTIDIS nanomechanical signature as a precise and clinically applicable predictor for effectively combining radiation and immunotherapies.

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MODULATION OF GUT COMMENSAL-INDUCED T EFFECTOR CELL PROGRAM IN CHECKPOINT BLOCKADE DEPENDENT CONTROL OF TUMORS SHARING ANTIGEN WITH GUT MICROBIOTA

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Although cancer immunotherapy has shown remarkable effectiveness across multiple cancer types, most cancer patients fail to respond. Recent findings have provided strong evidence that inter-individual differences in the intestinal microbiota may account for the significant heterogeneity in therapeutic efficacy of immune checkpoint therapies. However, a mechanistic understanding of how gut commensal bacteria influence the efficacy of immune checkpoint blockade remains elusive. Some direct and/or indirect factors may account for such profound effects of gut commensal bacteria on the efficacy of immune checkpoint therapies. Examples may include antigen mimicry between microbial antigens and tumor neoantigens, production of bacterial metabolites that enhance immune function at distal sites (e.g. to improve antigen presentation), induction of soluble immunomodulatory factors by gut microbes (e.g. serum amyloid A protein production by host cells), and/or microbe-elicited, tumor non-specific Teff cells (e.g. Th17, Th1/Th1*, and CD8+ T cells which traffic to the tumor site and secrete inflammatory cytokines to activate antigen-specific CTLs or NK cells and recruit other myeloid cell types). To better understand how microbiota can influence control of tumor growth, we are using gut-resident bacteria that induce stereotyped antigen-specific T cell effector programs, e.g. SFB (commensal that induces a non-pathogenic Th17 response), *H. hepaticus* (pathobiont that induces a regulatory T cell response at homeostasis and a pathogenic Th17 response upon blockade of IL-10 function), and attenuated *Listeria monocytogenes* expressing SFB antigen (attenuated pathogen that induces a strong Th1 response), to ask how specific gut-resident bacterial populations can modulate anti-tumor responses. As an experimental tool, we are asking whether coupling bacteria-specific responses to tumor recognition via expression of bacterial antigens in the tumors will alter immune checkpoint responses. Using an artificial mimicry model, with mouse B16-F10 melanoma cells expressing the immunodominant SFB-3340 protein, we investigated how gut colonization with SFB influences the effectiveness of immune-checkpoint blockade in restraining tumor growth. We found that anti-PD-1 inhibited growth of implanted B16-3340 tumors only if mice were colonized with SFB. Remarkably, whereas SFB antigen-specific cells in the small intestine were Th17 cells, those within the tumors had a Th1 phenotype. Using a combination of *Il17a-cre;Rosa-tdTomato* fate mapping and peptide-MHC tetramer staining of the T cells, we found that a substantial proportion of the tumor-associated SFB-specific Th1 cells were derived from Th17 cells. These presumptive gut-educated Th1 cells produced high amounts of IFN γ and TNF α , which are likely required to promote effector functions of CD8+ TILs. Additional mechanistic studies, with single cell TCR repertoire analysis and examination of a potential role of IL-23 in the phenotypic switch, will be presented. Our results suggest that the immune modulatory qualities of intestinal commensal microbes can be harnessed to elicit effective anti-tumor T cell responses.

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DEFINING IMMUNE DESERT, EXCLUDED, AND INFLAMED MELANOMA SUBTYPES WITH A GENE EXPRESSION CLASSIFIER REFLECTING INTRA-TUMORAL IMMUNE RESPONSE AND STROMAL PATTERNS

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The spatial distribution of infiltrating lymphocytes within a tumor defines several histologically and clinically distinct immune subtypes - desert, excluded, and inflamed.

This classification system considers the presence and location of tumor-infiltrating leukocytes. Desert tumors are poorly immune-infiltrated and lack the of pre-existing antitumor immunity. Excluded tumors have a dense extracellular matrix and retain TILs in the reactive stroma. Inflamed tumors are characterized by considerable infiltration of TILs, often not properly functioning. Although universal in nature and potentially compliant as an immunotherapy biomarker, this subtyping system needs more evidence to reach consensus in the academic and clinical network. Also, robust classification of immune subtypes still requires deeper experimental evidence across various cancer types. Here, we aimed to investigate, define, and validate the immune subtypes in melanoma by coupling transcriptional and histological assessment of lymphocyte distribution in tumor parenchyma and stroma.

We used the transcriptomic data from The Cancer Genome Atlas melanoma dataset to screen for desert, excluded, and inflamed immune subtypes. We defined the subtype-specific genes and used them to construct a subtype assignment algorithm. We validated the two-step algorithm via qPCR in the 96 real-world melanoma patient cohort with histologically defined tumor immune subtypes.

One of the primary outcomes of our study was the recognition of desert, excluded, and inflamed immune melanoma subtypes both transcriptionally *in silico* as well as transcriptionally and histologically *ex vivo*. To develop an algorithm for rapid immune subtyping *ex vivo*, we took advantage of publicly available data and constructed the immune subtyping classifier based on the expression of seven genes. Four of them – CD2, CD8B, CD53, and IRF1 are associated with immune response. Another three genes – COL5A2, INHBA, and TNFAIP6 – represent the reactive stroma compartment. The accuracy of a classifier encompassing expression data reached 79% in a real-world validation cohort.

We also report the different distributions of major oncogenic melanoma alterations (BRAF, NRAS, KIT) among different immune subtype groups. NRAS mutation was more frequent in the immune desert subtype. BRAF mutation was more characteristic of excluded and inflamed subtypes, as confirmed in both TCGA and patient cohorts. Almost none of the inflamed tumors had alterations in KIT.

Our findings suggest that melanoma tumors can be classified into transcriptionally and histologically distinct desert, excluded, and inflamed subtypes. Gene expression-based algorithms can assist physicians and pathologists in the rapid assessment of tumor immune microenvironment while serving as a tool for clinical decision making. The proposed classifier could be validated on a larger scale to determine its clinical utility and performance in immunotherapy-treated patients.

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PRECLINICAL MODELS OF HORMONE RECEPTOR-POSITIVE BREAST CANCER: B6BC, A NEW MOUSE CELL LINE RESISTANT TO PD1 BLOCKADE

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Despite the recent implementation of targeted-therapies to complement hormone therapy and chemotherapy, hormone receptor-positive (HR+) breast cancer (BC) is still quantitatively the most deadly female mammary carcinoma. Contrasting with other BC subtypes, immunotherapies have been inefficient so far to treat HR+ BC and long-term responses are still difficult to achieve. This immunotherapeutic-desert is partly explained by the difficulty of modeling HR+ BC in representative preclinical immunocompetent mouse models. HR+ tumorigenesis can be induced by combining a progesterone analogue (MPA) with a carcinogen (DMBA). However, MPA/DMBA tumors generally present a long latency and are overall more difficult to handle than syngeneic cell-line derived transplantable models. Strikingly, despite C57BL/6 is the most commonly used mouse strain, especially for the generation of transgenic variants, there were so far no HR+ BC cells transplantable to C57BL/6 immunocompetent hosts. Thus, we decided to establish such cell lines.

Mammary carcinogenesis was induced using MPA/DMBA in C57BL/6 females. Established tumors were recovered, dissociated to single cell suspension and serially passage in culture to generate eight cell lines. Then cell lines were selected for hormone (estrogen + progesterone) receptor positivity and transplantability into C57BL/6 immunocompetent females.

One cell line, named B6BC, fulfilled the abovementioned criteria and generated estrogen receptor-positive invasive mammary carcinomas with features of epithelial-to-mesenchymal transition when transplanted in C57BL/6 hosts. B6BC tumors partially and transiently responded to hormone therapy and immunogenic chemotherapy, however the latter one was not influenced by the depletion of T cells. Indeed, B6BC tumors were scarcely infiltrated by T lymphocytes and did not respond to PD-1 targeted therapy. However, as determined by single-nucleus RNA sequencing and high-dimensional leukocyte profiling, B6BC tumors were abundantly infiltrated by myeloid cells (mostly expressing Spp1) and mildly reduced their growth upon blockade of the pan-myeloid marker CD11b, suggesting tumor sustainment by CD11b+ myeloid cells. Of note, none of the tested in vivo systemic treatments (hormone therapy, chemotherapy and immunotherapy) achieve curative responses in B6BC TT.

B6BC is the first cell line transplantable to C57BL/6 mice and generating mammary carcinomas that recapitulate the complex tumor microenvironment of locally advanced HR+ BC naturally resistant to PD-1 immunotherapy.

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EXERCISE-INDUCED EXTRACELLULAR VESICLES MODULATE THE IMMUNE TUMOR MICROENVIRONMENT AND INFLUENCE CANCER PROGRESSION IN MURINE BREAST CANCER MODELS

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Breast cancer is the most common cancer in women worldwide, with rising global incidence. Breast cancer heterogeneity demands better prevention strategies and treatment options. Physical exercise has shown promise in reducing cancer risk and improving outcomes for BC patients. Understanding the molecular mechanisms behind exercise-induced effects can lead to biomarker identification and evidence-based exercise prescriptions. Aiming to uncover novel insights into exercise as a preventive and therapeutic measure against cancer, this study focuses on exploring the impact of exercise-induced extracellular vesicles on the immune tumor microenvironment and metastatic cancer progression using mouse models of breast cancer.

A treadmill-based animal exercise plan was designed, and mice were trained to collect plasma containing exercise-induced extracellular vesicles. Subsequently, extracellular vesicles were isolated from plasma to assess their quantity, size, and protein markers. Transplantable metastatic murine breast cancer models (4T1 and E0771) were established and treated with isolated extracellular vesicles in the prophylactic and therapeutic setting. Tumor growth was monitored. At the endpoint, the tumor immune microenvironment was assessed via flow cytometry and qPCR.

The mice underwent an exercise plan consisting of 10 sessions of 30 minutes each, at 60% of their VO₂ max. Plasma samples were collected from the mice, and extracellular vesicles were successfully isolated using size-exclusion chromatography. The results revealed that the running mice had significantly more exosomes compared to the sedentary control group. Additionally, a comparison between the two mouse strains showed that BALB/c mice had a higher abundance of extracellular vesicles compared to C57BL/6 mice.

In parallel, two murine breast cancer models were established: 4T1 in BALB/c mice and E0771 in C57BL/6 mice. E0771 showed a higher abundance of MHC I, PD-L1, and CD11b markers, while CD44 was found in both cell lines. 4T1 tumors grew faster ($p < 0.05$) than E0771. Both models exhibited heavy immune cell infiltration, with 4T1 having higher T cell infiltration, especially the CD4+ T helper subset. E0771's immune microenvironment remained stable during tumor development, while 4T1 showed dynamic changes, with decreasing T cell proportion over time.

Upon injecting extracellular vesicles (EVs) into tumor-bearing mice, both in prophylactic and therapeutic settings, notable effects on tumor growth were observed. The mice treated with EVs displayed a delay in tumor growth compared to the untreated control group. Moreover, at the study endpoint, the mice treated with EVs exhibited smaller tumor volumes. Additionally, the administration of EVs influenced the immune microenvironment within the tumors. There were noticeable differences in the proportions of both lymphoid and myeloid cell subpopulations, suggesting that EVs might have immunomodulatory effects on the tumor microenvironment.

Overall, our study provides valuable insights into the impact of exercise on EV production and their potential therapeutic effects in breast cancer treatment. The observed alterations in the immune microenvironment further support the notion that EVs may hold promise as a novel

immunomodulatory therapy for breast cancer. These findings promote future research and the development of targeted therapies utilizing EVs to improve cancer treatment outcomes.

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COUNTERACTING IMMUNE SUPPRESSION AND ENHANCING CYTOTOXIC T CELL ACTIVITY WITH ALLOGENEIC ARMORED CAR EXPRESSING INVARIANT NATURAL KILLER T CELLS TARGETING FIBROBLAST ACTIVATION PROTEIN IN AN ADVANCED SOLID TUMOR MODEL SYSTEM

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A significant challenge in solid tumor cancer is the inadequate response seen in well over 60% of patients to checkpoint modulators, often due to active immune suppression within the tumor microenvironment (TME). To overcome suppression and increase tumor control we genetically modified invariant Natural Killer T cells (iNKT) cells to express a Chimeric Antigen Receptor (CAR) targeting Fibroblast Activating Protein (FAP), with IL-15 as armor. FAP is overexpressed on immune-suppressive Cancer Associated Fibroblasts (CAFs), which form a major stromal component of most epithelial derived tumors. iNKT cells were selected for their natural resistance to exhaustion, tissue homing, selective cytotoxicity towards M2 macrophages and stimulation of dendritic cell maturation, which are key elements for eliciting an effective and durable anti-tumor immune response. In addition, manufacturing of iNKT cells has proven to be highly scalable and stable, and, due to the presence of an invariant T cell receptor, does not require TCR knockout for clinical application in order to prevent Graft versus Host Disease.

We've used a proprietary advanced A549-based orthotopic NSCLC xenograft mouse model to elucidate the impact of IL-15 armored FAP-CAR iNKT cells on cancer-associated fibroblasts (CAFs) and immune-suppressive mechanisms within the TME. A549 tumor cells are engineered to present NY-ESO1 on HLA-A*02:01, enabling recognition by co-administered NY-ESO TCR-expressing conventional T cells (NY-ESO T).

In this model murine fibroblasts are recruited to the tumor stroma and induced to become immune-suppressive CAFs. We observed that conventional NY-ESO1 T cells cannot effectively infiltrate the tumors in the lung and have limited effect on tumor control. FAP-CAR-IL-15 iNKT cells effectively kill FAP-overexpressing CAFs, and in mice where conventional NY-ESO1-specific T cells and FAP CAR iNKT cells have been co-administered we see dramatic increase in NY-ESO1 T cell infiltration, intra-tumoral proliferation and cytotoxic activity, leading to complete tumor eradication and long-term survival. We have further analyzed the Immune-suppressive mechanisms at play in this model and show that these A549 tumors and their CAF-rich stroma express immunosuppressive cytokines such as CXCL12. IL-15 armored FAP-CAR-iNKT effectively attack high-FAP CAFs and reduce immune suppression in the tumor, which we believe is instrumental in enabling the increased influx, proliferation, and tumor cell co-localization of NY-ESO1 T cells. We believe that this model recapitulates key immune suppressive mechanisms limiting clinical activity of cytotoxic T cells in non-small cell lung cancer, and shows how armored, FAP-targeted invariant natural killer T cells may counteract immune suppression and potentiate durable tumor control.

Our novel armored CAR-iNKT product effectively targets FAP-positive tumor cells and suppressive CAF subsets in the TME. By combining the potent natural activity of iNKT cells with the selective action of our FAP-CAR, we envision achieving significant tumor control and immune engagement, surpassing current treatment options for solid tumor patients

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DIFFERENCES IN ANTIGEN-SPECIFIC CD8 AND CD4 T-CELL RESPONSES ACCORDING TO PRIMARY TUMOR SITE AND ANTIGEN FAMILY IN HUMAN PAPILLOMAVIRUS-INDUCED EPITHELIAL CANCERS

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Human Papillomavirus (HPV) is responsible for more than 600000 new invasive cancers each year worldwide, resulting in more than 250000 deaths. Neoplasia caused by HPV are mainly cervical, anogenital, and oropharyngeal carcinomas. Among the HPV oncogenic high-risk subtypes, HPV-16 is the most common, and is responsible for half of cervical cancers and about 90% of HPV-induced oropharyngeal cancers. HPV carcinogenesis is largely driven by two viral early genes (E6 and E7, often referred to as HPV oncogenes), and thus, almost all of the therapeutic vaccines in development target these two oncoproteins. The aim of our study was to compare specific CD8 and CD4 T-cell responses to HPV-16 antigens, in 2 different tumor sites, namely cervical and oropharyngeal cancers.

we constituted a cohort of patients newly diagnosed with non-metastatic HPV-16-induced cancer. We collected a tumor biopsy and the peripheral blood mononuclear cells (PBMCs). Potential CD8 T-cell epitopes derived from HPV-16 proteins E2, E5, E6 and E7 and presented by a reference set of 27 human leukocyte antigens (HLA-A and B) covering 97% of the world population were predicted using the Immune Epitope Database (IEDB). A total of 241 predicted 9 to 10 amino acid long peptides were synthesized and used to assess circulating CD8 T-cell responses by intracellular cytokine staining following in vitro stimulation and short-term culture. Through a similar approach, CD4 T-cell responses were assessed using pools of 20 amino acid long peptides, overlapping by 10 amino acids and covering the full-length sequence of E2, E5, E6 or E7 proteins.

twenty three patients were included in the study (13 with cervical and 10 with oropharyngeal cancers). We were able to detect CD8 and CD4 antigen-specific responses not only against E6 and E7 oncoproteins, but also frequently against E2 and E5 proteins. The proportions of specific CD8 T cells were similar between the two tumor types, but CD4 T-cell responses were higher in oropharyngeal cancers, in particular for responses directed against E7. Interestingly, the immunogenic epitopes recognized by CD8 T cells were differently distributed depending on the tumor type, with epitopes for cervical cancers originating equivalently from E2, E5, E6 and E7, whereas for oropharyngeal cancers T cells targeted epitopes from E5, and secondarily from E6 and then from E2. Some epitopes were identified in several patients (up to 4 different patients), highlighting their strong immunogenicity, and sometimes across tumor types. Interestingly, using fluorescent HLA class I/peptide multimers and flow cytometry analysis, we showed that T cells specific for the same epitopes exhibited divergent exhaustion phenotypes according to the tumor type.

HPV antigens-specific CD8 and CD4 T-cell responses spontaneously arising in cancer patients show differences according to the primary tumor type/site, opening the way to the adaptation of immunotherapies, including immune checkpoints inhibitors and vaccination, adapted to each location.

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THE SUB-VENTRICULAR ZONE OF GLIOBLASTOMA PATIENTS IS A PRO-INFLAMMATORY TUMOR MICROENVIRONMENT CHARACTERIZED BY A MESENCHYMAL SIGNATURE

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The sub-ventricular zone (SVZ) is the most well-characterized neurogenic area in the mammalian brain. We previously showed that in 65% of glioblastoma (GBM) patients, the SVZ is a tumor area (T_SVZ) and a reservoir of cancer stem-like cells (CSCs) contributing to treatment resistance and to the emergence of the recurrent tumor that is inevitable in GBM patients. However, the sampling and the characterization of the T_SVZ in GBM patients pose several challenges, as this area is extremely small and needs to be objectively identified during tumor surgical resection.

Here, by using our previously published fluorescence-guided multiple sampling scheme we built a single-cell/single-nucleus RNA-sequencing-based microenvironment atlas of the T_SVZ in 15 GBM patients and performed a systematic comparison of copy number variations and cellular composition with matched tumor mass (T) samples and histologically normal SVZ (N_SVZ) samples.

Analysis of copy number variations of the T_SVZ and matched T samples revealed the presence of genetic alterations that are common in GBM. As expected, no genetic alterations were detected in the N_SVZ samples. Interestingly, cell type/cell state annotation and analysis of cancer cell signatures revealed that the T_SVZ of GBM patients is a reservoir of heterogeneous CSCs mainly characterized by a mesenchymal signature when compared with matched T samples. Moreover, the T_SVZ microenvironment is predominantly characterized by microglia and pro-inflammatory mediators.

Overall, our results show that T_SVZ is a distinct tumor microenvironment characterized by a mesenchymal signature and pro-inflammatory mediators. Single-cell interaction network analysis, spatial transcriptomics, and functional phenotyping assays are currently ongoing to dissect the cross-talk between CSCs and stromal/immune cells in the T_SVZ for the identification of novel therapeutic targets.

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GENOME-WIDE CRISPR SCREENS DECODE CANCER CELL PATHWAYS THAT TRIGGER GAMMA-DELTA T CELL DETECTION AND KILLING

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$\gamma\delta$ T cells are potent anti-cancer effectors with potential to target tumors broadly, independent of patient-specific neoantigens or HLA-background. Their tumor abundance has correlated with favorable patient outcomes across many different types of cancer, and $\gamma\delta$ T cells have been found to be important effectors against HLA class I defective tumors after immune checkpoint blockade. $\gamma\delta$ T cells can sense conserved cell stress signals prevalent in transformed cells, although the mechanisms behind the targeting of stressed target cells remain poorly characterized. V γ 9V δ 2 T cells – the most abundant subset of human $\gamma\delta$ T cells – recognize a protein complex containing butyrophilin 2A1 (BTN2A1) and BTN3A1. This cell surface butyrophilin complex is ubiquitously expressed in healthy tissue and is activated through BTN3A1 sensing of mevalonate pathway hyperactivity commonly found in tumor cells.

We used genome-wide CRISPR screens in Daudi cells (B cell lymphoma) to identify pathways that regulate: (1) BTN3A1 cell surface expression with a FACS-based screen, and (2) T cell activity with a functional $\gamma\delta$ T cell-Daudi co-culture killing screen. We validated our findings by generating Daudi cells knocked out for individual gene hits; mapping transcriptional regulator binding sites at the butyrophilin genomic locus using CUT&RUN; testing the effects of relevant metabolic pathways on BTN3A1 surface expression using metabolic agonists and antagonists; and quantifying the effects of modulating various metabolic and transcriptional regulators on V γ 9V δ 2 T cell killing of treated cell lines and patient-derived tumor organoids.

In addition to known components of the butyrophilin complex and the mevalonate pathway, both screens identified cell processes that modulate BTN3A1 expression: positive and negative transcriptional regulation (IRF1, CTBP1, ZNF217, RUNX1), intracellular trafficking, sialylation, N-linked glycosylation, OXPHOS, purine metabolism, and other metabolic pathways. Consistent with these results, we found upregulated BTN3A1 on cells undergoing an energy crisis due to glucose deprivation, glycolysis inhibition, or OXPHOS inhibition. After testing multiple metabolic regulators, we discovered that activation of the energy sensor AMPK upregulates both BTN2A1 and BTN3A1. Furthermore, AMPK activation in Daudi cells and patient-derived tumor organoids led to increased V γ 9V δ 2 TCR-mediated killing. Finally, genetic signatures based on the screens' hits significantly correlated with higher survival in low-grade glioma patients with high V γ 9V δ 2 T cell tumor infiltration.

Our study reveals multilayered regulation of BTN3A1 abundance and/or accessibility through transcriptional regulators, glycosylation and sialylation, iron-sulphur cluster formation, trafficking, metabolic sensing, and various metabolic pathways. We identified AMPK as a critical regulator of BTN2A1 and BTN3A1 expression in cells undergoing an energy crisis, thereby providing a mechanism of stress-mediated regulation of a key $\gamma\delta$ T cell-cancer cell interaction. Understanding this AMPK-dependent mechanism of metabolic stress-induced activation of V γ 9V δ 2 T cells opens new possibilities of enhancing the activity of these cells in cancer patients.

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APOPTOTIC PRIMING PREDICTS ENHANCED PERSISTENCE AND ANTI-TUMOR CYTOTOXICITY OF CAR T CELLS OVEREXPRESSING BCL-XL

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Introduction

Chimeric antigen receptor (CAR) T cell therapy has recently provided a promising treatment option for patients with hematologic malignancies; however, relapse remains a common hurdle in the setting of aggressive disease. A strong correlate identified in patients that relapse from CAR T cell therapy is poor persistence of their engineered T cells, placing a spotlight on the quality of the T cell product and how it can be improved. Programmed cell death plays a vital role in culling the natural T cell pool during infection and the role of apoptosis in CAR T cell therapy is only just beginning to be understood. Here, we sought to investigate the apoptotic priming, a measure of the likelihood of a cell to undergo apoptosis, of CAR T cells using BH3 profiling and explore whether overexpression of members of the Bcl-2 family of antiapoptotic proteins improves CAR T cell apoptotic priming and persistence.

Materials & methods

Coexpression constructs were used to generate CD19-targeted CAR T cells expressing Bcl-2, a mutant form of Bcl-2 found in patients with CLL resistant to the BH3 mimetic venetoclax (Bcl-2 G101V), Mcl-1 and Bcl-xL. BH3 profiling was used to determine the overall apoptotic priming as well as any antiapoptotic dependencies of wild type and Bcl-2 family overexpression CAR T cells. CAR T cell killing of NALM6 cells (B-ALL) and persistence were investigated in coculture assays in vitro with and without BH3 mimetics and in vivo.

Results

Overexpression of Bcl-2 and Bcl-2(G101V) significantly reduced the apoptotic priming of CD19 CAR T cells, though, Bcl-xL overexpression provided the greatest reduction in apoptotic priming in CD19-targeted CARs. Interestingly, both Bcl-2(G101V) and Bcl-xL overexpressing CD19 CAR T cells exhibited enhanced tumor control in vivo and CARs overexpressing Bcl-xL exhibited significantly improved in vivo persistence, suggesting a relationship between apoptotic priming and CAR T cell function. Of note, BH3 profiling revealed a reduced dependence on the antiapoptotic protein Mcl-1 to resist cell death in CAR T cells overexpressing Bcl-xL. This finding was consistent with in vitro coculture assays where Bcl-xL overexpressing CAR T cells exhibited enhanced persistence and anti-tumor cytotoxicity in the presence of the Mcl-1 inhibitor AZD5991, demonstrating that BH3 profiling reveals apoptotic dependencies in CAR T cells that can be exploited with BH3 mimetics to further improve CAR T cell function.

Conclusion

In summary, we found that overexpression of antiapoptotic proteins, namely Bcl-xL, reduces the apoptotic priming of CAR T cells and that reduced apoptotic priming strongly correlates with improved CAR T cell persistence and anti-tumor cytotoxicity with and without BH3 mimetics. These findings suggest that investigating CAR T cell apoptotic priming by BH3 profiling may be useful when engineering novel CAR T cell products aimed at improving CAR T cell persistence and killing.

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HUMAN CYTOKINE-INDUCED MEMORY-LIKE NK CELLS DISPLAY INCREASED REACTIVITY AGAINST NON-SMALL-CELL LUNG CANCER AND ARE EFFECTIVE IN REDUCING STEMNESS AND TUMORIGENICITY

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Non-small cell lung cancer (NSCLC) is diagnosed at an advanced stage in nearly 70% of patients and remains a leading cause of cancer death in adults worldwide. Despite the implementation of targeted therapies and immunotherapy, involving immune-checkpoint inhibitors (ICIs), the 5-year survival rate, particularly of patients at an advanced stage, has only sought moderate improvement. Indeed, only around 10–15% of patients exhibit sustained long-term benefits in response to ICIs, and in around 30% of cases, tumor progression occurs after an initial response. Tumor spreading and metastasis are considered the major therapeutic hurdles in NSCLC; thus, novel treatment options effective in controlling these events are highly desirable.

Natural killer (NK) cells are innate lymphoid cells that protect against infection and mediate anti-tumor responses. Different approaches are under study to improve their potential efficacy in anti-tumor therapies, by enhancing their effector functions, persistence, and tumor targeting and infiltrating properties. In this framework, cytokine-induced memory-like (CIML) NK cells, differentiated after a pre-activation with IL-12, IL-18, and IL-15, have been shown to display increased persistence and effector functions in-vivo in leukemia patients, and superior anti-tumor activity in the context of melanoma and ovarian carcinoma, both in-vitro and in human xenograft mouse models. These encouraging data stimulate further studies exploring the therapeutic potential of CIML-NK cells in a wider number of solid tumors, with particular attention to their ability to mitigate tumor malignancy by targeting cancer stem cells (CSCs) and metastatic disease.

The reactivity of CIML-NK cell derived from the peripheral blood of healthy donors was tested against NSCLC, employing four cell lines (A549, H3122, SW900, and H661) representative of the three main histotypes of NSCLC cultured in 2D or 3D conditions. The CSC content and the tumorigenicity of co-cultured tumor cells was analyzed in-vitro and in-vivo in NSG mice.

Our findings showed an increased reactivity of CIML-NK cells over control NK cells (cNK, i.e. exposed to low dose IL-15 for in-vitro survival) in terms of both cytotoxic degranulation and IFN- γ production, and against NK cells conventionally activated with IL-2 (IL-2-NK cells) in terms of cytotoxic degranulation. Remarkably, the superior functionality of CIML-NK cells was observed in response to both adherent cells and tumor spheroids, even if tumor cells derived from spheroids overall displayed increased resistance to NK cells' effector functions. In line with these findings, CIML-NK cells exhibited a superior and sustained killing of NSCLC tumor spheroids, as measured by Incucyte and Annexin-V-based killing assays, compared to both cNK and IL-2 NK cells.

The effect of CIML-NK cells on CSCs, a tumor subpopulation mediating tumorigenesis, metastatic spreading, and therapeutic resistance, was also evaluated in NSCLC spheroids. Our data revealed that CIML-NK cells were particularly effective in reducing spheroids' CSC content and limiting the tumorigenicity of spheroid-derived NSCLC cells both in-vitro and in-vivo in an NSCLC xenograft model in NSG mice.

These findings demonstrate that in-vitro induction of memory-like differentiation endows NK cells with enhanced responses against NSCLC. In addition, this work provides evidence that CIML-NK cells can efficiently control the stemness and tumorigenicity of NSCLC cells and thus, represent a potential therapeutic tool against NSCLC.

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HOST SEQUENCE EVOLUTION AND HOMOLOGY ANALYSES REVEAL SPECIES SUSCEPTIBILITY TO POTENTIALLY ZONOTIC SARBECOVIRUSES.

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Viruses play significant roles in cancer and immunity-associated diseases. Some viruses are directly carcinogenic (e.g. HPV, HTLV) while others weaken the immune system (e.g. HIV), which can be a prerequisite of cancer emergence. Cancer patients can be more susceptible to and suffer from long-term consequences of viral infections. For example, recent reports suggest cancer patients suffer from post-acute sequelae following SARS-CoV-2 (SCV-2) infection at higher rates than the general population. The SCV-2 pandemic demonstrates the ongoing need for more effective virus surveillance tools to control outbreaks before they emerge and afflict vulnerable populations. Current approaches target species known to harbor viruses with suspected or established zoonotic potential. However, sampling bias inherent in these approaches may omit relevant host species. We hypothesized that host receptor sequence changes resulting from evolutionary arms races between viruses and their hosts could reveal animal lineages harboring potentially zoonotic viruses and identify sites critical for host receptor interactions with viral entry proteins. Further, host receptor sequence homology across animals can be leveraged to identify host species with significant virus susceptibility.

We focused on the SCV-1 and -2 receptor ACE2 as a model because extensive virological and genomic data is abundant. We performed evolutionary sequence analyses of vertebrate ACE2 orthologs to determine which species have historically been impacted by ACE2-utilizing viruses. Using known direct ACE2-spike residue interactions as a guide, we employed a novel sequence homology analysis with multiple reference species to identify a pool of species that are likely susceptible to SCV-like infection. To test these susceptibility predictions, we infected 293T cells, transiently expressing ACE2 from putatively susceptible species, with VSV-luciferase reporter viruses pseudotyped with SCV-2 variant and SCV-like glycoproteins.

Consistent with existing virological data, our ACE2 sequence analyses indicate SARS-like viruses have persisted in the horseshoe and leafnose bat lineage for approximately 64 million years. These data imply that SCV-like coronavirus glycoproteins have persistently targeted specific residues that are critical for modern coronavirus entry. We found that our infection assays were generally consistent with our computational susceptibility predictions. These data also suggest that SCV-2 adaptation within a host population can alter SCV-2 host tropism.

Our study illustrates how host receptor sequence analyses can identify potential virus reservoir lineages and predict host species susceptibility to viral entry. Future work will combine host receptor evolutionary sequence analyses with homology predictions to generate an integrated pipeline that can be applied to further viral entry receptor proteins without the need for structural data. The insights gained from this study highlight how analysis of mammalian genome sequences can inform virus surveillance efforts to prevent zoonotic infection of vulnerable populations, such as those afflicted with cancer.

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DEVELOPMENT OF NEXT-GENERATION RETINOID PATHWAY IMMUNOTHERAPEUTICS THROUGH DUAL TARGETING OF TUMOR AND MYELOID ALDEHYDE DEHYDROGENASES

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Aldehyde dehydrogenase (ALDH) expression and enzymatic activity has long been established as a pro-tumorigenic feature of many cancers, yet the identification of specific isoforms responsible for tumorigenesis, the mechanism of action of this isoform(s), and viable therapeutic strategies to target this pathway have long remained absent. Whereas one of the well-established functions of the ALDH1 family is the conversion of retinaldehyde into retinoic acid to activate nuclear retinoid signaling, the retinoid pathway is paradoxically hypothesized as a cell-intrinsic tumor suppressor pathway.

Here we resolve this long-standing paradox by identifying that ALDH1a3 is enriched in tumor cells and is complemented by ALDH1a2 expressed in immunosuppressive myeloid cells. Both isoforms work in concert to drive retinoid-mediated differentiation of T cells toward immune suppression and tumor immune escape. Next, we developed a series of first-in-class, orally-available antagonists of ALDH1a2 and ALDH1a3 to show that these enzymes exclusively function to oxidize all-trans retinal into all-trans retinoic acid in solid tumors.

Application of these first-in-class oral therapeutics to in vivo efficacy experiments reveal potent single agent activity and nearly 50% complete response rates when combined with other standard-of-care immunotherapies. Large mammal pharmacokinetic, pharmacodynamic and toxicology studies then allowed the selection of a clinical candidate molecule that will be entering first-in-human testing in 18 months.

Findings of this study resolve prior contradictions in the field through the development of highly specific and potent ALDH1a2/1a3 inhibitors to show that the retinoid pathway is a key immune-suppressive pathway in multiple solid tumor types. In light of the preponderance of randomized clinical data showing that the retinoid pathway is pro-tumorigenic, early clinical trials will focus on defined cancer populations with amplification of ALDH1a2/1a3.

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BACTERIAL CGAS-LIKE ENZYMES PRODUCE 2',3'-cGAMP AND ACTIVATE PROGRAMMABLE RECEPTORS FOR BIOTECHNOLOGY

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The mammalian innate immune system uses cyclic GMP–AMP synthase (cGAS) to rapidly synthesize the cyclic dinucleotide (CDN) 2',3'-cGAMP during antiviral and antitumor immune responses. 2',3'-cGAMP activates downstream immune signaling via stimulator of interferon genes (STING) resulting in protective inflammatory responses. Bacteria encode ancestral cGAS-like enzymes (CD-NTases) that are encoded in innate immune systems that protect against viral predation by bacteriophages. In contrast to human cGAS, all known bacterial CD-NTases produce distinct cyclic oligonucleotides unique to bacteria. Here we discover bacterial cGAS-like enzymes that produce 2',3'-cGAMP to initiate potent antiviral signaling in response to bacteriophage infection. We further identified that the cognate transmembrane receptor binds 2',3'-cGAMP with nanomolar affinity and forms an ion channel that restricts phage replication. The cGAMP-binding domain from this channel is part of a widespread family of CDN receptors and is fused to diverse effector domains involved in antiviral conflict. The cGAS-STING pathway plays an indispensable role in immunity against pathogens and cancer. Consequently, there is a significant interest in development 2',3'-cGAMP-responsive biological tools to study and manipulate cGAS-STING for host-directed immunotherapy. To this end, we reasoned that the bacterial 2',3'-cGAMP receptor may have utility as a biological sensor that couples CDN-binding to an effector output. We took the bacterial receptor that we discovered and replaced the pore-forming domain with an endonuclease domain to engineer a chimeric biosensor for 2',3'-cGAMP. The chimeric nuclease was functional in-vitro and degraded DNA in response to 2',3'-cGAMP and did not cross-react with other cyclic dinucleotides. These experiments provide proof-of-concept that the bacterial 2',3'-cGAMP receptor can be used to program an enzyme to respond to a specific CDN with high fidelity. The ability to couple 2',3'-cGAMP-binding to a direct enzymatic output may have broad use for study or manipulation of mammalian cGAS-STING signaling. Our experiments provide proof-of-principle that bacterial immune receptors can serve as a platform for generating new synthetic biology with direct application for the study of human immunology. Collectively, this study reveals the existence of a 2',3'-cGAMP signaling system beyond the mammalian cGAS-STING pathway, and provides a new biological toolkit of 2',3'-cGAMP synthases and receptors that can be engineered for biological discovery and molecular medicine.

- bacteriology, bacteriophage, innate immunity, enzymology, electrophysiology, protein engineering
- We identified bacterial cGAS-like enzymes that produce 2',3'-cGAMP to protect against bacteriophage.
- We discovered that the 2',3'-cGAMP-receptor in bacteria is a novel ion channel.
- We engineered the bacterial 2',3'-cGAMP receptor to an enzymatic domain and created a new biosensor for 2',3'-cGAMP
- First identification of 2',3'-cGAMP-based immune signaling exists outside of mammalian cGAS-STING.
- Bacteria represent a potential reservoir for 2',3'-cGAMP which may have implications in microbiome-based cGAS-STING-directed immunotherapy.
- Bacterial cGAS-like enzymes that produce 2',3'-cGAMP may have utility in cGAS-STING-directed immunotherapy (first 2',3'-cGAMP synthases beyond metazoan cGAS).
- Bacterial 2',3'-cGAMP receptors can be engineered to elicit effector responses and may represent a new platform for 2',3'-cGAMP-activated biotechnology.

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PROFILING TUMOR INFILTRATING IMMUNE CELLS FOR BETTER UNDERSTANDING TUMOR STATUS IN SOFT TISSUE SARCOMAS

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The tumor microenvironment is closely associated with tumor development and progression, which is made up of tumor cells, blood vessels, immune cells, fibroblast, and so on. Especially, immune cells within tumor microenvironments have been attracted in clinicians and researchers for unexpected therapeutic responses. Even in sarcoma, which is rare, and heterogenous leading to few options for cure, immune therapy has been tried and shown to be promising in some patients with therapy against PD-1 previously.

Here, we established platform flow cytometry-based immune cell panel with 17 markers and profiled thirty tissues obtained from patients diagnosed as soft tissue sarcomas.

Overall, infiltrated leukocytes and CD3+ T cells were remarkably increased, whereas NKT and CD4+ T cells were decreased in tumor tissues compared to adjacent normal tissues. M1 macrophage was also increased in tumor tissues. High CD3+ T cell population was positively correlated with CD8+ T cells and PD1+ CD8+ T cell population. Especially, PD1+CD8+ T cells are usually expressed exhaustion-related conditions and low levels of cytotoxic immune cells. The correlation analyses between immune cell groups and tumor characteristics including tumor grades, sexuality, subtypes and age were performed.

This study provides resources to understand immune cell population of soft tissue sarcoma, and will gain a fundamental matrix to study immunotherapy.

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INVESTIGATING THE INTERPLAY BETWEEN SENESENCE AND T CELL IMMUNITY

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Cellular senescence is a stress-induced program characterized by two major components: a stable proliferative arrest and a senescence-associated secretory phenotype (SASP). The SASP is characterized by the secretion of a plethora of soluble factors that can influence the tumor microenvironment (TME) and that has been shown to either favor or disfavor an antitumoral immune response in different contexts. Senescence is a common outcome of anticancer therapies, yet the precise mechanisms by which senescence alters adaptive anti-tumor immune responses remain largely unexplored.

Senescence-inducing therapies only trigger senescence in a (sometimes relatively small) fraction of cancer cells, yet these therapies can lead to substantial, immune mediated, tumor regressions, we hypothesize that induction of senescence can favor bystander elimination of non-senescent cells; this can be possible as the cytokines secreted by senescent cells are able to recruit immune cells to the tumor microenvironment and to potentiate their activity against senescent and non-senescent cells.

In order to investigate this effect, we have developed a genetic strategy to induce senescence in a tumor only in a defined fraction of cancer cells and to track in vivo which cells senesce and which cells do not. Additionally, we have established strategies for expressing different T cell epitopes in senescent and non-senescent cells; by doing this we are able to directly interrogate how T cells specific for senescent and non-senescent cells will behave in response to induction of senescence in a fraction of cancer cells.

We are currently investigating, by imaging and flow cytometry how T cells specific for senescent and non-senescent cells are affected by the presence of senescent cells in the tumor microenvironment.

Results of our research can give us a better understanding of how different anticancer therapies can induce an antitumoral immune response.

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UNDERSTANDING THE MECHANISTIC ROLE OF THE INTESTINAL MICROBIOME ON CAR T CELL THERAPY OUTCOME

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CAR T cell therapy has rapidly become a standard of treatment against aggressive hematological cancers, and multiple myeloma. There are now six FDA approved CAR T cell-based therapies that show remarkable tumor-eradication and long-term survival. However 60% of patients fail to achieve a successful outcome, and instead relapse after a few months or experience toxicity due to deleterious immune activation in the form of cytokine release syndrome or immune effector cell-associated neurotoxicity syndrome. Thus, mitigating these iatrogenic toxicities while still preserving high on-target efficacy has become a fundamental part of the ongoing development of this immunotherapy. The microbiome has been found to play a fundamental role in the immune system of the host, greatly impacting the induction, training, and overall function of T cells. Retrospective studies show that microbiome dysbiosis correlates with worse overall survival of CAR T cell therapy recipients and toxicity presentation in clinic. My research aims to elucidate the mechanistic role of the intestinal microbiota that promotes CAR T cell therapy response.

We have established two pre-clinical models of dysbiosis: 1) derivation of humanized intestinal microbiota avatars through fecal microbiota transplants (FMTs) from responders and non-responder patients of CAR T cell therapy ; and 2) antibiotic-mediated intestinal dysbiosis as often seen in clinic. The antibiotic model consists of giving SPF mice a broad-spectrum antibiotic cocktail of ampicillin (500mg/L) and Enrofloxacin (250mg/L) 8 days before CAR T cell infusion (n=68). The avatar model is an established tumor model in which germ-free mice are undergo a fecal microbiota transplant (FMT) via gavage of 200uL of patient stool slurry. Each avatar experiment consists of half of the mice being colonized by fecal matter from a patient who achieved a complete response to CAR T cell therapy and one who did not achieve a complete response by day 100 (n=15). Kaplan-Meier curves were used to assess overall survival, and evaluated by log-rank test for significance. Fecal samples were collected before and after any microbiota perturbation, and weekly after CAR T cell infusion. All stool was then submitted for occurred at D10. Results were compared between the dysbiotic groups and those that did not experience dysbiosis with a vehicle of regular drinking water or with intestinal colonization from a complete responder of CAR T cell therapy.

We have demonstrated that intestinal dysbiosis modeled via administration of a broad-spectrum antibiotic cocktail consisting of ampicillin and enrofloxacin, leads to worse overall survival of mice exposed when compared to mice who were not exposed to antibiotic cocktail (p<0.0001). We also found that antibiotic-exposed mice display decreased CAR T cell abundances at day 10 in tumor liver site (p=0.0196), and bone marrow (p=0.02). Serum analysis also reveals antibiotic-exposed mice to have lower quantities of inflammatory cytokines such as IL-12p70 (p=0.0144), IL-17A (p=0.0271), GM-CSF (p=0.0056). Ongoing metabolomics analysis is underway. In our humanized microbiome avatars, mice inoculated with non-responder FMT had worse overall survival than mice inoculated with complete responder FMT (p=0.0069). Metagenomic shotgun sequencing revealed that responder FMT maintained a higher alpha-diversity than non-responder FMT avatars at baseline and in the following 2 weeks post-CAR T cell infusion. Current analysis is looking into metabolite biomarkers, and cellular differences.

We propose to elucidate the mechanistic role by which the intestinal microbiota drives CAR T cell therapy response. The central hypothesis for this project is that strategic microbiota modulation can enhance therapy response by abating dysbiosis and thereby preserving favorable intestinal taxa that promote improved effector function of circulating CAR T cells.

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HIGH-THROUGHPUT BASE EDITING MAPS FUNCTIONAL ALLELES TO TUNE HUMAN T CELL ACTIVITY

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CRISPR-enabled genetic screening has emerged as a powerful tool to discover genes that control T cell function and has nominated candidate target genes for immunotherapies. However, new approaches are required to probe the function of specific nucleotide sequences within key genes. While the broad-brush approach of gene activation and interference has been tremendously enlightening, precision editing of specific nucleotide sequences within key genes can provide further insights. DNA base editors are powerful tools to introduce targeted mutations with high efficiency. Here, we develop a high-throughput approach for large-scale base editing mutagenesis screens in primary human T cells with the goal of pinpointing nucleotides encoding amino acid residues that tune activation responses.

We generated a pooled library of ~117,000 sgRNAs targeting both ABE and CBE base editors targeting 385 genes at every possible site within their coding sequence. These genes have functions for T cell activity, differentiation and were identified by extensive literature search, previous CRISPR knockout, activation and interference screens as well as by unbiased gene ontology assessment. A pool of activated and edited T cells was screened for their capability to produce key markers of T cell activity including cytokine production and upregulation of activation induced surface markers.

We systematically identified protein domains and specific amino acid residues that regulate T cell activation and cytokine production and discovered a broad spectrum of alleles with variants encoding critical amino acid residues (in PIK3CD, VAV1, LCP2, PLCG1 and DGKZ and other key genes), comprising both gain-of-function and loss-of-function mutations. We validated the functional effects of diverse alleles and further demonstrated that base edit hits could positively and negatively tune T cell cytotoxic function. Additionally, our screens nominated a spectrum of PIK3CD alleles that quantitatively tuned T cell responses. In some cases, these alleles overlapped with naturally occurring patient mutations, but we also discovered synthetic alleles that tune T cell activity. Finally, we performed higher-resolution screening using a base editor engineered with relaxed PAM requirements (NG versus NGG), revealing specific structural domains and protein-protein interaction sites that can be targeted to tune T cell functions. Base editing screens in primary immune cells provide biochemical insights with potential to accelerate immunotherapy design.

Conducting these functional studies directly in the cell types relevant to disease pathogenesis is crucial to accurately defining variant effects on the biological processes active in disease. Base editing mutagenesis screens complement an existing pipeline of functional genetic technologies in human immune cells. Now we can use the power of pooled sgRNA perturbation libraries coupled with base editors to introduce targeted mutations throughout these key genes and discover functional allelic variants. Looking forward, complementary technologies can be deployed for deeper mutagenesis at sites nominated by base editing screens, including pooled knockins or EvolvR to test the full complement of possible synthetic mutations to identify optimal variants to engineer into T cell products. Taken together these studies will nominate alleles that can be engineered into immune cell therapies, facilitate design of engineered protein and RNA therapies, and accelerate small molecule discovery campaigns by prioritizing promising functional pockets in target complexes. Base editing mutagenesis will deepen our understanding of molecular mechanisms in human immunology and provide a transformative tool for immunotherapy development.

P551

DC-THERAPY IS REQUIRED TO EXPAND CD8 T CELL RESPONSE TO LOW AND HIGH MUTATIONAL BURDEN LUNG CANCER REFRACTORY TO IMMUNE CHECKPOINT BLOCKADE

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Clinical data identified correlations between tumor mutational burden and response to immune checkpoint blockade (ICB) in non- small cell lung cancer (NSCLC). However, since the fraction of patients responding to ICB remains modest, it is important to explore therapies that promote neoantigen cross-presentation in low and high TMB settings. Here we generated a novel model of NSCLC expressing controlled levels of neoAgs to compare ICB to therapy targeting cross-presenting type 1 DCs (cDC1). We found that antibodies to PD-L1 are unable to induce tumor regression regardless of the neoAgs abundance. In contrast, amplification and activation of cDC1 by Flt3L and anti CD40 (DCs-therapy) induced control of poorly immunogenic tumors and eradication of tumors enriched in neoAgs. Consistently, cDC1 density correlates to CD8+ T cell scores in low and high human NSCLC. Single-cell RNA sequencing reveals molecular remodeling induced by DCs-therapy including expansion of immunogenic lung cDC1 and proliferation of cytotoxic CD8+ T cells with reduced exhaustion. We conclude that boosting cDC1 activity is critical to leverage neoAgs content for therapeutic advantage in low and high mutational burden lung tumors that do not respond to ICB.

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P552

LNP MEDIATED IN VIVO REPROGRAMMING OF TUMOR ASSOCIATED MACROPHAGES

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Solid tumors make up 90% of adult cancers, however immunotherapy and cell therapy treatments such as checkpoint blockade and CAR T treatments have demonstrated heterogeneous success. It is well known that tumor associated macrophages are a major constituent of immunosuppression within the tumor microenvironment (TME), and are strongly associated with poor prognosis and tumor progression in many types of solid tumors such as lung cancer. However, depletion alone of macrophages within the TME does not restimulate the immune landscape and interventions to repolarize them into an immunostimulatory state are either systemic, resulting in potential toxicity, or depend on ex vivo macrophage engineering, which require high doses often unattainable within the clinic. We propose utilizing lipid nanoparticle (LNP) delivery: an emerging class of medicine that has demonstrated widespread success within COVID-19 vaccines, autoimmunity treatments, and gene therapy. LNPs are nonimmunogenic and deliver transient RNA payloads to cells such as APCs, hepatocytes, and stromal cells throughout the body. While biodistribution has been studied, the field has not yet been able to achieve cell specificity or an understanding of how LNPs affect transfected cell state. Our objective is to engineer novel LNPs and payloads to specifically reprogram macrophages within the TME while decreasing off target effects, either through enhancing transfection or through cell specific payload activation.

Here we have screened lipid formulations on bone marrow derived macrophages and dendritic cells to determine their delivery efficiency, their effect on the cell state, and any changes in the myeloid cell's capacity to prime T cells. We identified 3 top LNPs through said in vitro screens that performed better than Moderna and Pfizer formulations and injected them in vivo within C57/B6 mice at steady state alongside a clinical control. Using flow cytometry, we measured the delivery of the mCherry payload within myeloid and lymphoid cell types and selected the top lipid formulation. Simultaneously, we utilized bone marrow derived macrophage cultures and both steady state and lung tumor models to validate our potential payloads and measure their impact on the endogenous immune response.

Using a panel of myeloid activation markers, we found two classes of lipid formulations within our screen: silent and stimulatory. Of our top 3 hits within the screen, two were stimulatory causing an upregulation of the costimulatory markers on bone marrow derived macrophages and dendritic cells and one was silent, inducing little to no change in cell state. Surprisingly, the effect of the lipid formulation on the myeloid cell state was not correlated with the myeloid cells' capacity to prime T cells, with lipids in both the silent and stimulatory groups enhancing OT1 T cell activation upon coculture with LNP treated macrophages and OVA peptide. We tested these formulations via systemic injections into C57/B6 mice at steady state and settled upon Formulation #3 as the most effective within myeloid cells due to its high efficiency in vivo, low effect on myeloid cell state, and immunostimulatory impact within the T cell co-culture. Lastly, we validated initial immunostimulatory payloads, demonstrating robust delivery and initial endogenous immune activation in vitro and in vivo in lung tumor settings.

Within this work, we developed myeloid-targeting LNPs that effectively deliver within the lung under both steady state and tumor bearing conditions. Additionally, we show for the first time that LNP formulation has a large effect on both myeloid cell state and T cell priming, and that said effects do not seem interdependent. Lastly we demonstrate initial efficacy of payloads activating the innate and adaptive immune system within the TME and lay a foundation for future work.

P553

INCREASING PLEXITY OF IMAGING MASS CYTOMETRY FOR TUMOR TISSUE ANALYSIS

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Imaging Mass Cytometry™ (IMC™) is the leading platform for high-plex tissue imaging. IMC allows for detailed assessment of cell phenotype and function using 40-plus markers simultaneously at subcellular resolution on a single slide. A comprehensive IMC panel containing structural, functional, and immune markers enables us to reveal the complex heterogeneity of tumor tissues as well as the tumor microenvironment (TME). Driven by an increase in the number of antibody markers and the addition of mRNA markers, there is an increasing demand for larger panels. In addition, increasing the number of investigated target markers on a single tissue enriches spatial characterization that may facilitate a more accurate prediction of disease progression and preclinical outcome measures in clinical research projects using tumor biopsies or tissue microarrays (TMAs). Therefore, to increase the plexity of IMC panels, it is essential to expand the number of available metal channels. Here, we demonstrate the incorporation of conjugated antibodies with yttrium (89Y) and indium (115In), two low-mass metals, for IMC application. These metal tags have been previously tested as putative channels for IMC application.

We performed IMC analysis of various tissue types stained with panels of conjugated antibodies including the novel 89Y- and 115In-conjugated antibodies. At least 3 different regions of interest (ROIs) were assessed for each of the investigated tissue types. We compared images for the 89Y- and 115In-conjugated antibodies with the images generated using Maxpar® catalog antibodies of the same clones, with a focus on marker specificity and background signal. Compared with the lanthanide-conjugated catalog antibodies, the 89Y- and 115In-conjugated antibodies showed equivalent specificity and staining quality.

Our results open a new avenue to assign markers to 89Y and 115In, which enables a larger list of potential targets to be investigated in any IMC study. Expanding the number of markers to 40-plus in Imaging Mass Cytometry will improve the imaging results necessary to identify novel cell signatures (phenotype and interactions) in the TME.

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- New channels allow marker assignment to 89Y and 115In metals, opening new research avenues.
- 89Y- and 115In-conjugated antibodies showed equivalent specificity and staining quality compared with the lanthanide-conjugated catalog antibodies of the same clone.
- Moving existing markers to new 89Y and 115In metals (161Dy and 169Tm in this poster) opens 2 existing channels for additional flexibility in user-defined markers.

- Expanding the number of channels/markers to 40-plus in Imaging Mass Cytometry will improve the imaging results necessary to identify novel cell signatures (phenotype and interactions) in the TME.
- More channels allow users to answer more questions with protein markers or RNA (other applications).

P554

HIGH-PLEX CO-DETECTION OF RNA AND PROTEIN TO EXPLORE TUMOUR-IMMUNE INTERACTIONS UTILIZING RNASCOPE WITH IMAGING MASS CYTOMETRY

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The next breakthroughs in immuno-oncology will be driven by high-plex tools that decipher the spatial arrangement of different cell types within the tumor microenvironment (TME). Imaging Mass Cytometry™ (IMC™) is a proven tool for the study of complex cellular interactions in the TME. It utilizes CyTOF® technology for simultaneous assessment of 40-plus protein markers at subcellular resolution without spectral overlap or background autofluorescence, thus providing unprecedented insight into the organization and function of the TME. Despite this, some protein targets are challenging to include in IMC as they have very few or no commercial antibodies available. Moreover, although cellular identity can easily be deciphered through detection of protein targets, knowledge of the cell's

transcriptome improves understanding of cellular function and activation state. Here, we present a robust and reliable workflow that combines the highly sensitive and specific RNAscope™ technology for RNA detection with the multiplexing capability of IMC to visualize key RNA and protein markers in the same tumor samples. The RNAscope HiPlex v2 assay was combined with protein detection using IMC to evaluate expression of both RNA and protein targets in formalin-fixed, paraffin-embedded (FFPE) tumor tissue microarray (TMA).

STEP 1 – Labeling Detection Oligonucleotides With Metal Tags for IMC

STEP 2 – Modified RNAscope HiPlex Flex Assay v2 With Metal-Labeled Probes

STEP 3 – IMC Antibody Staining Overnight

FFPE human lung cancer TMA was processed using the RNAscope HiPlex Assay v2 procedure (found under 3-Step Procedure Overview). Slides were stained with RNAscope HiPlex 12 Positive Control Probe - Hs (ACD, Cat. No. 324311) to identify 12 housekeeping genes.

- Here, we provide a simple 3-step procedure for the co-detection of RNA and protein targets within the same FFPE tumor samples.
- With our procedure, an investigator can visualize 40-plus markers, 12 of which can be used for RNA detection.
- Using RNA co-detection with IMC, we were able to identify proliferating cancer cells with high transcriptional activity. We also identified PD-L1-rich cancer cells, activated T-reg cells, and activated macrophages.

P555

SPATIAL MULTIPARAMETRIC INVESTIGATION OF MOUSE AND HUMAN BRAIN TUMOR MICROENVIRONMENT IDENTIFIES CRITICAL NEURO-ONCOLOGICAL PROCESSES IN BRAIN NEOPLASMS

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Brain cancer research presents challenges that require comprehensive assessment of the structural and cellular organization of the tumor microenvironment (TME). Lack of readily available tissue samples, insufficient multiparametric assessment, and tissue autofluorescence further limit identification of key neuro-oncological processes that dictate disease progression. Imaging Mass Cytometry™ (IMC™) offers unprecedented insight into the TME by uncovering the spatial distribution of 40-plus distinct molecular markers without data artifacts caused by autofluorescence. Here, we present a deep phenotypic spatial analysis of various mouse and human brain tumors and identify cellular composition and activation of immuno-oncological processes within the TME.

Our high-plex brain cell phenotyping antibody panel is designed for imaging application on formalin-fixed, paraffin-embedded tissues. These neural research-specific panels consist of human and mouse cross-reactive clones and are compatible with Maxpar® Human and Maxpar OnDemand™ Mouse Immuno-Oncology IMC Panel Kits. They enable flexible panel design for brain-specific research goals, such as brain tumor classification, and assessment of neuronal inflammation, degeneration, and development. We applied the high-plex antibody panel on tissue microarrays containing a variety of human and mouse brain tumors. Normal brain tissues were used for comparative analysis as controls. The Hyperion™ Imaging System was utilized to digitize images from the tissues followed by quantitative analysis to assess the cellular composition of normal and cancerous brain TME.

We identified major cell populations that make up human and mouse brain matter, such as neurons, astrocytes, microglia, and oligodendrocytes. Various tumor cell phenotypes, resident and infiltrating cells, and resting and activated microglia were detected in multiple tumor subtypes. Additionally, we assessed vascular coverage, extracellular matrix composition, and activation of immune cells within the TME. Subsequent single-cell analysis provided a comprehensive and quantitative assessment of the brain TME in our samples. Our phenotypic analysis resolved the brain TME to the single-cell level and provided insights into the spatial complexity of neuronal neoplasms. We quantified myeloid and lymphoid immune cell infiltration across normal, astrocytoma, and glioblastoma tissues.

A 39-parameter human antibody panel and a 36-parameter mouse antibody panel, both designed to highlight central features of normal and diseased brain tissue, are presented in this poster (middle section).

These panels contain antibodies that identify major cell populations in normal brain and the specific states of tumor and immune cell populations in the diseased brain. The respective high-parameter panels were applied on human and mouse normal and tumor tissues. Tissue slides were prepared and stained using optimized antibody dilutions and were imaged using the Hyperion™ Imaging System at 200 Hz with 1 µm pixel size.

Qualitative data analysis, multiplexed image rendering, and single-channel image extractions were performed using MCD™ Viewer

Our data demonstrates performance of markers with high specificity and signal intensity and absence of autofluorescence data artifacts in human and mouse normal and diseased brain tissue. High-parameter human and mouse neuro-oncology panels were successful in determining the spatial landscape of normal and neoplastic brain tissues.

- Neuro phenotyping panel for Imaging Mass Cytometry consists of 7 cross-reactive markers to successfully identify the cellular composition of human and mouse neuronal tissue.
- Applications of 39-parameter human neuro-oncology panel and 36-parameter mouse neuro-oncology panel distinguish critical pathophysiological changes in human and mouse glioblastoma TME composition.

P556

IDENTIFYING PATHOPHYSIOLOGICAL FEATURES OF MOUSE TUMORS USING IMAGING MASS CYTOMETRY

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An obstacle in predicting therapeutic drug efficacy is the ability to quantitatively evaluate the multi-parametric post-treatment response in the tumor microenvironment (TME). Identification of immunological processes that dictate tumor growth, metastasis, and immune response is essential for selecting promising drug candidates for further clinical evaluation. Imaging Mass Cytometry™ (IMC™) is a vital and proven high-plex imaging technology that enables deep characterization of the complexity and diversity of tumor tissue without disrupting spatial context. The Hyperion™ Imaging System utilizes IMC technology to simultaneously assess 40-plus individual structural and functional markers in tissues, providing unprecedented insight into the organization and function of the TME. Here, we showcase the Maxpar® OnDemand™ Mouse Immuno-Oncology IMC Panel Kit for application on a variety of mouse tumor tissues.

We compiled the antibody panel to quantitatively assess IO-related processes and applied it to a tissue microarray (TMA) containing a large variety of mouse tumors. We digitized high-plex data from mouse tissues using IMC and generated images demonstrating the detailed layout of the TME. We further conducted single-cell analysis to identify specific populations of tumor and immune cells in the TME.

This approach successfully identified pathophysiological processes such as immune cell infiltration and activation, signaling pathway activation, biomarkers of epithelial-to-mesenchymal transition (EMT), metabolic activity, growth, and the tissue architecture of the TME. Single-cell analysis of several highly relevant tumor types separated distinct cellular clusters representing tumor, immune, stromal, and vascular cells. Activation of cellular processes associated with signaling, growth, and metastasis were identified in tumor cells. In addition, cytotoxic and inflammatory activation in lymphoid and myeloid immune cell subtypes were detected.

Application of IMC based multiparametric analysis successfully identified the spatial landscape of the TME at single-cell resolution.

A 33-parameter antibody panel, designed to highlight central features of the mouse TME, is presented in this poster. This panel consists of a combination of 4 inter-compatible mouse IMC panel kits, with each kit assembled to reveal critical insights about tumor biology. The full panel was applied on mouse tumor tissue microarray (TMA) containing a wide variety of tumor types as well as on normal mouse TMA. Normal and tumor TMA slides were prepared and stained using optimized antibody dilutions. All antibodies were titrated and tested on positive control tissue (spleen, colon, lung) alongside tumor samples. Tissue slides were ablated using the Hyperion™ Imaging System. Qualitative data analysis, multiplexed image rendering, and single-channel image extractions were performed using MCD™ Viewer. IMC and singlecell analysis workflow is summarized in figures below.

The analysis presented showcases the ability of the Maxpar OnDemand Mouse Immuno-Oncology IMC Panel Kit to uncover the spatial landscape of the TME on a cellular level. Our results show the localization of specific tumor and immune cell populations in 4 distinct tumor types. Overall, data shown here provide evidence of panel kit performance on mouse tumor tissues and reveal the potential of IMC based multiplexed analysis to accelerate preclinical drug discoveries and precision medicine.

Preclinical cancer therapeutics often fail in the clinic due to unavailability of holistic data about their precise effect on tumors.

By unleashing the power of IMC technology, it is possible to generate high-quality high-plex

quantitative data with respect to key tumor parameters, enabling researchers to create comprehensive tumor atlases and make high-confidence decisions about therapeutic drug targets for further clinical evaluation

P557

UNCOVERING T CELL CLONALITY IN RESPONSE TO IMMUNE CHECKPOINT INHIBITORS IN TRIPLE NEGATIVE BREAST CANCER

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Triple negative breast cancer (TNBC) is an aggressive subtype of breast cancer associated with poor prognosis. The immune checkpoint inhibitors (ICIs) are approved for use in TNBC, but fail to recapitulate the remarkable responses achieved in other cancer types. TNBC can display a number of features that have been previously associated with response to ICI, including PDL1 expression and robust intratumoural T cell infiltration. However, only 38% of patients with increased PDL1 expression will experience tumor shrinkage and PDL1 expression does not predict response to ICI in the neoadjuvant setting. These observations provide solid reasoning to study the immune response to TNBC and ICI therapy directly, rather than extrapolate what is known from other cancer types. Understanding why TNBC is recalcitrant to ICI therapy is crucial for improving TNBC patient outcomes.

Our aims were as follows

1. Generate a single cell map of TNBC ICI response and resistance
2. Uncover T cell clonal dynamics in two models of TNBC ICI response and resistance

We hypothesised that

1. The cellular milieu of the tumour or tumour draining lymph node can be associated with ICI response
2. T cell clonal expansion can be associated with response to ICI therapy in TNBC.

Single cell and spatial transcriptomics allow us to interrogate the cancer cells, the tumour microenvironment and the immune system concurrently. Murine models of TNBC enable experimental flexibility and a sampling approach that would be near impossible to achieve in human samples. We used two well-established, syngeneic mouse models of TNBC: the EMT6 model, which responds robustly to ICI, and the 4T1 model, which does not. Both of these models express similar levels of MHC I, PDL1 and are well infiltrated with T cells.

To generate a snapshot of the response to ICI as it was occurring, mice were treated with two doses of either combination ICI (anti-PD1+anti-CTLA4), monotherapy (anti-PD1 or anti-CTLA4), or isotype control antibodies prior to sample collection. The tumour and tumour draining lymph nodes (TDLN) were collected and processed for 5' single cell RNA sequencing using the 10x Genomics Chromium system. A section of the tumour was taken prior to dissociation for 10x Visium spatial transcriptomics analysis.

Comparing the control treated samples revealed a strikingly similar cellular composition of both models, disproving our first hypothesis and suggesting a cellular biomarker of ICI response may be challenging to derive. We then focused on the T cell compartment, comparing combination ICI and control antibody samples. Remarkably, even at the T cell level, the cellular composition of control treated samples from both EMT6 and 4T1 tumour bearing mice were essentially indistinguishable. Analysis of the TCR repertoire was more fruitful. CD8+ effector cell clonal expansion was observed in the repertoire of ICI treated EMT6 tumour bearing mice, but not in control treated mice or 4T1 tumour bearing mice, supporting our hypothesis. In all models and conditions, varying degrees of T regulatory and T follicular helper cell clonal expansion was observed. Within EMT6 ICI treated samples, we found that CD8+ effector cell clonal expansion was driven by a dominant clone and this clone was found in the primary tumour and the TDLN.

To the best of our knowledge, we have generated the first single cell map of murine TNBC response to ICI that includes T cell repertoire data. This dataset incorporates multiple tissues, timepoints and treatment arms from two murine breast cancer models with polar opposite responses to ICI.

Intriguingly, both models shared a very similar immune milieu, but single cell TCR profiling revealed that CD8+ effector cell clonal expansion can indeed be associated with response to ICI in TNBC. This provides important evidence for CD8+ clonal expansion as a biomarker of ICI response. Analysis of this data set is ongoing, and in future will incorporate spatial transcriptomic analyses.

P558**ADOPTIVE CELL THERAPY WITH GENETICALLY ENGINEERED T-CELLS FOR OVARIAN CANCER**

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Epithelial ovarian carcinoma (EOC) is the deadliest gynecologic disease threatening women's health. Although several studies have demonstrated a positive correlation between the prognosis of EOC and the extent of tumor-infiltrating effector T lymphocytes (TILs), the clinical benefit of immunotherapies that promote TILs-such as immune checkpoint inhibitors (ICIs)-is limited by the presence of multiple immunosuppressive mechanisms within the ovarian tumor microenvironment (TME). Indeed, during cancer progression, tumor-specific T cells have been shown to display increased, chronic expression of multiple inhibitory receptors (IRs), including PD-1, LAG-3, and TIM-3, which causes their functional exhaustion and unresponsiveness. We hypothesize that protecting T cells by gene editing from immunosuppression in the tumor microenvironment can maximize their therapeutic potential upon adoptive transfer.

We characterized ovarian cancer cell lines and primary cultures derived from EOC patients' ascites using polychromatic high dimensional flow cytometry to evaluate the expression of selected tumor-associated antigens and membrane-bound IR ligands. In addition, we evaluated the expression of membrane-bound and secreted IR ligands on primary cultures after 48h interferon γ (IFN γ) treatment by flow cytometry and ELISA assay. We selected the most suitable TCRs for ovarian cancer cells from our established TCR library. We focused on HD1-TCR, specific for the WT-1 (37-45) epitope presented on HLA-A*02:01. We tested different T cell products all engineered to express the HD1-TCR upon Knock out (KO) of the endogenous TCR genes, and harboring gene disruption of three distinct IRs, namely LAG-3, TIM-3 or 2B4: [HD1-TCR; LAG3KO HD1-TCR; TIM-3KO HD1-TCR; 2B4KO HD1-TCR]. To functionally characterize the putative advantage provided by the IR disruption on the anti-tumor efficacy of the engineered T cells, HD1-TCR and HD1-IRKO gene-edited T cells were challenged with WT-1pos HLA-A*02:01pos cancer cell lines, primary cultures derived from patients' ascites and tumor derived organoids (PDOs). As readout for the functional assays, we evaluated cytokine secretion, T cell activation, and apoptosis induction through IncuCyte live cell imaging and flow cytometry analysis.

We identified a high and consistent expression of WT-1 antigen, while a low percentage of tumor cells expressing membrane-bound IR ligands was observed. Of interest, an increased expression of the IR ligands upon a 48h exposure of primary cultures to IFN γ , was evaluated. We assessed T cell functionality by co-culture of engineered T cells with tumor cells. We observed a similar ability of our T cell products in killing ovarian cancer cell lines. On the contrary, we observed a significant increase in the activation profile of 2B4KO HD1-TCR compared to HD1-TCR edited T cells on 5 different WT-1pos HLA-A2pos primary cultures. More potent recognition was observed with TIM-3KO and 2B4KO HD1-TCR edited-T cells although data were variable between tumor samples. We hypothesized that the differences in benefit were due to differential IR ligands expression levels and therefore treated EOC primary cultures with IFN γ . When challenged with IFN γ treated EOC primary cultures, we observed an enhanced activation profile and killing ability with all T cell products. Of note, TIM-3KO HD1-TCR edited T cells showed more potent killing of EOC primary cultures expressing TIM-3 IR ligands (Galectin 9; HMGB1) compared to the other T cell products. Finally, when challenged for 48 hours with ovarian cancer-derived organoids, we observed an enhanced efficiency of TIM-3KO and

LAG-3KO HD1-TCR T cells in inducing apoptosis compared to HD1-TCR T cells without IR disruption.

HD1-IRKO T-cell products appear superior to HD1-TCR IR-competent counterparts in recognizing and killing WT-1pos HLA-A2pos primary ovarian cancer cells in vitro. The most consistent advantages were observed with TIM-3KO HD1-TCR.

P559

CHEMOKINES EXPRESSED BY ENGINEERED BACTERIA RECRUIT AND ORCHESTRATE ANTI-TUMOR IMMUNITY

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Tumors employ multiple mechanisms to actively exclude or suppress adaptive immune cells involved in anti-tumor immunity.

Strategies focused on overcoming these immunosuppressive or exclusion signals – through localized delivery of chemokines that directly recruit immune cells into the tumor microenvironment – remain limited due to an inability to target therapeutics specifically to the tumor. Synthetic biology enables engineering of cells and microbes for tumor localized delivery, offering therapeutic candidates previously unavailable using conventional systemic administration techniques. We have previously developed a synthetic gene circuit in bacteria that results in bacteria undergoing cycles of growth and synchronized lysis in vitro and in vivo, termed the synchronized lysis circuit (SLC).

Here, we utilized the SLC and engineered bacteria to produce and intratumorally release chemokines to attract adaptive immune cells into the tumor environment.

We first generated a strain of probiotic *E. coli* Nissle (EcN) producing the chemokine CXCL16 and found that lysate of EcN expressing an activating mutation of human CXCL16 (hCXCL16K42A) recruits activated mouse and human T cells in vitro. Furthermore, intravenous or intratumoral delivery of EcN co-expressing the SLC and hCXCL16K42A (SLC-hCXCL16K42A) led to the recruitment of activated T cells within tumors and offered therapeutic benefit in multiple mouse tumor models. The therapeutic efficacy of SLC-hCXCL16K42A was dependent on the presence of CD8+ T cells and the egress of T cells from secondary lymphoid tissues, suggesting that SLC-hCXCL16K42A acted through recruitment of CD8+ T cells into the tumor. Furthermore, we rationally targeted an additional step in the immune activation cascade – specifically, the presentation of tumor-derived antigens by dendritic cells – using a second engineered EcN strain expressing CCL20. This combined targeting approach led to the recruitment of type 1 conventional dendritic cells and effectively synergized with hCXCL16K42A-induced T cell recruitment to provide additional therapeutic benefit.

In summary, we engineered bacteria to cooperatively recruit and activate both innate and adaptive anti-tumor immune responses, offering a new cancer immunotherapy strategy.

P560

MULTIMODAL ANALYSIS OF THE TUMOR MICROENVIRONMENT IN GASTROESOPHAGAL ADENOCARCINOMA IDENTIFIES A STRONG CORRELATION OF T CELL TISSUE RESIDENCY WITH CLINICAL OUTCOME.

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Gastroesophageal adenocarcinoma (GEAC) incidence has significantly increased worldwide over the last 40 years and remains the third-leading cause of mortality worldwide. The high resistance to chemo- and radiotherapy as well as the overall poor clinical outcome makes GEAC a largely unmet clinical need. GEAC is a heterogeneous disease, encompassing several subtypes defined either at the molecular or histological level. The integration of markers predictive of antitumor immune response has proved valuable for patient classification and treatment selection in other malignancies, such as colorectal cancer. The addition of immune characterization has provided fundamental insights into disease pathogenesis and allowed the identification of new therapeutic targets like immune checkpoint blockade.

CD8⁺ T cells exert robust anti-tumor responses against several solid tumors, and their tumor infiltration is correlated with improved survival and better response to conventional therapy as well as immune checkpoint blockade. In GEAC the clinical relevance of specific immune profiles is still poorly understood, and studies focused on the extent of T cell heterogeneity in the tumor microenvironment are limited. Here, our aim is to provide a comprehensive view of the global immune landscape in GEAC, and to specifically characterize the phenotype of CD8 T cells in GEAC patients.

Using high dimensional flow cytometry, we quantified the infiltration of nearly all immune populations in matched healthy and tumor samples from 15 treatment naïve GEAC patients. Extended analysis of RNAseq data, proteomic data, and functional assays to validate this hypothesis are currently ongoing.

Our data show that T cells dominate the gastroesophageal immune microenvironment both within healthy and tumor biopsies, representing up to 94% of total immune cells. We observed an increased CD4/CD8 ratio in the tumor biopsies as compared to healthy biopsies. We observed no significant changes to the myeloid or B-cell compartment. A closer analysis of the T cell phenotype highlighted a reduction in effector memory T cells and a further reduction in CD8⁺(CD69+CD103+CD49a⁺) tissue resident (TR) cells. In contrast, circulating CD8⁺(CD69-CD103-CD49a⁻) T cells and central memory CD4⁺ T cells were increased in tumor samples. Importantly, TR CD8⁺ T cell tumor abundance was positively associated with patient survival, while an opposite correlation was observed for circulating CD4 and CD8 T cells.

These findings indicate that the local alterations in T cell phenotype, and even the selective exclusion of TR CD8 T cells, could have decisive effects on the general antitumor immune response.

P561

STROMAL LOCALIZATION OF INACTIVE CD8+ T CELLS IN METASTATIC MISMATCH REPAIR DEFICIENT COLORECTAL CANCER

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The determinants of metastasis in mismatch repair deficiency with high levels of microsatellite instability (MSI-H) in colorectal cancer (CRC) are poorly understood. Here, we hypothesized that distinct immune and stromal microenvironments in primary tumors may discriminate between non-metastatic MSI-H CRC and metastatic MSI-H CRC.

We profiled 46,727 single cells using high-plex imaging mass cytometry and analyzed both differential cell type abundance, and spatial distribution of fibroblast and immune cells in primary CRC tumors with or without metastatic capacity. We validated our findings in a second independent cohort using immunohistochemistry.

High-plex imaging mass cytometry and hierarchical clustering based on microenvironmental markers separated primary MSI-H CRC tumors with and without metastatic capacity. Primary tumors with metastatic capacity displayed a high stromal content and low influx of CD8+ T cells, which expressed significantly lower levels of markers reflecting proliferation (Ki67) and antigen-experience (CD45RO) compared to CD8+ T cells in non-metastatic tumors. CD8+ T cells showed intra-epithelial localization in non-metastatic tumors, but stromal localization in metastatic tumors, which was validated in a second cohort.

We conclude that localization of phenotypically distinct CD8+ T cells within stroma may predict metastasis formation in MSI-H CRC and may pave the way for developing novel immunotherapeutic strategies.

P562

INHIBITION OF THE HISTONE METHYLTRANSFERASE EZH2 ENHANCES PROTUMOR MONOCYTE RECRUITMENT IN HUMAN MESOTHELIOMA SPHEROIDS

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Diffuse pleural mesothelioma (DPM) is a highly aggressive cancer with a long latency period and dismal prognosis. The histone methyl-transferase Enhancer of zeste homolog 2 (EZH2), a well-known oncogenic driver in different malignancies, is often overexpressed in DPM and associated with worst outcome. Accordingly, pharmacological EZH2 inhibition showed significant anti-tumor activity in DPM models overexpressing EZH2 because of BAP1 loss. Nevertheless, the response rate of patients with relapsed or refractory BAP1-inactivated mutations to the EZH2 inhibitor tazemetostat was modest, indicating that a better understanding of the mechanisms that sensitize cancer cells to EZH2 inhibitors are urgently needed. In particular, the direct and indirect effects of EZH2-dependent epigenetic reprogramming on the tumor microenvironment (TME) are hitherto unexplored. Macrophages are key orchestrators of both early tumor-promoting inflammation in response to asbestos fibers and immunosuppression at the advanced stage of MPM. Therefore, we evaluated the effects of EZH2-dependent epigenetic reprogramming on accumulation and activation of macrophages in association with mesothelioma cell responsiveness to tazemetostat.

We cultured human biphasic MSTO-211H cells in low adherence conditions to generate a three-dimensional (3D) multicellular spheroid (MCS) and we evaluated whether MCSs were able to recruit primary human monocytes (confocal microscopy analysis) and to induce their differentiation toward a TAM-like phenotype (gene expression analysis by RT-PCR). We used MCS to evaluate the effects of EZH2-dependent epigenetic reprogramming on TAM accumulation (confocal microscopy) and activation (RT-PCR), hence the impact of TAMs on mesothelioma cell responsiveness to EPZ-6438 in terms of tumor cell proliferation and spreading.

We developed a 3D mesothelioma spheroid model that recapitulates in vitro, both monocytes' recruitment in tumors and their functional differentiation toward a TAM-like phenotype (Mo-TAMs). Along with an increased expression of genes for monocyte chemoattractants, inhibitory immune checkpoints, immunosuppressive and M2-like molecules, Mo-TAMs promote tumor cell proliferation and spreading. Prolonged treatment of MPM spheroids with tazemetostat enhances both the recruitment of Mo-TAMs and the expression of their protumor phenotype. Therefore, Mo-TAMs profoundly suppress the antiproliferative effects due to EZH2 inhibition in mesothelioma cells. M1-polarized monocytes release soluble factors capable of killing different mesothelioma cells cultured as monolayer, but not as 3D MCS.

Our findings indicate that TAMs are a driving force for mesothelioma growth, progression and resistance to tazemetostat. Therefore, to improve the therapeutic efficacy of pharmacological inhibition of EZH2, combination strategies targeting TAM with tazemetostat warrant to be evaluated. Given that tazemetostat enhances TAM accumulation, strategies of their reprogramming in M1 antitumor effectors would ideally work in synergism with EZH2 inhibitors. Additionally, different strategies to eliminate TAMs selectively have been significantly advanced toward the clinic and have been preliminarily evaluated as single agents in mesothelioma, thereby supporting their potential use in combination with tazemetostat.

P563

A HUMANIZED 3D LYMPH NODE MICROENVIRONMENT TO STUDY RESPONSE TO RETINOIC ACID SIGNALING INHIBITORS IN CHRONIC LYMPHOCYTIC LEUKEMIA

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The microenvironment of secondary lymphoid organs is composed of various cell types, including stromal cells, which play a critical role in providing signals to promote activation, proliferation, differentiation, and survival of normal and malignant hematopoietic cells, such as chronic lymphocytic leukemia (CLL). We have previously demonstrated the presence of a retinoic acid-dependent stromal leukemia crosstalk that promotes the progression of CLL in mice. Whether this signaling pathway also plays a role in human CLL is still unknown. In malignant lymphoid diseases, several studies have shown that assessing the response of neoplastic cells to pharmacological therapies targeting signaling pathways using conventional 2D cultures does not fully predict drug efficacy in patients. Therefore, three-dimensional (3D) immune platforms that mimic the microenvironment of lymphoid tissue, although still underdeveloped, are of great importance as they offer the possibility to study in vitro both pathogenic mechanisms and response to therapy under more physiological conditions. To this end, we created a humanized 3D lymph node-like microenvironment to study the role of retinoic acid signaling in human stroma-CLL crosstalk.

For our studies, we used dynamic 3D cultures of human lymph node stromal cells (fibroblasts) and primary chronic lymphocytic leukemia cells, flow cytometry, immunofluorescence analysis and confocal microscopy.

We first mimicked the composition and structure of the human lymph node (LN) using porous, biocompatible scaffolds on which human LN stromal cells were seeded and maintained in long-term culture using a millifluidic bioreactor system. Immunofluorescence analysis performed at different time points of culture showed that the stromal cells grew efficiently and formed a LN-like reticular cell network with extracellular matrix deposition. We then performed 3D long-term cultures with the addition of human primary CLL cells. Immunofluorescence analysis combined with confocal imaging and flow cytometry showed that CLL cells adhered to the stromal cells, were homogeneously distributed, and survived in culture for up to four weeks. To test the role of retinoic acid (RA) signaling in stroma-leukemia crosstalk, we performed experiments with an inhibitor of RXR-alpha, a RA nuclear receptor that we previously found to be overexpressed in human CLL as compared to control B cells. The results showed that targeting RXR-alpha signaling impacted on CLL adhesion to stromal cells and caused the release of neoplastic cells from 3D lymphoid microenvironment.

By replicating a LN-like 3D microenvironment that mimics the spatial and cellular organization of stroma and leukemic cells in secondary lymphoid organs, we demonstrated a role for RA signaling in human CLL.

P564

NOVEL MECHANISMS OF MICROBIOTA REGULATION OF ANTI-TUMOR IMMUNITY

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The intestinal microbiota has been shown to influence the efficacy of cancer immunotherapy, supporting therapeutic manipulation of the microbiota as a new cancer therapy. However, a mechanistic understanding of how the microbiota influences anti-tumor immunity is essential for realizing this potential. Although it is well established that the microbiota generates metabolites that influence immune cell function, whether and how this metabolite mediated modulation influences anti-tumor immunity is poorly understood.

To that end, the Hang laboratory has demonstrated that enzymes produced by select commensal bacteria generate molecules that increase the efficacy of cancer immunotherapy. This confirms that the microbiota is a source of immunomodulatory compounds that directly influence anti-tumor immunity. To gain a fuller understanding of this interaction, it is important to characterize whether and how other metabolites derived from the microbiota influence immune cell function, specifically in the context of anti-tumor immunity. Here, we begin to investigate how microbiota derived bile acids influence anti-tumor T cell responses.

Using chemical probes, we identify several new candidate protein targets of bile acids. We assay the effect of bile acids on the function of these proteins.

Bile acids potentially regulate anti-tumor immunity through previously unappreciated pathways.

P565

T-CELL RECEPTOR REPERTOIRE ANALYSIS: A STRATEGY FOR THE DIAGNOSTIC & THERAPEUTIC POTENTIAL ENHANCEMENT IN LEUKEMIA

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Leukemia is a hematological malignancy of hematopoietic origin with an increasing prevalence at a global level. It has been ranked as the fifth type of cancer in Saudi Arabia among both genders according to the Saudi Cancer Registry (SCR). Although some types of leukemia are fatal, they are treatable and usually curable with early diagnosis and proper treatment regimens. It is important to understand the relationship between the disease pathogenesis and the host immune responses to minimize the use of suboptimal regimens, such as non-specific diagnosis. Changes in the immune repertoires have been reported in response to cancer. There is an increasing evidence that high throughput sequencing of TCR repertoire can serve as a rich source of potential diagnostic biomarkers and therapeutic agents as well as a potential biomarker of immune response monitoring in cancer patients. It can potentially provide valuable and compelling information on the immunological status of adaptive immunity not only during the disease but also post therapeutic interventions. Immune response tracking and monitoring is very important in cancer patients where relapse is the most common cause of treatment failure as seen in acute lymphoblastic leukemia (ALL). The main aim of this research is to fingerprint the T-cell immune responses at a higher resolution in a cohort of patients with acute lymphoblastic leukemia (ALL) at different treatment stages by profiling the T-cell receptor repertoires, using high throughput sequencing of immune repertoire technology and computational pipeline for quantitative analysis.

We isolated total RNA from PBMCs of 25 patients with ALL (newly diagnosed, n=8; remission, n=5; relapse, n=7) as well as normal healthy controls (n=5) and profiled their TCR repertoire using high throughput sequencing. Briefly, RNA samples were converted into cDNA, and then, they were modified to incorporate unique molecular identifiers, indices and sequencing adaptors. Then, TCR α - and β -chains were sequenced. The sequencing data were analyzed and processed using a computational tool that identified the numbers of sequenced TCRs, determined their frequency distribution and measured diversity in the TCR repertoire. The immune repertoire data were combined with the patient's clinical data.

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This study provides an insight into the adaptive immune status and determines the diversity of TCR repertoire in ALL patients. Characterizing the diversity of the TCR repertoire could be utilized to monitor the immune response in ALL patients. Profiling the immune repertoire could predict the disease severity and facilitate the development of effective treatment strategies

P566

FIBROBLASTIC RETICULAR CELLS INDUCE STEM CELL MEMORY T CELLS DIFFERENTIATION FROM ACTIVATED T CELLS

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An immune niche is defined as a special environment that provides spatial connections between different cellular subsets that, surrounded by extracellular matrix, cytokines, and chemokines produced by stromal cells, is essential for cell homeostasis, self-renewal, or differentiation (Jones DL, Nat Rev Mol Cell Biol, 2008). There is growing evidence that T cells require specific niches to maintain their long-lived memory functions and respond effectively to immunotherapy (Gattinoni L, Nat Med 2011; Di Rosa F, Trends Immunol, 2016). In secondary lymphoid tissues, different stromal cells provide distinct signals through the release of chemokines and cytokines to maintain T cell distribution and survival within the LN under physiological conditions and to support T cell differentiation under pathological conditions (Von Andrian UH, Nat Rev Immunol, 2003). Among them, fibroblastic reticular cells (FRCs) also produce in vivo survival and proliferation factors such as IL-7 and IL-15, which are known to promote the differentiation of T cells into stem cell memory T cells (Tscm), a cell type that has anti-tumour function after transplantation (Gattinoni L, Nat Med, 2011). Whether FRCs alone are able to promote Tscm differentiation or whether other cells are required for this process remains unclear. To clarify this, we investigated the contribution of FRCs isolated from different LN districts to induce the differentiation of T cells into Tscm cells.

LNs were harvested from newborn mice and stromal cell lines established. Antigen-specific T cells were derived from the spleens of Pmel-1 transgenic mice genetically modified with T cell receptors that recognize gp100, an antigen that is highly overexpressed by melanoma cells. Pmel-1 splenocytes or Pmel-1 naive T cells were activated in vitro in the presence of appropriate stimuli and then cultured in the presence of stromal cells. Flow cytometry was used to determine the phenotype of T cells at different time points during culture.

We first established stromal cell lines from peripheral and mesenteric LN districts. Flow cytometry analysis revealed that all FRC lines were positive for GP38/PDPN, PDGFR α , indicating a phenotype consistent with T-zone stromal cells. We then performed co-culture experiments with FRCs and Pmel-1 transgenic T cells recognizing gp100 melanoma antigen. After activation, Pmel-1 T cells were cultured with FRCs or stimulated with IL-7/ IL-15 to determine their phenotype. Flow cytometry performed 3 and 5 days after co-culture showed a higher frequency of Tscm in the presence of FRCs. Notably, FRCs from mesenteric LN were superior to FRCs from peripheral LNs in promoting Tscm, further supporting the notion that FRCs from different LNs districts may have distinct functions.

Our findings revealed that FRCs alone are efficient in supporting the generation of Tscm and provided evidence that FRCs from different LN districts may have different ability to promote T cell differentiation.

P567

ORGAN-ON-CHIP-BASED IN VITRO APPROACHES FOR CO-CULTURING 3D HUMAN CANCER TISSUES AND IMMUNE CELLS FOR MORE PREDICTIVE DRUG TESTING AND HUMAN DISEASE MODELING

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The human disease modeling for basic research and drug testing purposes is currently carried out through 2D cell culture in static conditions, and in vivo xenografts or genetically engineered animal models, but predictability, reliability, and complete immune compatibility remain important challenges. For this aim, novel 3D, fully humanized in vitro cancer tissue models have been recently optimized by adopting emerging technologies such as microphysiological systems (MPS) and 3D cell laden hydrogels. In particular, a novel Multi-In Vitro Organ (MIVO) MPS platform has been recently adopted to culture 3D clinically relevant size cancer tissues under proper physiological culture conditions to investigate the efficacy of anticancer treatments.

Biologically relevant cancer samples (up to 5 mm), have been developed by using a alginate base structure, resembling the extracellular matrix. The stiffness of such gels has been optimized to support a in vivo like tumor cells viability, cluster formation and migration. Ovarian and ovarian cell laden hydrogels (1 million cells/ml) have been cultured within the MIVO chamber, while either testing molecules (cisplatin) or human immune cells (Natural Killer cells, NK) respectively circulate in the MPS mimicking the blood capillary flow. The tumor cell proliferation and viability were investigated in such dynamic cell culture conditions to assess the cytotoxic efficacy of the treatment. When the systemic administration of cisplatin was simulated within the MPS, the anticancer drug efficacy was also tested and compared to the animal model. When NK cells were placed in circulation, their extravasation through a permeable barrier resembling the vascular barrier, and infiltration within the neuroblastoma cancer tissue were analyzed.

A human 3D ovarian model was developed and treated with Cisplatin in static conditions, within MIVO, and in the xenograft model. Similar tumor regression was observed in MIVO and in mice, while the static culture displayed an unpredictable chemoresistance, due to unreliable drug diffusion within the 3D matrix. A human 3D neuroblastoma cancer model with proper immunophenotype was optimized to develop a complex tumor/immune cell coculture as a paradigm of an immune-oncology screening platform. Importantly, a tumor-specific NK cell extravasation was observed under dynamic culture, with NK cells able to infiltration within the 3D tumor where they induced cancer cells apoptosis.

We generated a relevant human disease model, through the adoption of a MPS system, that can be efficiently employed as a drug screening platform but also for better investigating crosstalk among immune /tumor cells.

P568**EXPLORING FERROPTOSIS IN T CELLS: IMPLICATIONS FOR CANCER IMMUNOTHERAPY**

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Cancer immunotherapy has emerged in recent years as a promising approach to combat various malignancies. One of the most groundbreaking immunotherapeutic strategies developed for cancer treatment involves the use of T cells genetically engineered to express a chimeric antigen receptor (CAR). CAR-T cells can target and eliminate cancer cells when they encounter specific antigens. Although very effective, CAR-T therapies still face many challenges in the eradication of tumor cells. One of the reasons is a hostile, immunosuppressive tumor microenvironment (TME) characterized by elevated ferroptosis. Ferroptosis is an iron-dependent oxidative cell death driven by excessive peroxidation of membrane lipids. It has primarily been studied in the context of cancer cells, particularly those resistant to conventional modes of treatment. However, how immune cells respond to pro-ferroptotic signals is not clearly explained. Emerging data suggests that the TME may sensitize immune cells to ferroptosis, thus compromising their effector capabilities and establishing a significant metabolic barrier to effective tumor eradication. Thus, our research goal was to investigate the sensitivity of T cells and CAR-T cells to ferroptosis and to explore the impact of ferroptosis on their functions and antitumor cytotoxic activity.

T cells were isolated from peripheral blood mononuclear cells using magnetic separation and stimulated or not with anti-CD3/CD28 Dynabeads and IL-2. For CAR-T study, T cells were transduced with lentiviral vectors encoding CD19- or HER2-CAR. To assess the viability of T and CAR-T cells upon treatment with ferroptosis-inducing compounds (RSL3 and ML162), in subsequent weeks of cell culture, the propidium iodide flow cytometric assay was employed. Liproxstatin-1, a selective inhibitor of ferroptosis, was used to reverse ferroptotic cell death. Additionally, markers characteristic of ferroptosis, including reactive oxygen species level, membrane lipid peroxidation, and the labile iron pool, were analyzed by flow cytometry with specific fluorescence probes. The effect of RSL3 on CAR-T cell activation, their ability to produce cytokines and undergo degranulation was measured by flow cytometry. CAR-T cell cytotoxicity against target cells upon induction of ferroptosis was assessed by the impedance-based xCELLigence RTCA. Expression levels of key ferroptosis regulators, such as GPX4, FSP1, and ACSL4 were determined through Western blotting.

Our results reveal that long-term culture of stimulated T cells and CAR-T cells leads to the accumulation of pro-ferroptotic events such as increased lipid peroxidation, labile iron pool level, and ROS production. This potentially compromises T/CAR-T cells' antioxidant defense, rendering them susceptible to ferroptosis. We have also shown that inhibiting GPX4 activity, either alone or in combination with FSP1, induces ferroptosis in long-term stimulated T cells, underscoring their essential role in protecting against peroxidation damage. Furthermore, our findings indicate that induction of ferroptosis in CAR-T cells inhibits their function and antitumor cytotoxic activity, an effect that has not been described before.

In conclusion, our research supports the notion that ferroptosis plays an important role in regulating the functions of immune cells. While the induction of ferroptosis promotes tumor cell death, it can also cause potent immune suppression. Our observations demonstrate that ferroptosis not only affects the activity of cytotoxic T cells but also may impair the anti-tumor efficacy of adoptive CAR-T cell-based therapies. Therefore, the ambiguous role of ferroptosis in cancer, as well as diverse sensitivities and specific responses of all cell types within the tumor microenvironment should be thoroughly considered when designing novel therapeutic strategies.

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P569

SYNERGISTIC EFFECTS OF MESOTHELIN-DIRECTED CAR T CELLS AND A CD40 AGONIST IN SYNGENEIC MOUSE MODELS OF PDAC AND TNBC

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This study explores the synergistic potential of combining mouse mesothelin-specific chimeric antigen receptor (mmeso-CAR) T cells and a CD40 agonist (α CD40) to enhance CAR T cell and overall immune response against pancreatic ductal adenocarcinoma (PDAC) and triple-negative breast cancer (TNBC) in syngeneic mouse models.

The subcutaneous syngeneic PDAC model was established using a KPC-derived cell line, while for the syngeneic TNBC model, the E0771 C57BL/6 line transduced with mouse mesothelin was orthotopically implanted in the mammary fat pad. Mice received mmeso-CAR T cells, α CD40, or combinations of both. Therapeutic efficacy was evaluated in endpoint and time course models, monitoring tumor volume and histological changes over time, and assessing CAR T cell and immune cell distribution and activation in secondary lymphoid organs (SLOs): tumor draining lymph node (TdLN) and spleen, and the tumor microenvironment (TME). Our methodology encompassed real-time live cell assays, in vivo imaging, multiplex cytokine assays, multi-parametric flow cytometry, histology, RNAscope, and scRNAseq.

Combining mmeso-CAR T cells with α CD40 yielded improved tumor control and long-term survival outcomes against both tumor targets and notable memory response post re-challenges. In the PDAC model, α CD40 treatment induced significant tumor necrosis within 24 hours, with or without mmeso-CAR T cells. The necrotic effect persisted after seven days when combined with mmeso-CAR T cells, associated with a greater reduction in tumor weights and PanCK/mesothelin+ tumor areas. The early α CD40-mediated tumor necrosis was also observed in the TNBC model where mmeso-CAR T cells and α CD40 combination therapy confirmed to synergize in mediating a higher relapse rate compared with monotherapies. α CD40 treatment promoted the expansion of mmeso-CAR T cells and modulated their distribution and activation in SLOs and within the TME. The combination therapy engaged APCs, host T and NK cells, increasing recruitment and activation in SLOs and in the TME, associated with elevated blood levels of pro-inflammatory cytokines and chemokines. ScRNAseq analysis on immune cells from tumor and TdLN of the PDAC model confirmed the involvement of both CAR-dependent and independent antitumor responses.

This study unveils the synergistic antitumor effects of combining mmeso-CAR T cells with α CD40 in syngeneic PDAC and TNBC models. The combination therapy led to rapid and sustained tumor necrosis with increased infiltration and activation of immune cells in the SLOs and the TME and overall, a better tumor control. Our comprehensive characterization provided valuable mechanistic insights into the underlying synergistic mechanisms. Our evaluation of the mmeso-CAR T cells/ α CD40 therapy in two different syngeneic models of PDAC and TNBC confirmed the effectiveness in various solid tumors expressing mesothelin providing valuable mechanistic insights into the underlying synergistic mechanisms. These findings may open potential applications of meso-CAR T cells/ α CD40 combination therapy in the clinic.

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NEUTROPHILS MEDIATE PROTECTION AGAINST COLITIS AND CARCINOGENESIS BY CONTROLLING BACTERIAL INVASION AND DRIVING IL-22 PRODUCTION BY GAMMA DELTA T CELLS.

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Neutrophils play an important role in the innate immune response and represent the first line of defense against invading microorganisms. In addition to their antibacterial effector functions, neutrophils have emerged as a crucial cell type involved in modulating immune and inflammatory responses in various pathological conditions, including cancer.

Colorectal cancer (CRC) ranks as the third leading cause of cancer related deaths worldwide, and patients with ulcerative colitis (UC) are at increased risk of developing colitis-associated CRC (CAC). The pathogenesis of CAC involves multiple factors, such as genetic alterations, environmental conditions, including variations in intestinal microbiota and mucosal healing.

Neutrophils have been proposed to influence the pathogenesis of CAC; however, conflicting reports have been reported and their overall role remains poorly understood. These divergent results may be the result of different methodological approaches used to study the role of neutrophils, such as antibody-based depletion or genetically modified mouse models that alter neutrophil survival or their effector functions.

To address this issue, we decided to investigate the role of neutrophils using a genetic model of neutrophil deficiency (Csf3r^{-/-} mice), in which neutrophil production is dramatically reduced. Csf3r^{-/-} mice were challenged with classic models of dextran sodium sulfate (DSS)-induced colitis and azoxymethane (AOM)/DSS-induced CAC. The role of neutrophils was assessed by histological, cellular and molecular analyses coupled with adoptive cell transfer and correlative analyses using human datasets.

Our findings revealed that neutrophil deficiency was associated with increased susceptibility to colitis and CAC. Csf3r^{-/-} mice showed increased body weight loss, intestinal dysbiosis associated with increased bacterial invasion and an altered inflammatory response. Histological analysis showed a reduced number of healing ulcers in Csf3r^{-/-} mice, indicating that neutrophils sustained the regenerative capacity of colonic epithelial cells. In the intestine, the IL-23/IL-22 axis plays a crucial role in antimicrobial defense and mucosal healing. Interestingly, increased susceptibility of Csf3r^{-/-} mice was associated with altered expression of inflammatory cytokines, including decreased expression of IL-22, as well as its upstream regulator IL-23. Neutrophil adoptive transfer in Csf3r^{-/-} mice was sufficient to completely restore IL-22 and IL-23 tissue concentration to the levels observed in control mice. We set out an in vitro model to assess the impact of neutrophils on the production of IL-23 by macrophages. We found that neutrophils did not produce IL-23, but amplified IL-23 expression by macrophages.

We characterized by flow cytometry the immune infiltrate in the colon lamina propria of Csf3r^{-/-} and Csf3r^{+/+} mice upon DSS-induced colitis. We found altered polarization of gd T cells towards reduced expression of aryl hydrocarbon receptor (AhR) and IL-22.

We analysed public datasets of RNA sequencing of rectal biopsies from treatment-naïve patients (pediatric patients (GSE109142) and adult patients (GSE87473)) with ulcerative colitis (UC). In both, the expression of CSF3R was positively correlated with IL23, IL22, and AHR expression. Moreover, patients with higher CSF3R expression exhibited enrichment of gene signatures associated with epithelial cell development and proliferation, and antimicrobial activity.

The results presented in this study highlight the importance of neutrophils in maintaining intestinal homeostasis in response to inflammatory insults. We propose a model in which neutrophils control intestinal inflammation and CAC susceptibility by shaping the composition of the gut microbiota and activating an IL-22-dependent tissue repair pathway.

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DISSECTING THE THERAPEUTIC MECHANISM OF ANTI-PD1 THERAPY IN THE CHRONICALLY INFECTED OR TUMOR BEARING BRAIN

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While achieving great success in many cancer types, PD1 immunotherapy has limited efficacy for the treatment of metastatic brain tumors and fails in primary brain tumors for unknown reasons. There is an underwhelming understanding of CD8 T-cell subsets and responses to PD1 blockade in the context of the brain that must be addressed to better understand this disparity.

To address this, we utilize the Clone 13 strain of lymphocytic choriomeningitis virus (LCMV) which results in chronic viral infection of peripheral organs that disseminates to the brain. We are also studying primary pediatric high-grade gliomas (pHGG) and the systemic immune response using an immunocompetent genetically engineered mouse model (RCAS-Tva) to better understand the potential points of failure with anti-PD1 therapy and how this can be improved upon gleaned insights from the LCMV system.

Using LCMV CI13 we demonstrate infectious viral antigen can persist in the brain for at least 250 days while clearing from other peripheral organs in 40 days. Sampling from the olfactory bulb, cortical hemisphere, sub-ventricular zone, and cerebellum demonstrates region-specific viral loads exist, with over a log fold difference between regions. Interestingly, viral clearance in each region was significantly accelerated with systemic anti-PD1 therapy. Using LCMV specific tetramers and flow cytometry, we find a high presence of PD1+CD101+TIM3+ terminally differentiated CD8 T-cells in the brain that persist for the majority of infection. There is minimal presence of PD1+TCF1+ stem-like CD8 T-cells. Compared to untreated mice, anti-PD1 treatment significantly reduces the presence of CD101+TIM3+ “exhausted” CD8 T-cells, increases the presence of CD101-TIM3+ and IL18Ra+ effector CD8 T-cells, and increases GZMB, IFN γ , and TNF α production in virus-specific CD8+ T-cells. Additionally, anti-PD1 therapy increased the presence of TCF1+ CD8 T-cells, Ly6C+ inflammatory monocytes, Ly6G+ neutrophils, and CD11C+ dendritic cells. Flow cytometry analysis of the blood demonstrates therapeutic anti-PD1 gains access to the brain parenchyma via binding to circulating virus-specific CD4+ and CD8+ T-cells which then migrate into the brain out of circulation while carrying therapeutic antibody. This phenomenon may explain lack of therapeutic efficacy of anti-PD1 therapy in pHGGs, as we find very few PD1+CD8+ T cells in the blood and secondary lymphoid tissues of tumor-bearing mice. As expected, anti-PD1 therapy in pHGG tumor-bearing mice renders no survival benefit.

We are currently investigating the transcriptional and phenotypic profile of virus-specific CD4+ and CD8+ T-cells in the brain with and without immunotherapy using single cell RNA sequencing and flow cytometry. We are also analyzing the effect anti-PD1 therapy has on the blood brain barrier utilizing leakage assays and immunofluorescence. These findings have important implications for the design and treatment of chronic viral infections and cancer within the brain using immunotherapy and provide a foundation for developing or improving checkpoint blockade therapies.

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THE SYMPATHETIC NERVOUS SYSTEM AND IMMUNE CELL ACTIVITIES IN RHYTHMIC ANTI-TUMOR RESPONSES

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β -adrenergic signaling is a major component of the sympathetic nervous system and is known to control leukocyte migration. It has further been suggested to increase tumor burden by exerting pro-tumoral effects. In the tumor microenvironment (TME), β -adrenergic receptors are expressed on stromal cells, endothelial cells, as well as immune cells, all important players controlling tumor growth. However, whether β -adrenergic signaling can regulate tumor burden in a time-of-day-dependent manner and how sympathetic innervation modulates the TME in the early stages of tumor growth are unclear. In this study, we demonstrate the effect of chemical and genetic sympathectomy on tumor size and composition of leukocytes within the TME. We further show that the depletion of leukocyte subsets exhibits similar effects on tumors as sympathetic denervation experiments. Together, these data point toward the role of the sympathetic nervous system in affecting tumor size via the regulation of leukocyte properties.

Keywords: Sympathetic Nervous System, Tumor-immunology, β -adrenergic signaling