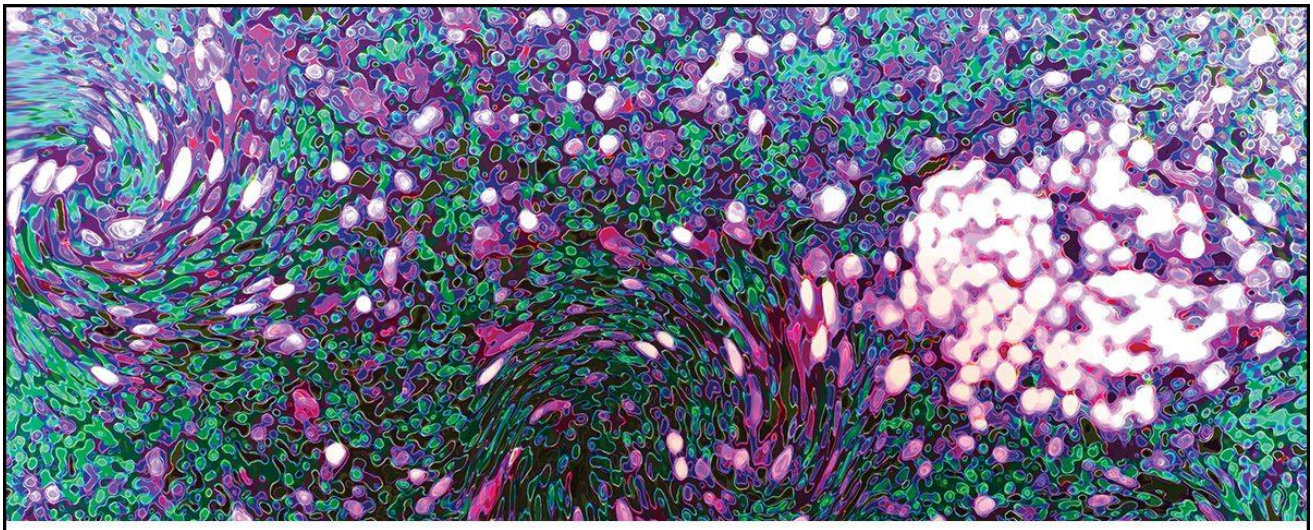

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ABSTRACTS*

current as of September 21, 2022

Invited Talks
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Invited Talks

IA01: Dissecting and modulating reactive anti-tumor immunity in human cancers

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Reinvigoration of tumor-specific T cells by cancer immunotherapies, in particular PD-1/PD-L1 blocking agents, has been the most important innovation in the treatment of patients with cancer. Nevertheless, durable clinical benefit is currently limited to a small number of patients. At present, the immunological alterations that occur in human cancers upon PD-1 blockade are not well understood. Using patient-derived ex vivo tumor models, we investigate how tumor immune composition and architecture influence immunotherapy response and how distinct treatments can change immune activity in a tumor. The observed immunotherapy-induced changes can then be linked to the inherent qualities of a tumor and its infiltrating immune populations, thereby contributing to the identification of determinants for effective response to immunotherapy and to the development of novel treatment strategies.

IA02: LAG-3: The third checkpoint inhibitor

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Immunotherapies targeting the PD1/PDL1 pathway have had a major impact on cancer treatment. However, only a proportion of patients respond, and an even smaller proportion exhibit a long-term, durable cure. Several mechanisms of resistance and potential combinatorial approaches will be discussed. Lack of response to inhibitory receptor (IR) blockade therapy and increased disease burden has been associated with circulating, peripheral $CD8^+$ T cell exhaustion, which is defined by poor T cell function linked to increased IR expression (eg: PD1, LAG3, etc). LAG3 is the third IR to be targeted in the clinic, consequently garnering considerable interest and scrutiny. However, persistent antigen exposure in the tumor microenvironment results in sustained PD1/LAG3 expression, contributing to a state of exhaustion manifest in impaired proliferation and cytokine production. Previously published and emerging data from both mouse models and clinical trials suggest that combinatorial blockade of PD1 and LAG3 can lead to increased efficacy in patients with melanoma. However, how LAG3 works (mediates its inhibitory signaling) and how LAG3 and PD1 combine mechanistically to limit the function of intratumoral T cells remains unclear.

IA03: Pyroptosis: from antibacterial immunity to antitumor immunity

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Pyroptosis, originally observed in bacteria-infected macrophages, is a proinflammatory form of cell death executed by the gasdermin family of pore-forming proteins. Among the family, GSDMD (gasdermin D) is cleaved by canonical inflammasome-activated caspase-1 as well as bacterial LPS-activated caspase-11/4/5 (the so-called noncanonical inflammasome pathway). The cleavage unmasks the pore-forming domain in GSDMD that then perforates the plasma membrane to lyse the cells. GSDMD-mediated pyroptosis plays a key role in all inflammasome-mediated immune defenses, including those against various bacterial infections. Mouse genetic analyses reveal that the caspase-11/GSDMD pyroptosis axis could stimulate effective humoral immunity during *Shigella flexneri* infection. Using a novel bioorthogonal chemical biology approach that allows controlled delivery of an active gasdermin into tumors in mice, we observe that pyroptosis of < 15% tumor cells could clear the entire 4T1 mammary tumorgraft, which is absent in immune-deficient mice or upon T-cell depletion. Such effect is also observed in other syngeneic models, suggesting that pyroptosis-induced inflammation remodels the tumor microenvironment and stimulates robust antitumor immunity. We further discover that cytotoxic lymphocytes, including natural killer cells and CD8+ T lymphocytes, kill GSDMB-positive cancer cells through inducing pyroptosis, and the pyroptosis is mediated by granzyme A (GZMA) cleavage and activation of GSDMB. IFN-γ upregulates GSDMB expression and promotes pyroptotic killing of cancer cells including that by CAR-T and TCR-T cells. GSDME, another gasdermin that is often silenced in cancers, is known to be an efficient pyroptotic substrate of caspase-3. Accordingly, others further discover that cytotoxic lymphocytes can also confer pyroptotic killing of target cells through the GZMB/caspase-3 axis. Thus, gasdermin-executed pyroptosis serves as a cytotoxic lymphocyte killing mechanism, playing an important role in cancer immunotherapy.

IA04: Immune function of extracellular matrix in tumor microenvironment and bone marrow

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Among the ECM components, matricellular proteins are capable of regulatory function. They comprise Osteonectin/SPARC, Osteopontin, Tenascin, Thrombospondins and other “sibling” molecules.

SPARC can be produced by almost every cell under stress condition, attempting to restore homeostasis. Not surprisingly it is often and persistently overexpressed in non-healing wound, such us tumors. We have studied SPARC KO mice, which are largely healthy in normal cage condition, beside osteopenia and cataract, using bone marrow (BM) chimera such to distinguish its function when originating from marrow versus radio-resistant host cells.

In the BM, SPARC is expressed by mesenchymal stroma cells (MSC) lining the endosteal and vascular niches. In a model of thrombopoietin-induced myelofibrosis, SPARC deficiency in the

radioresistant BM stroma compartment impairs myelofibrosis while enhances myeloproliferation. Similarly, under the myeloproliferative spur due to Apc^{min} or cytoplasmic nucleophosmin (NPM) mutation, exacerbated myeloproliferation occurs transplanting mutant HSC into Sparc-null but not WT recipients. Initial B lymphocytes development is stroma dependent. HSC transplanted into SPARC-null recipients have defective B cells maturation that can be corrected through a second transplant of first recipient HSC into a WT host. The impaired number of mature B cells in SPARC-null recipients depend on apoptosis induced by P-selecting over-expressed by MSC of null- but of WT-mice. These data indicate that absence of SPARC repress B lymphopoiesis in favor of myelopoiesis, granulopoiesis in particular. Also, SPARC null mice reduce collagen assembly with consequences on (i) collagen brake on immune response via binding to the inhibitory LAIR-1 receptor and on(ii) reduced scaffold/compartmentalization of secondary lymphoid organ structure. Expressed in hematopoietic early precursors, LAIR-1 is downregulated along granulocyte differentiation but promptly re-expressed in activated mature granulocytes when its binding to collagen prevents neutrophils extracellular traps (NET) formation. The organized microarchitecture of the spleen remains conserved even in presence of autoreactive CD3+/B220+ lymphoid cells of Fas deficient $Fas^{lpr/lpr}$ mice, expanding within the follicle. In the $Fas^{lpr/lpr}$ $Sparc^{-/-}$ double mutants the loss of collagen (both type I and IV) allow CD5 B cells to take contact with cells normally segregated from them including neutrophils no longer inhibited via LAIR-1 and undergoing NETosi, a condition that favor transformation of CD5 B cells into a CLL like lymphoma.

No humans are *SPARC*-deficient, therefore we tested whether any of the above phenotypes occurs under SPARC downregulation. An autoimmune SLE like condition can be induced by repeated immunization with DCs pulsed with NETs. In mice developing autoimmune Abs, including anti-ANCA, their BM show downregulation of SPARC expression in MSCs and show increased inflammatory conditions (TNF and IFNg up, Tregs down) and local NETs formation. This result found confirmation in 12 BM of autoimmune patients biopsied because of unexplained cytopenia. Thus, the challenging experiment was testing whether vaccination induced SPARC downregulation phenocopies SPARC deficiency in exacerbating the mild myeloproliferation of NPM^+ mice and the result was fully confirmatory. Also in human, AML patients with NPM1 mutation have significantly more NETs in their BM than non-mutated counterparts, which are rather characterize by increased SPARC expression. While the WT protein is located in nucleoli and nuclear membrane, the mutated NPM shows aberrant cytoplasmic localization and decorate the DNA threads in case of NETosis. Having shown that proteins associated with the DNA threads are processed and presented by DC to induce SLE and vasculitis we tested the possibility of using NETs from NPM mutated AML blast as possible vaccine for patients carrying such mutation in surrogate mouse models. NPM^+NET/DC immunization controls NPM^+-induced myeloproliferation directly in transgenic mice and in a competitive setting in which WT mice transplanted with a 1:1 mixture of HSC from NPM^+ (CD45.1) and WT (CD45.2) donors, showed selective impairment of the CD45.1 myeloblasts upon vaccination. For a short-term readout of vaccine efficacy, the leukemic cell line C1498 was transduced with the human mutant NPM1 and injected into NPM^+ transgenic mice, a setting in which NPM^+NET/DC vaccination should brake tolerance to be effective. Indeed, vaccination significantly delayed transplanted leukemia growth and induced CD8 CTL, as tested in vivo against splenocytes pulsed with NPM peptides ($CSFE^{high}$)versus equal number of splenocytes pulsed with unrelated peptides ($CSFE^{low}$). This result underscores the possibility of using

leukemic blasts as source of NETs, which display the cell antigenic repertoire onto the DNA threads along with the adjuvant functions of DNA and associate DAMP proteins.

In sum, SPARC can impinge on immune regulation with some similarity in cancer and autoimmunity.

IA05: Novel subsets of tumor-promoting macrophages

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Macrophages are incredibly versatile cells of innate immunity with diverse functions including immune responses, wound healing, and tissue homeostasis. This functional heterogeneity is reflected in their phenotype and gene expression. Macrophages also display hallmark plasticity, whereby they respond to their microenvironmental requirements by modulating their functions and altering their phenotype. Circulating monocytes or embryonic precursors give rise to tissue macrophages. Thus, ontogeny and tissue residency both determines macrophage phenotype and function. These concepts are also valid in the tumor microenvironment where recent studies underscore diverse functions of macrophages that include both pro- and anti-tumor effects. A common method to assign macrophage phenotype is based on identifying the repertoire of cell surface markers through flow cytometry (FCS). This method, however, is limited by reagents and the capabilities of the cytometer. Applications of single cell RNA sequencing (scRNA-seq) overcomes many of these limitations and provides and unbiased window into macrophage heterogeneity. Thus, we performed scRNA-seq profiling of intratumoral leukocytes, which revealed a rich diversity of macrophages whose numbers and phenotype varied between different types of tumor and treatment modality. Nonetheless, we identified several distinct subsets that were conserved across tumor types. One such subset is uniquely characterized by gene expression suggestive of high intracellular iron and heme metabolism. We labelled these macrophages as iron-rich tumor-associated macrophages or iTAMs, and isolated iTAMs by using their ferromagnetic properties. iTAMs supported tumor growth through angiogenesis and immunosuppression, and uniquely expressed high levels of endothelin receptor B (EdnrB). Genetic deletion of EdnrB in iTAMs reduced tumor growth and vascular density. We also identified transcription factor Bach1 as the negative regulator of iTAMs and EdnrB expression. Heme is a known inhibitor of Bach1 and, correspondingly, heme exposure induced EdnrB and iTAM signature genes in macrophages. Solid tumors commonly hemorrhage and macrophages specialize in phagocytosing the extravasated erythrocytes to degrade heme and recycle iron. Thus, our findings support a potential role of intratumoral hemorrhage in regulating tumor grown via induction of EdnrB expressing iTAMs. Ongoing research in our laboratory is examining the location of iTAMs within tumor, their existence in normal tissue, and how Edn signaling regulates iTAM function.

IA06: Context-dependent regulation of T-cell exhaustion by extracellular nutrient availability

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T-cell function within tumors is restricted by a conserved set of transcriptional, epigenetic, and functional alterations known collectively as T-cell "exhaustion." This program is accompanied by alterations in nutrient uptake and metabolism, but whether altered nutrient uptake is a driver or consequence of T-cell exhaustion remains unknown. The objectives of this study were to determine wither distinct nutrients are required for establishment and maintenance of the exhaustion program and if so, to determine the mechanisms by which extracellular nutrients contribute to this process.

In this study, we demonstrate distinct requirements for extracellular glucose and amino acids in establishing and maintaining chronic antigen-dependent T-cell dysfunction. First, we found that intratumoral T-cells exhibit decreased translation rates due to chronic antigen-dependent insufficient amino acid availability. Mechanistically, we show that chronic activation of T-cell receptor signaling increases translational demand, and that this demand cannot be met when extracellular amino acids are limiting, leading to decreases in both global translation rate and inflammatory cytokine production. This defect is observed in chronically stimulated T-cells, but not in bone marrow-derived macrophages. Furthermore, we demonstrate that suppression of translation during chronic TCR stimulation is associated preferential uncharging of $tRNA^{Gln}$ but cannot be overcome by either blocking GCN2-dependent eIF2a phosphorylation or constitutive mTORC1 activation. Finally, we show that $tRNA^{Gln}$ uncharging is driven at least in part by high rates of glutamine anaplerosis in chronically stimulated T-cells. Consequently, blocking glutamine hydrolysis by inhibiting glutaminase restores translation rates in chronically stimulated T-cells and maintains cytokine production under conditions of amino acid limitation.

Second, we found that glucose limitation significantly impaired the development of terminal T-cell exhaustion. This impairment was associated with a loss of expression of cytotoxic molecules including interferon gamma and granzyme B. Impairment of chronic antigen-driven terminal T-cell exhaustion could be reversed by the addition of cell permeable alpha-ketoglutarate (aKG), but not acetate, suggesting that aKG-dependent chromatin modifications are required to promote terminal T-cell exhaustion. Indeed, we found that glucose availability was a key determinant of genome-wide aKG-dependent demethylation of both repressive (H3K27me3) as well as activating (H4K3me3) histone marks. Finally, we found that promoting constitutive glucose uptake through the Glut1 transporter was sufficient to activate components of the T-cell exhaustion program in the absence of persistent antigen and accelerated terminal T-cell exhaustion and death during chronic antigen exposure.

Taken together, our results establish distinct roles of extracellular nutrients in the context-dependent regulation of intratumoral T-cell function and demonstrate how re-balancing glucose and amino acid availability can potentiate anti-tumor immunity.

IA07: Therapeutic targeting of intratumoral dendritic cells

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$XCR1^+$ type I dendritic cells (cDC1s) are necessary for anti-tumor immunity, but therapies designed to target these cells are currently lacking. By evaluating expression of immune regulators on mouse cDC1s, we identified TIM-3 as a potential target for therapy, with blockade of TIM-3 enhaning response to paclitaxel chemotherapy in mouse models of triple-negative and luminal B breast cancer. Enhanced efficacy was mediated by TIM-3 blocking antibodies increasing expression of the chemokine CXCL9 by cDC1s, resulting in improved spatial localization with $CD8^+$ T cells and enhanced effector function. CXCL9 expression was a result of cGAS-STING pathway activation, as TIM-3 was responsible for inhibiting the endocytosis of extracellular DNA by human and mouse cDC1s through its ability to bind HMGB1. As the presence of HMGB1 was sufficient for DNA endocytosis, we evaluated the release of HMGB1 by tumors cells in response to different chemotherapies. In addition to paclitaxel and docetaxel, 5-fluorouracil induced the loss of nuclear HMGB1 by human and mouse breast cancer cell lines, and TIM-3 blockade improved the efficacy of 5-fluorouracil therapy. Cumulatively, these studies describe the mechanism by which TIM-3 blocking antibodies activate tumor cDC1s, and use this information to identify rationale combination therapies for future evaluation.

IA08: Lesion-to-lesion heterogeneity in response to pembrolizumab therapy

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Background: Disease progression is often considered a binary state reflecting presence or absence of response. However, meaningful heterogeneity between metastatic sites of a given patient may exist and may impact therapeutic outcomes. To characterize the heterogeneity of progression with immunotherapy, we evaluated lesion-level dynamics of pembrolizumab-treated patients across three tumor types.

Patients and methods: Individual metastatic lesion dynamics were analyzed retrospectively in patients with advanced melanoma, non–small cell lung cancer (NSCLC), and gastric or gastroesophageal junction (G/GEJ) cancer who received pembrolizumab in KEYNOTE-001 or KEYNOTE-059. Primary progression was defined as radiologic progression per RECIST v1.1 occurring at the first on-treatment study scan (~9-12 weeks, + 2-week window) and secondary progression as progression occurring beyond the first scan (~14 weeks and beyond). The change in sum of target lesions and of individual lesions was examined, as were patterns and timing of progression.

Results: 9239 individual lesions from 1194 patients were analyzed. Among patients with primary progression (39% [200/511] of patients with melanoma, 41% [179/432] with NSCLC, 61% [154/251] with G/GEJ cancer), most patients (51-63%) had a mixture of growing, stable, and shrinking lesions. Despite overall primary progression, a minority of patients (19-25%) had tumor growth at every metastatic site and 17-32% had ≥1 shrinking lesion. Among patients with

secondary progression (22% [113/511] of patients with melanoma, 27% [117/432] with NSCLC, 18% [44/251] with G/GEJ cancer), few patients had rebound growth (>20% increase in diameter from nadir) in all lesions whereas the majority (74-84%) had sustained regression in ≥1 lesion.

Conclusions: Lesion-level heterogeneity at the time of disease progression was common in pembrolizumab-treated patients, with many patients demonstrating ongoing disease control in a subset of tumor sites. These results may inform clinical decision making, trial design, and tumor sampling in the future.

IA09: Modeling immune response in ovarian cancer: Opportunities and challenges

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Ovarian cancer remains a leading cause of death among gynecologic malignancies, with high grade serous histology being most common. Prognostic significance of tumor-infiltrating lymphocytes has been demonstrated in ovarian cancer in a number of studies. Preclinical models of ovarian cancer such as ID8, which is based on transformed mouse ovarian surface epithelial cells, have demonstrated responsiveness to immune checkpoint inhibitors (ICI) generating rationale for evaluation in humans. Despite these findings, the activity of single-agent PD-1/ PDL1 inhibitors in ovarian cancer have been very modest to date without clear established biomarkers. Unlike many cancers that are commonly responsive to ICI such as melanoma and lung cancer, ovarian cancers exhibit few single nucleotide variations (SNVs) and are largely driven by large-scale structural variations (SVs) including rearrangements, amplifications, and deletions. Distinct SV-associated mutational processes have been defined that stratify HGSOC into four major genomic instability phenotype subgroups: two homologous recombination deficiency (HRD) subtypes (BRCA1- and BRCA1-like-associated tandem duplications (HRD-dup); BRCA2- and BRCA2-like-associated interstitial deletions (HRD-del)), foldback-inversion (FBI) bearing tumors, and CDK12-associated tandem duplicators (TD). Analyses of the tumor microenvironment composition of these subtypes demonstrate significant differences in the numbers and phenotypes of tumor-infiltrating T cells, with HRD tumors associated with most pronounced immune activation.

Based on improved understanding of ovarian cancer genetics, a number of novel genetically engineered ovarian cancer models based on common genomic alterations in ovarian cancer such as mutations in *TP53, BRCA1/2*, and amplification of *MYC* have been recently developed. These models have confirmed that HRD tumors, such as those driven by *BRCA1/2* alterations, are associated with increased immune infiltration. These studies have also demonstrated rationale for combinatorial therapy of ICI with DNA-damaging agents such as platinum chemotherapy and PARP inhibitors, thus re-invigorating an interest in ICI in ovarian cancer. Despite these findings, clinical studies to date failed to demonstrate significant improvement in clinical outcomes in patients with ovarian cancer treated with chemotherapy and PD-L1 inhibitors, including patients with HRD tumors. Deep multi-site analyses of tumors from newly diagnosed OC patients reveal high degree of inter-site tumor immune heterogeneity and evidence of acquisition of adaptive immune resistance mechanisms such as common loss of heterozygosity at the HLA locus, findings that are not captured in animal models. These findings highlight that the evolution of

cancer genetics and tumor microenvironment is not well-recapitulated by short-term animal models in genomically-unstable tumors such as high grade serous ovarian cancer and demonstrate the need to take these features into account when translating pre-clinical findings to patients.

IA10: Engineering next-generation CAR-T cells for cancer immunotherapy

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The adoptive transfer of T cells expressing chimeric antigen receptors (CARs) has demonstrated clinical efficacy in the treatment of advanced cancers, with anti-CD19 CAR-T cells achieving up to 90% complete remission among patients with relapsed B-cell malignancies. However, challenges such as antigen escape and immunosuppression limit the long-term efficacy of adoptive T-cell therapy. Here, I will discuss the development of and clinical data on next-generation T cells that can target multiple cancer antigens and resist antigen escape. I will also present recent work on tuning CAR signaling activities via rational protein design to achieve greater *in vivo* anti-tumor efficacy. This presentation will highlight the potential of synthetic biology in generating novel mammalian cell systems with multifunctional outputs for therapeutic applications.

IA11: Leveraging tumor-associated alterations in O-glycosylation for cancer immunotherapy

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Chimeric antigen receptor (CAR) technology allows T cells to be genetically engineered with a novel immune receptor (as well as additional pro-inflammatory or effector molecules) that redirect T cell specificity based on the targets of monoclonal antibodies.

Previously, we designed a CAR-T cell therapy that targets the glycopeptide epitope Tn-MUC1 and demonstrated anti-tumor efficacy against a variety of tumor histotypes, including T cell lymphoma, pancreatic cancer, breast cancer, prostate cancer, and other cancer histotypes. Now, we extend the targeting of Tn-glycopeptides to an oncofetal form of fibronectin, recognized by the antibody FDC-6. Tn-fibronectin-targeting CAR T cells exhibit similar efficacy against PC3 prostate tumors as CAR T cells targeting cell surface antigens; however, the mechanism of action of cytotoxicity appears to be different, suggesting that T cells targeting extracellular antigens may represent a novel platform for inducing anti-tumor efficacy. We found that loss of IFNγ signaling as a potential tumor escape mechanism of CAR T cells and identified TLR2/6 and TLR4 agonists as compounds that can synergize with CAR T cells to kill tumor deficient in IFNγ signaling.

We also investigated the influence of the truncated O-glycan Tn antigen on tumor growth as well as the composition of the tumor immune microenvironment. Tn+ PDAC tumors exhibit increased tumor growth and an increased infiltration of immunosuppressive myeloid cells. RNA sequencing of WT/parental PDAC and Tn+ PDAC revealed differential gene expression in biological pathways related to myeloid recruitment and differentiation, cell cycle, hypoxia,

epithelial-to-mesenchymal transition, and extracellular matrix remodeling. These complementary tumor immunology and immunotherapy studies suggest that differentially glycosylated epitopes are relevant targets to alleviate drivers of immunosuppression in the tumor microenvironment as well as reduce tumor burden.

IA12: Individualized mRNA neoantigen vaccines for pancreatic cancer

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Although immunotherapies that target anti-tumor T cells are landmark oncologic breakthroughs, they are ineffective in the ~80% of tumors that are immunologically "cold". Pancreatic ductal adenocarcinoma (PDAC) exemplifies this challenge – most PDACs (~91%) are cold, are infiltrated by few T cells, and are almost completely insensitive (<5% response rate - RR) to current immunotherapies. This low RR is partially attributed to PDACs having a low mutation rate that generates few neoantigens, that render PDACs weakly antigenic, with fewer infiltrating T cells. However, we recently reported that most PDACs in fact harbor more neoantigens than previously predicted. Furthermore, we have also found that neoantigens may stimulate anti-tumor T cells in PDAC, as patients with tumors enriched in immunodominant "high quality" neoantigens exhibit delayed recurrence and longer survival.

In this talk, using recent improvements in our neoantigen quality model that selects immunodominant neoantigens, I will highlight further recent evidence that despite being a lowly mutated tumor, mutation-derived neoantigens are immunodominant antigens in PDAC. I will also outline results from a recently completed clinical trial to target neoantigens with personalized mRNA vaccines in PDAC patients, as evidence that neoantigens are bonafide clinically actionable targets in PDAC, and more broadly in lowly mutated tumors.

IA13: Cancer immunogenomics research with the CRI iAtlas web portal

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The number, size, and complexity of 'omics datasets in cancer immunology are growing rapidly. There is a need for tools that harmonize these datasets and make their analysis broadly accessible to researchers, including those without computational expertise and/or a team of bioinformaticians to acquire, curate, process, and analyze the data. This need is especially acute in translational research. With the increased volume of genomics data from studies involving treatment with immune checkpoint inhibition (ICI) and other immunotherapies, researchers remain unable to make full use of results for foundational studies or biomarker development due to lack of comprehensive access to data or the ability to compare features and outcomes across datasets. In parallel with the growth in publicly available datasets, the set of tools and methods for immunogenomics feature extraction continues to grow as well. As such, there is a need for

making these tools and results on publicly-available datasets readily available to the research community. The Cancer Research Institute (CRI) iAtlas[1] (www.cri-iatlas.org) is a comprehensive web platform for interactive data exploration and discovery in immuno-oncology, originating in a study by The Cancer Genome Atlas (TCGA)[1–3]. iAtlas provides topic-oriented analysis modules, incorporating best practices in genomics and immunogenomics methods to generate visualizations and statistics for studying interactions between tumors and the immune microenvironment.

iAtlas currently holds genomic and immunogenomic feature data from 15 ICI trials encompassing 1,142 samples, augmenting the 11,535 patient samples from TCGA[1–3] and the Pan-Cancer Analysis of Whole Genomes[5] consortia. For each dataset, sequencing data FASTQ files have been processed with a standardized bioinformatics workflow[4], mapped to de-identified clinical metadata, and incorporated into the iAtlas database. For efficient access, all data have been incorporated into a relational database, and programmatic access has been made available through an application programming interface (API). Docker images using Common Workflow Language descriptors are provided so that researchers can run iAtlas workflows on their own data if desired. Computational notebooks are provided to illustrate and explain iAtlas code, plots, and functionality and to facilitate integration of iAtlas data with data sourced from a researcher's own study. iAtlas was initially developed to host immunogenomic feature data from The Cancer Genome Atlas Pan-Cancer Atlas Project[3]. Since that time, the number of datasets and set of available iAtlas modules have been vastly extended and numerous improvements have been made to the codebase.

iAtlas provides 17 interactive analysis modules to explore immune-cancer interactions, immunotherapy treatment, and outcomes in 12,677 patient samples. Users can define sample cohorts and sample groups and run comparative analyses based on any available categorical or numerical variable. Six modules are dedicated to ICI studies: dataset overview, immune readouts, immunomodulators, clinical outcome, regression analysis, and a machine learning module to enable identification of factors associated with response to therapy. We added modules to explore how germline variation and copy number alterations relate to immune response and how receptor-ligand interactions mediate interactions among tumor and immune cells. A compendium of in-development immunotherapy drug targets[6], results of a study of germline genetic contribution to immune response in cancer[7], and a tool for immune subtyping[8] are included. iAtlas serves as a repository and resource for harmonized data on immune response in cancer and response to immunotherapy, enabling researchers to readily test hypotheses and access data through multiple modalities: an interactive web portal, data download, tools[8], and computational workflows and notebooks.

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IA14: Gaining insight into tumor immunity by combining new highly multiplex 2D and 3D imaging with analytical tools

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Methods for deep analysis of single cells from dissociated tissues are powerful tools for understanding cell state, whether at the genomic, epigenomic, or biochemical level. However, the study of isolated cells does not provide key information about how these cells function within either healthy or malignant tissue setting. The process of dissociation obscures the spatial organization that is crucial to information exchange between the cellular elements and these cells with acellular matrix components, as well as the distribution of vascular, lymphatic and neuronal elements. While computational methods are beginning to make progress in imputing some of these relationships from dissociated cells, we are still far from being able to re-assemble detailed tissue architecture using such technologies to correctly position individual cells and hence, relate their state to their surroundings. At present, optical imaging, in combination with new interpretative tools that often harness machine-learning-based methods, is the approach best suited to achieving the needed spatial mapping. Towards this end, we and others have developed multiplex imaging pipelines and analytical tools for high-content imaging. Our efforts have focused on creating methods (Histo-cytometry, IBEX, Ce3D, Ce3D-IBEX) that can be widely employed and are not constrained by pre-selection of antibody panels available from commercial entities, can be used with the diverse microscopes available at most academic centers, work with both archival FFPE samples and optimally fixed material, and that enable high content imaging in both thin sections and in thicker material to provide 3D views of tissue architecture. To accompany these imaging technologies we have also invested in developing new analytical pipelines (RAPID, SPACE) that are similarly open source in nature and, where possible, instantiated as software with easy to employ graphical user interfaces (GUI). Using these tools, we have investigated several cancer models and clinical conditions. As examples I will discuss immunotherapeutic treatment of a mouse model of pancreatic ductal adenocarcinoma (PDAC) and human follicular lymphoma (FL).

The PDAC model involves a transplantable clonal derivative of a tumor generated in the KrasG12DTrp53R172HPdx-1-Cre (KPC) GEM model. Tumors derived from this clone show substantial T cell infiltration in the absence of therapy. Treatment with a combination of anti-CD40 agonist antibody, anti-CTLA-4, and anti-PD-1 induces tumor growth arrest and regression. This treatment effect is associated with a loss of conventional Foxp3+ CD4+ T cells (cTregs), with remaining cTregs concentrated on the tumor margin away from other CD4+ and CD8+ T cells in the tumor interior. Surprisingly, the loss of cTregs is seen using anti-CD40 alone, even though these T cells lack expression of the target molecule. Given the known importance of CD40 in regulating dendritic cell function, anti-CD40 was used in BATF3-deficient animals or CD11c-DTR mice treated with DT. When the cDC1 or total dendritic cell populations were eliminated, cTreg loss was no longer observed. Since CD40 engagement induces IL-12 production by cDC1, a role for IL-12 and IFNg in Treg loss was explored, and both cytokines were found to be necessary for the depleting effect of anti-CD40. Prior work in gut inflammation models indicated that these cytokines could induce Tregs to acquire Th1-like properties. To

probe whether conversion of Tregs into type 1 effectors might explain the loss of Foxp3+ CD4+ T cells in the PDAC model, we employed Foxp3eGFP-Cre-ERT2 x Gt(ROSA)26Sortm(CAG-tdTomato)/Hze lineage tracing mice. Anti-CD40 treatment markedly increased the frequency of lineage+Foxp3- CD4+ T cells (Ex-Tregs) and these Ex-Tregs transcribed the IFNg gene in situ. Together, these findings indicate that anti-CD40 both reduces the suppressive activity of Tregs and their proximity to effector T cells in the TME while at the same time enhancing the pool of type 1 effectors making IFNg. These results provide a new picture of how immunotherapy can alter the balance of immune inhibitory and immune effector activities within a tumor, potentially turning a poor prognostic situation (high Treg numbers) into one that might, with the proper treatment, make a positive contribution to tumor elimination.

The FL study was designed to develop a deeper knowledge of the TME to look for patterns that might be used to predict rapid progression vs. indolence as well as provide clues for improved therapy of the early progressors. The combined use of transcriptional profiling and highly multiplex imaging (IBEX) together with new analytical tools for assessing complex phenotypes has provided intriguing leads for future study. The findings to date have highlighted the extent of myeloid and stromal under-sampling from RNA sequencing (RNA-seq), while revealing that early progressors have lymph nodes showing distinctive features involving fibroblast/matrix components, along with putative glycosylated BCR:DC-SIGN interactions that may reflect trans-cellular signaling driving lymphoma cell survival and proliferation.

To increase our capacity to map the spatial architecture of the TME, we have developed a new method that combines our clearing technology (Ce3D) with the iterative imaging of IBEX to achieve greater than 20-plex imaging of samples hundreds of microns thick. Such 3D imaging reveals aspects of biology that are largely invisible in thin sections, such as the connectivity of blood vessels, the paths of lymphatic vessels, and the relationship of nerves to other elements in the tissue.

For data processing, RAPID is a machine learning-based tool that enables either pixel or object-based analysis of multiplex imaging data with a user-friendly GUI. It outperforms the current standards for cell segmentation, while enabling pixel-based processing more suitable for acellular components or highly irregularly-shaped cells. SPACE is a new technology for deep analysis of spatial organization in tissues that starts with raw, pixel- or object-based data processed in RAPID and then works across scales to identify non-random arrangements of cells, without the limiting assumption that all features of interest derive from physically neighboring pairs of cell types.

In summary, we have developed technologies that enable highly multiplex analyses of 2D and 3D tissue space in a manner that avoids commercial restrictions on experimental design, along with computational platforms that can process such complex data sets in a convenient manner so that investigators can interact with and learn from their data directly. The value of these imaging methods has already been demonstrated in multiple animal tumor models and with clinical samples.

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IA15: Harnessing B cell and tertiary lymphoid structure function in ovarian cancer for improved anti-tumor immunity

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B cell infiltration is common within the tumor microenvironment of patients with high grade serous ovarian cancer (HGSOC), and ~15% of patients contain B and T cell-rich tertiary lymphoid structures (TLS), which organize adaptive immunity and correlate with immunotherapeutic response in solid tumors. Further, increased B cells, plasma cells, and TLS correlate with improved prognosis in HGSOC. However, current immunotherapies have exhibited a marginal 10% response in ovarian cancer patients, highlighting the need for synergistic or complementary strategies. Our research aims to identify the underlying stromal and tumor-intrinsic factors promoting TLS formation and maturation in HGSOC to identify key factors that could boost the poor immunotherapeutic response in patients. We hypothesize that pro-tumorigenic cancer associated mesenchymal stem cells (CA-MSC) hinder TLS, while BRCA mutant tumors promote TLS via DNA damage response.

Novel multiplex immunofluorescence panels were designed to capture immune phenotypes and consensus markers of TLS maturity. Digital image analysis was used to analyze TLS presence and maturity and CA-MSC ratios. These features were also correlated with clinical annotations. Nanostring Digital Spatial Profiling (DSP, 18,000 gene whole transcriptomic analysis) was performed on 16 HGSOC samples to provide a spatially resolved transcriptional signature of TLS, B cells, and stroma.

We developed a new TLS scoring strategy, which relies on bioinformatic analysis of multispectral immunofluorescence and spatial transcriptomics. This approach was applied to a large cohort of HGSOC patient samples. We reported the frequency, composition, and maturity of TLS across HGSOC anatomical sites (fallopian tube, ovary, omentum), noting an unexpected enrichment of mature TLS in tumors residing within the fallopian tube. Additionally, our analysis demonstrated increased TLS number and size in patients with BRCA mutations. We also quantified CA-MSC burden within tumors, allowing correlation of CA-MSC to MSC ratio with TLS presence. Our preliminary analyses indicate that CA-MSC are increased when TLS are not present. Finally, our DSP experiments generated transcriptional signatures of TLS, TLS-resident versus non-resident B lymphocytes, and TLS-adjacent versus TLS-distal stromal signatures.

Our work reveals stromal and tumor-intrinsic factors involved with TLS formation and maturation, revealing plausible strategies for promoting the development of these important ectopic structures, with the ultimate goal of increasing immunotherapy efficacy, and extending patient survival.

IA16: From the clinic to the lab: Investigating mechanisms of response and resistance to immune checkpoint therapy

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Immune checkpoint therapy has revolutionized cancer treatment, but there are many unanswered questions pertaining to mechanisms of response and resistance. In an attempt to address some of these questions, Dr. Sharma focuses her effort on a "reverse translation" process. She studies human immune responses to generate hypotheses related to mechanisms of tumor rejection, which she tests in appropriately designed pre-clinical models, and subsequently uses the new data to design novel clinical trials to improve outcomes for patients with cancer. For example, Dr. Sharma designed and conducted the first neoadjuvant (pre-surgical) trial with immune checkpoint therapy (anti-CTLA-4) in 2006. These studies were conducted in patients with localized bladder cancer and led to translational research projects in pre-clinical models, which identified the ICOS/ICOSL pathway as a novel target for cancer immunotherapy strategies. This initial clinical trial also provided the first safety data for administration of immune checkpoint therapy in the neoadjuvant setting, which led to many other neoadjuvant studies. The clinical trial also led to the first anti-tumor responses with immune checkpoint therapy in patients with bladder cancer, which led to many other clinical trials in patients with bladder cancer, including clinical trials with anti-PD-1 (nivolumab) that were led by Dr. Sharma and her colleagues to enable FDA-approval of nivolumab for patients with metastatic bladder cancer. In addition, Dr. Sharma demonstrated for the first time that human tumors express VISTA as an immunosuppressive pathway, which acts as a resistance mechanism to immune checkpoint therapy. Clinical trials targeting VISTA are now ongoing. Dr. Sharma was also the first to demonstrate that anti-CTLA-4 plus inhibition of the EZH2 epigenetic pathway can improve anti-tumor responses, which led her to design a new clinical trial with this combination. This clinical trial is currently accruing patients. In addition, Dr. Sharma demonstrated for the first time that the unique immunologic niche within a specific organ, such as bone, impacts response to treatment with immune checkpoint therapy. Dr. Sharma will present data from many of the clinical trials and the associated pre-clinical studies investigating mechanism of response and resistance to immune checkpoint therapy.

Proffered Talks

PR01/A015: TGF-β inhibits the function of progenitor-exhausted CD8+ T cells in response to PD-1 blockade

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The anti-tumor activity of anti-PD-1 depends on a subset of progenitor exhausted $CD8^+$ T cells (T_{PEX}) with stem-like properties. Whether specific environmental cues can limit their functionality in response to anti-PD-1 remains to be fully elucidated. We have originally characterized T_{PEX} in human cancers[1] and have recently shown that their developmental trajectory is transcriptionally, epigenetically and clonally distinct from that of more functional memory T cells[2]. We now show that the effector differentiation potential of T_{PEX} in human cancer is specifically inhibited by the immunosuppressive cytokine transforming growth factor beta (TGF-β), thereby explaining, at least in part, why TGF-β is a major mechanism of resistance to immune checkpoint blockade. In human lung cancer, intratumoral levels of *TGFB1* mRNA highly correlated with T_{PEX} abundance, suggesting a functional relationship. Treatment of purified T_{PEX} with TGF-β in vitro stabilized the exhausted phenotype by increasing the levels of *PDCD1* and *TOX*, among others, and by inhibiting effector differentiation via downregulation of several functional molecules. Single cell RNA-sequencing coupled with computational prediction of cell:cell communications revealed that TGF-β massively shaped the repertoire of T_{PEX} interactions in the tumor microenvironment, promoting crosstalk with subsets of cancer associated fibroblasts previously reported to be highly immunosuppressive and that are further shown to be abundant in patients not responding to anti-PD1 treatment.

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[This proffered abstract will also be presented as a poster in Poster Session A.]

PR02: Circulating Tex cells track non-small cell lung cancer evolution and predict response to immunotherapy

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Neoantigen specific CD8 T cells are re-programmed to a state of dysfunction in the non-small cell lung cancer (NSCLC) tumour microenvironment (TME). The ability to track this program via liquid biopsy could generate new tools for immune monitoring of tumour development, recurrence, or immunotherapy sensitivity. However, T cell dysfunction in the circulation is poorly characterised and the dynamics of this process during lung cancer evolution remain undefined.

Here we identify tumour specific CD8 Tex cells in the NSCLC TME and track these clones to post-mortem where they infiltrate multiple metastatic sites, raising possibility that tumour specific Tex cells recirculate during primary disease and are maintained long-term in a circulating niche.

Using independent data sets of scRNAseq we find that a subset of *PDCD1+TCF7*lo*TOX+CXCR4+GZMK+* circulating Tex-like CD8 T cells in NSCLC share T cell receptors (TCRs) with the intra-tumoural Tex pool. In a combined longitudinal study of both NSCLC patients (n=146, TRACERx study) and untreated HIV-1 infected subjects (N=64, Protocol C study, International AIDS Vaccine Initiative [IAVI]) we discover that circulating CD8 Tex cells are consistently expanded in humans harbouring chronic tumour or viral antigen and respond to dynamic changes in antigen load. Although these populations contract upon antigen withdrawal (i.e. surgical resection or anti-retroviral therapy), we find that circulating Tex cells undergo clonal re-expansion during NSCLC recurrence with preserved clonal architecture, suggesting a Tex-driven recall response. Commensurate with this hypothesis scRNAseq analysis of 81 patients with metastatic NSCLC from the Samsung Medical Centre revealed that a higher fraction of circulating pre-therapy Tex cells predicted response and durable clinical benefit to anti-PD1. Analysis of circulating Tex cells in responders revealed a conserved transcriptional program characterised by co-ordinated expression of pro-inflammatory cytokines, lung homing markers and a discrete subset of T cell exhaustion-associated loci.

These data provide a basis for the long-term maintenance of Tex cells from diagnosis to death in NSCLC and define transcriptional and phenotypic signatures to track these cells in the blood for liquid immune monitoring and immunotherapy stratification.

PR03: Critical Role of Macrophages in Organizing Adaptive Immunity against Tumors

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In solid tumors, CD8 T cells often lack adequate numbers, potent stimulation and are driven to a dysfunctional state following a predictable differentiation path. Apart from T cell-intrinsic factors extrinsic ones, such as the myeloid cell-driven immune organization decisively dictate the anti-tumor function of CD8 T cells. Specifically, the role of tumor-associated macrophages (TAMs) in this context are yet to be fully explored beyond the typical and almost certainly oversimplistic view that they are 'immunosuppressive'. This is in part because we have lacked nuanced tools to investigate the subsets of TAMs and their context-dependent role and in directing the immune response in cancer. We addressed this challenge by developing and using a macrophage reporter and depletion tool largely specific to well-differentiated TAMs and thereby discovered their critical role in organizing the adaptive immune niche vital for CD8 T cell-mediated anti-tumor function.

Remarkably till now, a viable method to specifically deplete TAMs, without global depletion of virtually all macrophages and monocytes did not exist. We alleviated this problem by generating a novel mouse strain, in which conditional (lox-Stop-lox flanked) Venus (a YFP variant) and DTR sequences are knocked into the endogenous CD206 locus. Thus, an intercross of this allele with Csf1r-Cre (to specify monocyte-derived lineage) provides a 2-allele gating system to limit and robustly penetrate reporter and depletion function to TAMs, specifically those expressing CD206 and thought to be pro-tumor. Timed and specific depletion revealed an anti-tumor role for these TAMs in the adaptive immune organization in the tumor microenvironment. Specifically, TAMs orchestrate the Flt3l-dependent cDC1 infiltration into the tumor indirectly by enhancing NK cell numbers and that of Cxcr3-driven CD8 T cells directly by secretion of Cxcl9 and Cxcl10. Importantly, these effects were prominent only when depletion was performed from an early stage, and not when it was begun late, even though TAMs were obliterated in both cases. Notably, these functions were mediated, perhaps surprisingly by subsets of $CD206^{hi}$ well-differentiated TAMs, showing them to be more than simply of the pro-tumor or "M2" phenotype. Thus, notwithstanding their suppressive role in inducing CD8 T cell exhaustion, TAM depletion did not lead to tumor control in a B16-derivative model. In fact, these organizational functions of TAMs were critical for anti-tumor immunity and their early depletion prevented tumor control in an MC38-derived model responsive to adoptive transfer of antigen-specific T cells.

Overall, this study introduces and uses a novel CD206+ macrophage visualization and depletion tool, which revealed a prominent role for $CD206^{hi}$ TAMs in assembling the adaptive immune machinery for CD8 T cell-mediated anti-tumor function. These findings add context and nuance to the current paradigm of myeloid targeting as a strategy for cancer immunotherapy.

PR04/A082: Antibiotics disrupt the ileal MAdCAM-1/α4β7 axis, compromising tumor immunosurveillance during PD-1 blockade

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INTRODUCTION: Studies have shown that antibiotics (ATB) compromise the outcome of immune checkpoint inhibitors (ICI) in patients. Yet, mechanisms underlying their immunosuppressive effects remain unknown. Given the impact of gut microbiota on ICI efficacy in various solid cancer types, such as Non-Small Cell Lung Cancer (NSCLC), melanoma or urothelial cancers, we hypothesized that gut immune cells might interfere with immune responses in cancer. Gut-specific homing relies on leukocyte expression of the integrin α4β7 and its binding to the addressin MAdCAM-1, which is expressed in gut-associated lymphoid tissue (GALT). Our goal was to understand whether the intestinal MAdCAM-1/α4β7 axis is involved in PD-1 blockade efficacy and its relationship with gut microbiome.

METHODS: To understand the role of the gut microbiota on MAdCAM-1 expression in the intestine, dysbiosis was induced by broad-spectrum ATB in mice and microbial recolonization of the intestine following ATB cessation was studied. Also, oral gavages with bacteria of interest or feces from Responder or Non-Responder (R/NR) patients to ICI were performed in mice. MAdCAM-1 expression in the gut was assessed by ELISA, flow cytometry and immunohistochemistry (IHC) in mice and by RT-PCR in mice and humans. The dynamics of intestinal cell migration to the tumor microenvironment (TME) were assessed using *in vivo* cell tracing models and blocking MAdCAM-1 with an antibody. To unveil the characteristics of those migrating cells, single cell RNA sequencing was performed on the labelled cells migrating from the intestine to the tumor microenvironment (TME). Finally, the soluble form of MAdCAM-1 in plasma (sMAdCAM-1) was measured in two cohorts of NSCLC patients treated with ICI.

RESULTS: We showed that ATB decreased ileal and soluble MAdCAM-1 levels in mice and patients. Recolonization after ATB was accompanied by an overrepresentation of *Enterocloster* genus in mice. Similarly, an overrepresentation of *Enterocloster* genus was highlighted in the gut microbiota of NR patients to ICI. We emphasized the fact that the ileal MAdCAM-1 expression depends on the gut microbiota composition: increased by bacteria found in responder patient feces, such as *Akkermansia muciniphila* and decreased by *Enterocloster* genus after oral gavage in mice. Also, disruption of ileal MAdCAM-1 led to the loss of anti-PD-1 efficacy. Thanks to *in vivo* cell tracing models, we demonstrated that this mechanism relies on the emigration of immunosuppressive gut tropic α4β7 T cells to the TME. This emigration from the gut of immunosuppressive T cells, such as Treg cells, was reinforced by the loss of MAdCAM-1 in the intestine or its blockade by an anti-MAdCAM-1 antibody. Finally, the assessment of sMAdCAM-1 levels in NSCLC patients predicted resistance to ICI in two independent cohorts.

CONCLUSION: Our findings uncover the pivotal role of the small intestine and gut microbiota in hosts facing a carcinogenesis, in which GALT, involving the expression of MAdCAM-1, controls the emigration of immunosuppressive gut tropic cells. Intestinal dysbiosis characterized by an outgrowth of bacteria from the *Enterocloster* genus contributes to the loss of MAdCAM-1 in the intestine and the accumulation of α4β7 Treg cells in the TME which is associated with decreasing anti-PD-1 efficacy. Remarkably, sMAdCAM-1 levels reflecting its expression in the gut could be a novel in-clinic biomarker to predict ICI resistance in patients with lung NSCLC treated with ICI.

[This proffered abstract will also be presented as a poster in Poster Session A.]

PR05/A052: Mechanisms regulating Tertiary Lymphoid Structures in the CNS

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Glioblastoma (GBM) is an aggressive brain tumor characterized by a low-overall immunogenicity and an immune-suppressed microenvironment. A potential strategy to enhance anti-tumor immunity is via the induction of tertiary lymphoid structures (TLSs). We have recently observed that TLS can form in association with glioblastoma, but the mechanisms regulating their formation in the brain are still not clear. Here, we have characterized TLS in murine glioma and investigated cellular and molecular mechanisms potentially involved in their formation. TLS containing B cells, T cells, dendritic cells and fibroblasts formed around veins close to meninges and ventricles in murine glioma models, and were surrounded by extracellular matrix. In addition, perivascular cuffs (PVCs) with a similar cellular composition as TLS formed around veins in peritumoral regions. Early TLS contained a higher relative proportion of fibroblasts and dendritic cells, while large TLS were dominated by B cells and T cells in glioma bearing mice. TLS formation was associated with intratumoral expression of pro-inflammatory cytokines, and was preceded by expression of Cxcl13 in Pdgfra+ fibroblasts located in the meninges and surrounding large veins. Pdgfra+ Cxcl13+ cells were present in early TLS, indicating that they may act as lymphoid tissue organizer (LTo) cells. In conclusion, TLS and PVCs have a similar cellular composition in glioma, and their specific location in the brain can be explained by the anatomic location of Pdgfra+ fibroblasts which are likely to act as LTo cells.

[This proffered abstract will also be presented as a poster in Poster Session A.]

PR06: Identifying the role of tRNA modifications in T cells

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Cancer immunotherapy has shown remarkable promise to treat cancer patients by enhancing their own immune system; however, unfortunately only a small subset of patients effectively responds to immunotherapy. More than ever, a better understanding of immune cells to improve anti-tumor immunity is urgently needed to improve immunotherapy for all patients. In particular, $CD8^+$ cytotoxic T cells (CTLs) in the tumor microenvironment are very important with directly killing tumor cells. In most tumors, CTLs reside in dysfunctional states leading to defective proliferation, activation, and killing ability leading to ineffective immunotherapy. To support CTLs' rapid proliferation, activation and killing function, robust protein synthesis is critical to generate important biological molecules. However, the intricate details involved with protein synthesis in T cells still remains relatively unknown. In our studies, we aim to understand the critical steps involved during protein synthesis by targeting tRNA modification enzymes, which are a key component of the translational process for robust anti-tumor immunity. To test this hypothesis, we decided to assess the function and necessity of enzymes involved in critical tRNA modifications. Among all, we selected ADAT1 as a key target enzyme to analyze its function in T cell biology.

ADAT1 is an tRNA specific modification enzyme which converts adenosine to inosine by a deamination reaction at position 37 of tRNAs. Upon TCR engagement, activated mouse T cells rapidly increased expression of ADAT1 within a time frame where T cells undergo rapid translation in preparation for clonal expansion. To understand the role of ADAT1 *in vivo*, we generated global ADAT1 knockout (KO) mice using CRISPR-Cas9 technology by deleting multiple exons, which encode critical portions required for its enzymatic activity. Using various *in vivo* murine tumor models such as MC38 (colon), B16 (melanoma), and EL4 (lymphoma), we observed that ADAT1 KO mice display delayed tumor growth compared to wildtype (WT) mice. To elucidate the intrinsic effects of ADAT1 in $CD8^+$ T cells, we adoptively transferred OVA antigen specific $CD8^+$ T cells (OTI) from WT and ADAT1 KO mice into naïve WT mice bearing OVA expressing tumors and monitored tumor growth over time. Interestingly, we observed markedly slower tumor growth in recipients with ADAT1 KO OTI T cells compared to WT OTI T cells. Since good quality long-lived T cells are highly desired for durable and successful adoptive cell therapy, we wanted to know whether ADAT1 deficiency may help enhance longevity and reduce exhaustion of T cells. Using flow cytometry analysis, we observed that ADAT1 deficient T cells reduced exhaustion markers and enhanced stem-cell like properties. Additionally, we intravenously infected WT and ADAT1 KO mice with *Listeria monocytogenes* expressing OVA to analyze antigen specific OVA $CD8^+$ T cells. On Day 7 of the peak acute response, non-OVA and OVA specific ADAT1 KO $CD8^+$ T cells displayed a central memory-like phenotype defined by increased $CD62L^+CD44^{high}$ expression. Additionally, during the memory phase, we also observed a greater number of ADAT1 KO CD8+ T cells compared to WT $CD8^+$ T cells. This data demonstrated that ADAT1 KO $CD8^+$ T cells display a long-lived memory cell phenotype.

In summary, we observed that ADAT1 expression is increased upon T cell activation and is important in controlling tumor growth in a T cell intrinsic manner. Our data demonstrates that ADAT1 is important in memory T cell formation implicating ADAT1 as a potential therapeutic target to improve immunotherapy for patients.

PR07/B122: ESCRT-dependent STING degradation curtails steady-state and cGAMP-induced signaling

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STING is an intracellular sensor of cyclic di-nucleotides involved in response to pathogen- or self-derived DNA that induces protective immunity, or if dysregulated, autoimmunity. STING trafficking is tightly linked to its activity. We aimed to systematically characterize genes regulating STING trafficking and to define their impact on STING responses. Based on proximity-ligation proteomics and genetic screens, an ESCRT complex containing HGS, VPS37A and UBAP1 was found to be required for STING degradation and signaling shutdown. Analogous to phosphorylated STING creating a platform for IRF3 recruitment, oligomerization-driven STING ubiquitination by UBE2N formed a platform for ESCRT recruitment at the endosome, responsible for STING signaling shutdown. A UBAP1 mutant that underlies human spastic paraplegia and disrupts ESCRT function led to STING-dependent type I IFN responses at the steady-state, defining ESCRT as a homeostatic regulator of STING signaling.

[This proffered abstract will also be presented as a poster in Poster Session B.]

PR08: Role of XCR1+ dendritic cells in the generation and maintenance of stem like CD8 T cells

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CD8 T-cell exhaustion is a key feature of the development of therapeutic resistance and disease progression in chronic viral infections and cancer. $CD8^+$ T-cells are responsible for mediating the adaptive immune response by killing infected or cancerous cells, however under persistent antigen exposure, $CD8^+$ T-cells become non-functional and exhausted. Subsets of $CD8^+$ T-cells,

including stem-like $CD8^+$ T-cells, have been shown to restore cytotoxic functions after therapeutically targeting the molecular mechanisms responsible for their dysfunction. Our lab has demonstrated the stem-like $CD8^+$ T-cell subset is responsible for regenerating effector populations after checkpoint blockade, highlighting their importance in anti-viral and anti-tumor immunity. Mechanisms governing the generation and maintenance of stem-like $CD8^+$ T-cells need to be further explored to identify factors controlling these processes as this population can be therapeutically harnessed for reinvigorating the immune response. Using lymphocytic choriomeningitis virus (LCMV) clone 13 to model chronic viral infection, we have identified a relationship between $XCR1^+$ dendritic cells (DCs) and stem-like $CD8^+$ T-cells that promotes the stem-like state of $CD8^+$ T-cells during chronic viral infection. Stem-like $CD8^+$ T-cells highly express XCL1, the ligand for XCR1 found on type 1 DCs. Interestingly, we demonstrate via immunofluorescence that $XCR1^+$ DCs are found within the T-cell zones of the spleen, in close proximity to stem-like $CD8^+$ T-cells and are minimally infected with LCMV antigen. Flow cytometry and IV labeling indicates the expression of costimulatory molecule CD80 is differentially expressed on red pulp/marginal zone $XCR1^+$ DCs as compared to white pulp $XCR1^+$ DCs, suggesting that $XCR1^+$ DCs maintain the stem-like state of CD8 T-cells by reducing their exposure to viral antigen and minimizing their activation and differentiation into effector CD8 T-cells. Using XCR1-DTR transgenic mice, we show that depletion of $XCR1^+$ DCs upon LCMV Clone 13 infection results in a reduction of $TCF1^+$ stem-like CD8+ T-cells and an increase in $TIM3^+TCF1^-$ CD8 T-cells. Depletion of $XCR1^+$ DCs in CD4 depleted LCMV Clone 13 chronically infected mice results in increased viral antigen presence in the white pulp/T cell zones of the spleen and a reduction in $TCF1^+$ CD8 T-cells. This was also met with an increase in $Ki67^+$ virus-specific CD8 T-cells, again suggesting $XCR1^+$ DCs maintain the stem-like state of CD8 T-cells by maintaining their quiescence and preventing their differentiation. The findings from these studies will directly impact our understanding of $CD8^+$ T-cell biology and aid the identification of novel therapeutically exploitable mechanisms for the treatment of chronic viral infections and cancer.

PR09: A vascular-restricted, tumor-induced neutrophil population drives vascular occlusion, pleomorphic necrosis, and metastasis

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The presence of necrosis in the primary tumor is associated with poor prognosis in cancer and promotes the selection of metastasis-competent cells, but is considered to be a passive process, occurring when tumor growth outpaces nutrient supply.

Here we report that tumor necrosis can be actively driven by a neutrophil subpopulation present in tumor-bearing mice and patients with cancer. Using whole mount tissue clearing to evaluate the three-dimensional architecture of tumors, we identified mouse models of cancer with either 1) a classical central necrotic core or 2) a novel pleomorphic necrotic architecture. The pleomorphic necrotic architectures were also found in biopsies of triple-negative breast cancer patients. Since the pleomorphic necrotic structures were reminiscent of the tumor vascular bed, we hypothesized that they could be actively caused by vascular occlusion. Indeed, we found that

blood flow was interrupted by intravascular neutrophil aggregates associated with neutrophil extracellular traps (NETs) in the models showing pleomorphic necrosis. Genetic and pharmacological inhibition of NET-formation reduced neutrophil intravascular aggregation and necrosis in the primary tumors, and reduced metastasis to the lungs. We found this correlated with a reduction in the amount of peri-necrotic, hypoxic tissue area where cancer cells had undergone changes associated with cell motility and invasion potential. To understand what drove the intravascular aggregation of neutrophils and pleomorphic necrosis, we compared the transcriptional and phenotypical properties of cancer cells in the models with pleomorphic and classical central core necrosis. We found that the tumors showing pleomorphic necrosis elicited changes in the hematopoietic compartment, inducing a myeloid skew in hematopoietic stem and progenitor cells that was at least partially dependent on CXCL1 secretion by cancer cells. This led, in mice bearing tumors with pleomorphic necrosis, to the appearance of a novel $Ly6G^{High}$ $Ly6C^{Low}$ neutrophil subpopulation. Compared to $Ly6C^{High}$, the $Ly6C^{Low}$ neutrophils exhibited increased NET formation and, interestingly, the inability to extravasate in response to inflammatory challenges. Using transcriptomic analyses, we defined a signature for these "vascular-restricted" $Ly6C^{Low}$ neutrophils and identified a neutrophil cluster with a similar signature in the blood of breast cancer patients.

Necrosis correlates with worse prognosis but is considered a passive and non-targetable process. Here, we show that pleomorphic tumor necrosis is caused by a vascular-restricted, tumor-induced neutrophil subpopulation, and that it can be targeted to reduce metastatic spread.

PR10: Objective clinical response by KRAS mutation-specific TCR-T cell therapy in previously treated advanced Non-small cell lung cancer (NSCLC)

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Introduction

Solid tumors harbor clonal cancer gene mutations that generate tumor neoantigens. These can be presented by human leukocyte antigen (HLA) molecules to T-cell receptors (TCRs) expressed on T cells. We have developed non-viral TCR-T cells based on the *Sleeping Beauty* transposon/transposase system, which is faster and more cost-effective than other gene transfer technologies. These TCR-T cells are generated from a library of validated TCRs with defined specificity to frequent HLA alleles and hotspot mutations in *KRAS, TP53* and *EGFR*, which are common in solid tumors.

Study Design

This is a first-in-human phase 1/2 study of autologous TCR-T cell therapy in patients with non-small cell lung (NSCLC), colorectal, endometrial, pancreatic, ovarian and bile duct cancer. The phase 1 portion of the study explores up to four TCR-T dose levels. The primary objective of the

phase 1 portion is to define the incidence of treatment related dose limiting toxicities (DLTs) and the recommended phase 2 dose to be explored in disease specific expansion cohorts.

Results

The first patient dosed in the phase 1 portion of the study was a 34-year-old female diagnosed with NSCLC. The patient had recurrence after left lower lobectomy and adjuvant platinum-based chemotherapy and progressed on three prior lines of systemic therapy including carboplatin/pemetrexed/pembrolizumab, durvalumab/tremelimumab/selumetinib and a SHP2 inhibitor with best response to prior therapy being partial response. The patient was germline for HLA-A*11:01 and we detected a $KRAS^{G12D}$ mutation in the tumor. This corresponded to the specificity of one of the ten TCRs within the TCR library. Autologous TCR-T cells were produced by *Sleeping Beauty* transposition at cGMP and were released based on identity, specificity, functionality and sterility. Seven days after start of inpatient lymphodepletion (cyclophosphamide [60mg/kg for 2 days] and fludarabine [25mg/m^2 for 5 days]), the patient received a product containing 95% transgenic TCR and contained a total dose of $9x10^9$ TCR-T cells (Day 0). The patient experienced grade 2 cytokine release syndrome (CRS) from days 0 to 4, which resolved with nasal cannula oxygen supplementation and did not require anti-IL-6 treatment. Grade 4 thrombocytopenia and grade 3 anemia occurred on Days 3 and 4, respectively, both attributed to lymphodepletion chemotherapy. The patient's cell counts started to recover allowing discharge on Day 11. TCR-T cellular kinetics demonstrated a short redistribution phase followed by rapid expansion reaching peak (Cmax) circulating TCR-T cells of 1,038 cells/μL by flow cytometry at Day 4, corresponding to $5x10^5$ copies/μg DNA by digital droplet PCR. TCR-T cell persistence was ongoing as of Week 12 with the patient's total $CD3^+$ cells comprising 22% TCR-T cells. Post-infusion serum cytokines demonstrated elevations of inflammatory cytokines (baseline vs peak) IL-8 (31.7 vs 595 pg/mL), TNFα (2.3 vs 4.0 pg/mL), GM-CSF (<limit of quantitation (LOQ) vs 18.9 pg/mL) and IL-6 (<LOQ vs 22.0 pg/mL), which correlated with onset and resolution of CRS. In contrast, IFN-γ, which was undetectable at baseline, increased following TCR-T infusion with a peak at Day 2 (606 pg/mL) and remained elevated through Day 10 (82.5 pg/mL). The patient was determined to have a confirmed partial response by RECIST v1.1 with regression of 46.3% in target lesions at Week 6, and 51.2% at Week 12. Confirmed regression of non-target lesions was also observed at weeks 6 and 12.

Conclusion

This is the first evidence of a confirmed objective response using TCR-T cells for treatment of advanced NSCLC. This is the first-in-human experience of *Sleeping Beauty* TCR-T cell therapy. No DLTs related to TCR-T cell product were observed, and treatment had a manageable safety profile. Significant persistence of TCR-T cells was observed at week 12. This clinical trial is open for enrollment of patients with advanced solid tumors harboring *KRAS*, *TP53* and *EGFR* mutations.

PR11: A TCR targeting a shared mutation mediates eradication of AML in vivo

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Acute myeloid leukemia (AML) is the most frequent leukemia in adults. Allogeneic hematopoietic stem cell transplantation still remains the major curative option. Due to high relapse rates as well as transplant-related morbidity and mortality, there is a large unmet medical need for this patient group, calling for improved therapeutic strategies. Adoptive therapy with T cells genetically modified to express receptors targeting antigens encoded by recurrent mutations could be an attractive option.

Treatment with tyrosine-kinase inhibitors has demonstrated that targeting of FLT3 gain-of-function mutations can provide significant survival benefits for patients. Here, we aimed to identify and characterize a T-cell receptor (TCR) targeting the recurrent D835Y driver mutation in the tyrosine kinase domain (TKD) of FLT3 ($FLT3^{D/Y)}$, and evaluate therapeutic efficacy of TCR-transduced T cells in disease-relevant *in vivo* models.

We previously demonstrated that healthy donor T cells provide a rich source of neoantigen-reactive T-cell receptors (*Stronen et al, Science 2016* and *Ali et al, Nat Protocols 2019*). Here, we used this technology to identify a TCR reactive to a peptide encoded by $FLT3^{D/Y}$ restricted by the prevalent MHC class I allele HLA-A*02:01.

We observed that T cells redirected with the $FLT3^{D/Y}$ reactive TCR ($TCR^{FLT3D/Y}$) recognized target cells pulsed with low peptide concentrations (10-100pM), indicating high functional avidity. TCR fingerprinting did not reveal cross-reactivity to naturally processed and presented peptides. Furthermore, no reactivity was observed against a panel of 26 cell lines of different tissue origin that expressed HLA-A*02:01, unless the cells were pulsed with the mutant peptide, indicating high specificity. $TCR^{FLT3D/Y}$ cells also eliminated patient-derived leukemia cells harboring the relevant mutation in vitro, while sparing the normal CD3+ T cells and CD19/CD20+ B cells in the same sample, and $HLA\text{-}A2^{pos}$ patient cells that carried different or no mutations in the same position.

Finally, the TCR was tested in five different mouse *in vivo* models including mice engrafted with patient-derived primary leukemia cells. First, since no leukemia cell line naturally expressing the

$FLT3^{D/Y}$ mutation was commercially available, we introduced it in the BV173 leukemic cell line, which was engrafted in NSG mice. All mice treated with $TCR^{FLT3D/Y}$ cells showed tumor clearance (bioluminescence), with tumor negative bone marrow at point of sacrifice. The *in vivo* efficacy of $TCR^{FLT3D/Y}$ cells against primary AML was next tested in PDX models from two different patients. Here, high levels of rapidly proliferating $CD34^-$ AML (model 1), versus slowly proliferating $CD34^+$ AML (model 2) were efficiently eliminated down to MRD negative levels as confirmed by ddPCR, with complete removal of $CD34^+$ cells in the BM of the $CD34^+$ AML. In a third PDX model, the $TCR^{FLT3D/Y}$ cells rejected patient AML cells in a setting resembling MRD. $TCR^{FLT3D/Y}$ cells thus efficiently eliminated leukemia with both high and low disease burden. Finally, we evaluated whether the $TCR^{FLT3D/Y}$ cells could eliminate *in vivo* leukemia-propagating cells. Patient AML cells were transplanted into NSG mice following in vitro co-culture for 48 hours with $TCR^{FLT3D/Y}$ or TCR^{1G4} (control) T cells or no T cells. After 28 weeks, all control mice showed leukemic engraftment, while no leukemia was observed at any time in any of the mice transplanted with AML cells co-cultured with $TCR^{FLT3D/Y}$ cells, demonstrating efficient removal of leukemia-propagating cells.

The large majority of neoantigens are exclusive to the individual cancer patient, hampering development of efficacious therapies specifically targeting them. Our data showing that T cells directed at a shared driver mutation eradicate primary acute myeloid leukemia *in vivo*, including leukemia-propagating cells, pave the way for off-the-shelf, neoantigen-specific cancer therapies.

PR12: Amino acid starvation response enhances oxidative metabolism and T cell anti-tumor activity

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The manipulation of T cell metabolism to enhance anti-tumor activity is an area of active investigation. Here, we report that activating the amino acid starvation response in effector CD8+ T cells using the General Control Non-depressible 2 (GCN2) agonist halofuginone (halo) enhances oxidative metabolism and effector function. Further characterization revealed that halo-treated CD8+ T cells increased expression of the large neutral amino acid (LNAA) transporter CD98 as well as the co-stimulatory marker 4-1BB. Mechanistically, we identified autophagy coupled with the CD98-mTOR axis as key downstream mediators of the phenotype induced by halo treatment. The adoptive transfer of halo-treated CD8+ T cells into mice bearing well-established tumors led to robust tumor control and curative responses. Halo-treated T cells also synergized *in vivo* with an 4-1BB agonistic antibody to control tumor growth in a preclinical tumor model resistant to immunotherapy. Lastly, we found that treatment of human T cells with halo could recapitulate the metabolic and functional phenotype observed in murine cells. We were able to generate activated human T cells transduced with a clinically relevant T cell receptor (TCR) that demonstrated the "halo phenotype" of oxidative metabolism, increased cytokine production and increased expression of 4-1BB and CD98. These findings demonstrate that activating the amino acid starvation response with the GCN2 agonist halofuginone can enhance T cell metabolism, effector function and anti-tumor activity thereby providing a novel strategy to enhance existing clinical cell therapy approached approaches.

PR13: Impact of Tet2-mutant clonal hematopoiesis on solid tumor immunology and response to checkpoint blockade

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Background: Clonal hematopoiesis (CH) is an age-related phenomenon characterized the by overrepresentation of mature blood cells arising from a single clone and can be detected in approximately 10-20% of healthy individuals over the age of 70. CH is caused by the acquisition of somatic, non-oncogenic mutations in hematopoietic stem cells that provide a selective advantage over the remaining hematopoietic pool. Emerging research indicates that CH plays an important role in a variety of non-hematological disorders, such as cardiovascular diseases and severe Covid-19 infections, by exacerbating the innate inflammatory response. However, the impact of CH in solid tumors and response to immunotherapies is unknown.

Methods: To assess the prevalence and role of CH in patients with solid tumors, we analyzed publicly available data from the MSKCC-IMPACT study. To mechanistically study the effects of CH on solid tumor biology, we established an orthotopic model of pancreatic adenocarcinoma (PDAC) in mice that harbor a CH clone heterozygous for a loss of function mutation in *Tet2* (*Tet2*$^{+/-}$ CH); the second most mutated gene seen in human CH. Briefly, lethally irradiated CD45.1 recipient mice were transplanted with 5x10^6 whole bone marrow cells at a 1:1 ratio of CD45.1 support marrow to either CD45.2 cells with a heterozygous mutation in *Tet2* (to establish the *Tet2*$^{+/-}$ CH mouse) or CD45.2 cells with no *Tet2* mutation (to establish the *Tet2*$^{+/+}$ WT controls). After 8 weeks to allow for bone marrow reconstitution, mice were orthotopically injected with mT4 cells, a *Kras*$^{+/G12D}$*TP53*$^{+/R172H}$*Pdx1-Cre* (KPC) derived model of PDAC. CH and WT mice were be randomized to receive 3 doses of either a vehicle control or ICB treatment (αCTLA-4 [100 μg/mouse] and αPD-1 [250 μg/mouse]) intraperitoneally. Single-cell (sc-) RNAseq was performed on tumor infiltrating lymphocytes from each group (n=3/group) while the remaining mice were observed for disease progression by luciferase imaging and overall survival (n=10/group).

Results: Analyzing CH frequencies in a cohort of patients with a variety of solid tumor, we observed that the prevalence of CH was approximately 5 times higher in patients with cancer when compared to healthy age-matched controls. Furthermore, patients with detectable CH clones had significantly worse overall survival. Together, this suggests that CH plays an important role in the predisposition for cancer and worsened prognosis for patients. *In vivo,* our sc-RNAseq data revealed that Ly6C+ monocytes and MHC-II+ macrophages cells present within the pancreatic tumors of mice with *Tet2*$^{+/-}$ CH were significantly enriched for both type I and type II interferon (IFN) signaling. Further, these IFN+ myeloid cells were ablated after ICB therapy in *Tet2*$^{+/+}$ WT mice but enhanced in mice with *Tet2*$^{+/-}$ CH. PDAC tumors from mice with *Tet2*$^{+/-}$ CH had approximately half the total number of infiltrating CD8 T cells at baseline when compared to those from *Tet2*$^{+/+}$ WT mice. Upon ICB treatment, CD8 effector cells only expanded in the tumors from *Tet2*$^{+/+}$ WT mice. Functionally, this translated to more rapidly progressing tumors, resistance to ICB, and reduced overall survival in mice with *Tet2*$^{+/-}$ CH.

Conclusions: CH is present in upwards of 30% of all patients with solid tumors and is associated with significantly worsened prognosis, yet the mechanistic role of CH in cancer immunology

remains unexplored. Our approach to model PDAC in the presence of *Tet2*$^{+/-}$ CH *in vivo* revealed distinct alterations in the tumor microenvironment that ultimately influenced tumor progression and response to ICB. Our proposed research lays the groundwork to bridge the fields of solid tumor immunology and clonal hematopoiesis to address novel mechanisms of immunotherapy resistance that will span cancer type and, ultimately, improve patient care.

PR14/B037: Dysregulation of ILC3s unleashes progression and immunotherapy resistance in colon cancer

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Background and Objectives: Chronic inflammation is recognized as a causative factor in the development of cancer and recent paradigms suggest that microbe-driven chronic inflammation is causally associated with the development and progression of cancer in the colon. Group 3 innate lymphoid cells (ILC3s) are a key immune cell population that regulates intestinal inflammation and immune dialogue with the microbiota. ILC3s are enriched in the gastrointestinal tract and critically influence immunity, inflammation, and tissue homeostasis, yet their role in cancer remains elusive. Further, the ability to identify and comprehensively characterize tumor-infiltrating ILC3 in human cancers is still under investigation. In this study, we hypothesize that ILC3s are involved in the regulation of adaptive immunity, the control of host-microbiota interactions, and the progression or therapeutic responsiveness of tumors in the context of CRC.

Methods: To mechanistically interrogate the influence of ILC3 on colon cancer we performed a comprehensive phenotypic and transcriptional analysis of tumor-infiltrating ILC by flow cytometry and RNA-Seq on resected-colorectal tumors, polyps, and matched-adjacent non-malignant tissues from a cohort of 72 patients. Further, we characterized ILC3s in mouse models of chemically induced (AOM-DSS) or spontaneous ($APC^{min/+}$ and $CDX2^{Cre}$-$APCmin^{f/f}$) colon cancer. We further generated a new mice model with an ILC3-intrinsic deletion of MHCII ($MHCII^{\Delta ILC3}$) to determine the functional role of ILC3s during tumor progression and responsiveness to anti-PD-1 checkpoint blockade therapy. We have performed Fecal Microbiota Transfer (FMT) to specifically determine how the dialogue between ILC3s and the microbiota can modulate tumor responsiveness to immunotherapy.

Results: Here, we identify that colorectal cancer (CRC) manifests with altered ILC3s that are characterized by reduced frequencies, increased plasticity, and an imbalance with T cells. We evaluated the consequences of these changes in mice and determined that a dialog between ILC3s and T cells via major histocompatibility complex class II (MHCII) is necessary to support colonization with microbiota that subsequently induce type-1 immunity in the intestine and tumor microenvironment. As a result, mice lacking ILC3-specific MHCII develop invasive CRC and resistance to anti-PD-1 immunotherapy. Finally, humans with dysregulated intestinal ILC3s harbor microbiota that fail to induce type-1 immunity and immunotherapy responsiveness when transferred to mice. Collectively, these data define a protective role for ILC3s in cancer and

indicate that their inherent disruption in CRC drives dysfunctional adaptive immunity, tumor progression, and immunotherapy resistance.

Conclusion: Collectively, these data define a protective role for antigen presenting ILC3s in cancer and indicate that inherent disruption of this pathway drives progression and immunotherapy resistance in CRC. These results also have broad implications to our understanding of ILC3s and host-microbiota interactions in multiple cancer types and will pave the way towards targeted approaches to manipulate these pathways to improve cancer therapies.

[This proffered abstract will also be presented as a poster in Poster Session B.]

PR15/B010: Identifying and overcoming barriers that limit the therapeutic anti-tumor efficacy of individualized RNA-lipoplex vaccines

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The utility of RNA-based vaccines in preventing infectious disease has been well established in recent years, serving as a critical tool in combating the COVID-19 pandemic. Such breakthroughs provide an exciting opportunity to continue the advancement of research focused on driving neoantigen targeted anti-tumor immune responses in the treatment of cancer. These efforts are further encouraged given that the initial hurdles of patient personalization and manufacturing time have also been overcome. In spite of such progress, the exact requirements for vaccine-induced T cells to control tumor progression in vivo remain unclear. Here, we use luminex, flow cytometry, and single cell RNA and TCR sequencing to delineate the determinants for ongoing preclinical tumor growth control using an individualized mRNA-based vaccine. This vaccine consists of two mRNAs complexed with liposomes to form a RNA lipoplex (RNA-LPX) for intravenous injection. Each mRNA encodes for 10 unique MC38 tumor neoantigens and also functions as adjuvant through activation of toll-like receptor (TLR) 7/8 to promote potent immunity. RNA-LPX elicits broad T cell responses and induces a transient systemic inflammatory response, characterized by an increased concentration of multiple serum cytokines, including IL6, IFNα, and IFNγ.

We find that weekly RNA-LPX vaccination effectively controls tumor progression across a wide range of tumor burdens with more robust control observed in smaller, less immunologically mature tumors versus larger more established tumors. We also assessed the requirement for continued weekly vaccination by comparing tumor growth control in mice who had received either 2, 3, or 4 weekly vaccinations. As the duration of treatment increased, so did the response rate with 40%, 60%, or 73% of mice achieving partial or complete responses respectively. This data clearly establishes a strong therapeutic benefit for ongoing vaccination. Combined, these data suggest that the capacity of neoantigen specific T cells to limit tumor growth is not absolute and is dependent on either a permissive tumor microenvironment (TME) or a vaccine that provides ongoing T cell support. Consistent with this, following vaccination we see a significant increase in the number of neoantigen-specific CD8 tumor infiltrating lymphocytes (TILs). These

CD8 TILs are proliferative and harbor potent effector and cytotoxic functions, characterized by high expression of Ki67, PD1, TIM3, and granzyme B. Furthermore, we observe broad TME remodeling, including a shift in the magnitude and phenotype of tumor associated macrophage (TAM) and dendritic cells (DC). In particular, we see a decrease in CD206 expression by TAM and an increase in iNOS expression by both TAM and DC populations. Following cessation of vaccination, CD8 TILs lose proliferative capacity, corresponding with a rapid decline in the yield of CD8 TILs and reduced MC38 tumor growth control. Furthermore, single cell RNA and TCR sequencing analysis demonstrates that by 12 days after vaccination, T cells that are retained and clonally expanded in the tumor undergo a significant shift in their functional state. This may be a result of negative signaling via immune checkpoint inhibitors, lack of supportive cytokine signaling, and/or phenotypic shifts in the myeloid compartment. In support of this hypothesis, we evaluated RNA-LPX vaccination in combination with programmed death-ligand 1 (PD-L1) checkpoint inhibition and found that this combination therapy synergizes to promote anti-tumor immunity and further improve efficacy, especially of larger established tumors.

These data suggest that generation of a neoantigen-specific T cell response alone is not sufficient to maintain anti-tumor activity. Rather, ongoing T cell boosting, modulation of local immune milieu, and/or blockade of local suppressive signals are likely required to maintain T cell function at the tumor site and promote meaningful and lasting tumor regression.

[This proffered abstract will also be presented as a poster in Poster Session B.]

PR16: Intratumoral mregDC and CXCL13 T helper niches enable local differentiation of CD8 T cells following PD-1 blockade

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Here, we leveraged a large neoadjuvant PD-1 blockade trial in patients with hepatocellular carcinoma (HCC) to search for correlates of response to immune checkpoint blockade (ICB) within T cell-rich tumors. We show that ICB response correlated with the clonal expansion of intratumoral $CXCL13^+$ $CH25H^+$ $IL\text{-}21^+$ $PD\text{-}1^+$ CD4 T helper cells ($CXCL13^+$ Th) and $GranzymeK^+$ $PD\text{-}1^+$ effector-like CD8 T cells, whereas terminally exhausted CD39hi TOX^{hi} $PD\text{-}1^{hi}$ CD8 T cells dominated in non- responders. Strikingly, most T cell receptor (TCR) clones that expanded post-treatment were found in pre-treatment biopsies. Notably, $PD\text{-}1^+$ $TCF\text{-}1^+$ progenitor-like CD8 T cells were present in tumors of responders and non-responders and shared clones mainly with effector-like cells in responders or terminally differentiated cells in non-responders, suggesting that local CD8 T cell differentiation occurs upon ICB. We found that these progenitor CD8 T cells interact with $CXCL13^+$ Th cells within cellular triads around dendritic cells enriched in maturation and regulatory molecules, or “mregDC”. Receptor-ligand analysis revealed unique interactions within these triads that may promote the differentiation of progenitor CD8 T cells into effector-like cells upon ICB. These results suggest

that discrete intratumoral niches that include mregDC and CXCL13^{+} Th cells control the differentiation of tumor-specific progenitor CD8 T cell clones in patients treated with ICB.

Poster Session A

A001: Autologous tumor cell immunotherapeutic platform, with evidence of clinical activity in glioblastoma, induces in vitro immune responses in both glioblastoma and endometrial cancer

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Background:
Imvax is developing a novel personalized immunotherapeutic platform combining irradiated, patient-derived tumor cells and insulin-like growth factor type-1 receptor antisense oligonucleotide (IMV-001) in biodiffusion chambers (BDC; 0.1-micron pores). The combination product IGV-001 was recently evaluated in a newly diagnosed glioblastoma (GBM) phase 1b clinical trial [Andrews DW, 2021]. Median overall survival of highest exposure IGV-001-treated 'Stupp-eligible' patients [Stupp R, 2005] was 38.2 months compared with 16.2 months in recent standard-of-care-treated patients (p=0.044) [Andrews DW, 2021; NCT02507583]. We have now manufactured the endometrial cancer analog to IGV-001, IEC-001, using patient-derived endometrial tumors and developed an assay using matched peripheral blood mononuclear cells (PBMC), to evaluate the in vitro immunostimulatory activity of both IEC-001 and IGV-001, as a precursor to future translational studies.

Methodology:
Nine IEC-001 and three IGV-001 patient-matched PBMC co-culture assays were evaluated. Patient-derived tumor cells treated with IMV-001 were loaded into BDCs, spiked with more IMV-001, and irradiated (5-6 Gy). Co-cultures were established with direct IEC-001 or IGV-001-PBMC contact and via Transwell® to mimic BDC membranes separating product from immune cells. Flow cytometric analyses were conducted to evaluate dendritic cell maturation, T cell activation and memory subsets on days 3, 7, 14 &/or 21. Some co-cultures were re-stimulated on day 22 with fresh tumor and analyzed 7 days later.

Results:
Co-culture of PBMCs with either matched-IEC-001 or IGV-001 showed increased immunological activity. Increased dendritic cell maturation was observed in direct and indirect co-cultures, presenting greater expression of HLA-DR beyond that of PBMC controls (9/9 IEC-001 experiments). Elevated percentages of activated CD4+ and CD8+ T cells were observed for both co-culture conditions (direct and Transwell) after 7 days with sustained activation on day 14. Of note, CD107a and CD69 were upregulated in ~80% of IEV-001 cultures. Increased effector memory CD4+ T cell subset was also observed by day 21 in 67% of the experiments. Lastly, data shows that T cell activation and memory responses were potentiated upon rechallenge.

Conclusions:
Dendritic cell maturation, CD4 and CD8 T cell activation, and increases in central and effector memory T cell phenotypes in vitro support the immunostimulatory activity of Imvax's immunotherapeutic platform. In combination with the favorable clinical results obtained in GBM

using IGV-001 [Andrews DW, 2021], our data suggest a path forward to evaluate IEC-001 in future clinical studies.

A003: Clinical update with molecular and immunological biomarker's analysis of patients with locally advanced or oligometastatic melanoma receiving neoadjuvant ipilimumab/nivolumab.

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Introduction:
Locally advanced or oligometastatic melanoma patients (pts) represent a clinical unmet need due to frequent relapses after surgery and adjuvant treatments. Previous studies demonstrated neoadjuvant to be the optimal setting in order to address translational issues. We performed an open label, single arm and two sites sequential clinical study of neoadjuvant immunotherapy, surgery and adjuvant immunotherapy in locally advanced/oligometastatic pts, evaluating efficacy and safety, together with humoral, molecular and immunological biomarkers.
Methods:
Treatment schedule consisted in four primary cycles of inverted dose Ipilimumab 1 mg/kg and Nivolumab 3 mg/kg every 3 weeks, followed by radical surgery and adjuvant Nivolumab 480 mg every 4 weeks for 6 cycles. Primary objective was pathological complete remission (pCR) rate, while secondary objectives were: safety, feasibility and efficacy; QoL; identification of molecular/immunological biomarkers of response and resistance (somatic genetic drivers, tumor mutational burden, mutational signatures, predicted neoantigens, germline HLA typing, somatic HLA mutations and liquid biopsy); degree of immune activation and inflammation; longitudinal evaluation of the gut microbiome.

Results:
From March 2019 to April 2021, 43 pts were enrolled in the trial and, with an intent to treat of 35 pts, 34 completed the neoadjuvant phase, 30 received surgery and 28 completed the adjuvant phase. Four pts were withdrawn during the primary phase for progression (2), toxicity (1) and consent withdrawal (1). At surgery: 18/30 pts (58%) obtained pCR, 2/30 (7%) near pCR, 4/30 (13%) pPR and 7/30 (22%) pNR, reaching a rate of pCR/near pCR of 65%, thogh meeting the primary endpoint. Relapses occurred in 11 pts: 2 pts progressed during primary phase and did not undergo surgery; 9 pts progressed during adjuvant or follow-up phases and a total of 4 pts died due to disease progression. With a median follow-up of 28 months, 18 months PFS and OS were 77% and 85%, respectively. PFS was significantly better for responders (89) than non responders (57%). Treatment related toxicities were mainly G1-2 and only 6 pts (17%) developed G3-4 adverse events (AE): 3 transaminitis, 1 pneumonitis, 1 myocarditis, 1 CPK increase and 1 dermatomyositis. Translational studies on samples collected before, during therapy and at progression (whole exome sequencing and gut microbiota dynamics on longitudinal samples) showed some relationships with responses and developing resistances. In particular, longitudinal analysis reveals increasing difference in gut microbiota diversity between responders and not-responders over the course of therapy, suggesting that immunotherapy impacts the gut

microbiota differently between patient groups. Differential abundance also identifies taxa increased at end-of-neoadjuvant timepoints in responders, which correlated with subsets of circulating immune cells and soluble molecules with reported roles in antitumor immunity and disease regulation, thus shortlisting candidate taxa with potential role in response. Paired analysis of Luminex profiles at before vs end of neoadjuvant immunotherapy highlighted circulating inflammatory molecules responsive to treatment, including CX3CL1, CXCL10 and CXCL9. In particular, our data showed a positive correlation between circulating eosinophils, abundance of Firmicutes bacteria and IL5. Moreover, in responders, IL5 correlated with increased eosinophils with Christensenellaceae and Clostridium sensu stricto, while CXCL10 with Ruminococcus torques.

Conclusion:
Neoadjuvant immunotherapy with Ipilimumab/Nivolumab in locally advanced/oligometastatic melanoma pts is feasible and able to achieve an elevated pCR/near pCR rate at surgery (65%), which appears to be predictive of long-term relapse free survival. Overall, our translational data suggest potential mechanistic links between the gut microbiota and favorable response.

A004: Anti-GD2 CAR-NKT cells are safe and produce antitumor responses in patients with relapsed/refractory neuroblastoma

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Conventional T cells expressing chimeric antigen receptors (CARs) remain largely ineffective in solid tumors. Vα24-invariant natural killer T cells (NKTs), a subset of innate-like T cells, have antitumor properties that can be enhanced by transgenic expression of tumor-specific receptors. We have conducted a first-in-human clinical evaluation of autologous NKTs expressing a GD2-specific CAR with interleukin-15 in patients with relapsed/refractory neuroblastoma (NB) using standard 3+3 dose-escalation schema (NCT03294954). Objectives included assessing safety and antitumor activity of GD2-CAR-NKTs, as well as patient immune response. Twelve patients with a median age of 7.6 years (range 2-12) have been infused on four dose levels (DL; CAR+ NKTs/m^2): DL1 ($3x10^6$), DL2 ($1x10^7$), DL3 ($3x10^7$), and DL4 ($1x10^8$) following lymphodepletion chemotherapy. Patient-derived NKTs were expanded to produce $\geq 10^9$ CAR-NKTs with median 93.1% NKT purity (range 74.1-97.2%) and 60.1% CAR expression (range 20.2-87.7%) after 10.9 ± 2.5 days in culture. Four of 12 infused patients received two infusions. No dose-limiting or grade 2+ toxicities related to CAR-NKTs were observed, and reinfusions did not result in more lymphodepletion- or CAR-NKT-related toxicities. Antitumor responses were evaluated using International Neuroblastoma Response Criteria and quantified using changes in Curie scores. The overall response rate was 25% (3/12) and disease control rate was 58% (7/12) including four patients with stable disease (SD), two partial responses (PR), and one complete response (CR) that lasted 12 months. For correlative studies, we divided patients into non-responders (7 total) and responders (5 total; 3 PR/CR and 2 SD, 1 with 30% Curie score reduction and 1 with clearance of bone marrow metastases). Responders had a significantly higher area under the curve (AUC) for CAR-NKT frequency in peripheral blood over a four-week period post-infusion than non-responders, indicating a longer exposure to the therapeutic

cells in the former group (911 vs 261.3, p=0.032). Additionally, the frequency of central memory-like CD62L+ CAR-NKTs in infused products correlated with AUC (R^2=0.61, p=0.003) and was higher in responders than non-responders (71% vs 35.3%, p=0.002). Analysis of tumor biopsies from eight patients two weeks post-infusion revealed that CAR-NKT infiltration into tumor tissues quantified by qPCR correlated with expansion of the cells in the peripheral blood (R^2= 0.98; p<0.001). These results indicate that CAR-NKTs are safe, expand post-transfer, can localize to tumor sites and produce objective responses in NB patients.

A006: Daratumumab (anti-CD38) but not edicotinib (CSF-1R inhibitor) demonstrates target engagement within the primary prostate cancer, bone marrow and systemic circulation of patients with localized disease

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INTRODUCTION: Immunotherapies have had limited clinical benefit in patients with advanced prostate cancer. This has been attributed to a prostate tumor microenvironment (TME) characterized by few effector T cells and enriched in immunosuppressive cells, cytokines and signaling pathways. Furthermore, this immunosuppressive TME differs between primary and bone metastatic sites of prostate cancer. Rational strategies are required to overcome the immunosuppressive TME to improve clinical outcomes.

HYPOTHESIS: Daratumumab (anti-CD38 antibody) or edicotinib (colony-stimulating factor-1 receptor [CSF-1R] inhibitor) may alter the immune balance within the prostate TME to promote anti-tumor responses.

METHODS: This study (NCT03177460) was an open-label, single-center phase I clinical trial in patients with high-risk, localized prostate cancer who received 4-weeks of daratumumab or edicotinib followed by radical prostatectomy (RP). Treated and untreated control prostatectomy specimens and patient-matched pre- and post-treatment bone marrow and peripheral blood mononuclear cells (PBMCs) samples were evaluated. The primary object was to evaluate safety (frequency of adverse events [AEs]). The secondary objective was to determine efficacy (pathologic complete remission [pCR] rate). Finally, the exploratory objective was to study immunological changes within the primary tumor tissue, bone marrow, and peripheral blood in response to the study drugs.

RESULTS: Twenty-five patients were treated with daratumumab (n=15) or edicotinib (n=10) and underwent RP without delays. None of the patients enrolled in this study experienced Grade 4 or 5 AEs. Grade 3 AEs with daratumumab were observed in three patients (12%; infusion reaction, n=2; urticaria, n=1), and no Grade 3 AEs occurred with edicotinib. Neither daratumumab nor edicotinib induced pCR or declines in serum prostate-specific antigen (PSA) levels in any of the treated patients. Treatment with daratumumab demonstrated evidence of target modulation, with consistent depletion of $CD38^+$ immune cell subsets ($CD4^+$ and $CD8^+$ T

cells, $CD4^+CD25^+FoxP3^+$ regulatory T cells [T_{reg}], $CD11b^+CD14^+HLA\text{-}DR^+$ myeloid cells, and $CD3^-CD19^-CD56^+$ NK cells) in primary prostate tumors, bone marrow, and peripheral blood. There were no consistent changes in $CSF\text{-}1R^+$ immune subpopulations within the primary prostate cancer, bone marrow, or systemic circulation with edicotinib treatment.

CONCLUSIONS: Daratumumab and edicotinib were safe in patients with high-risk localized prostate cancer but did not induce pCRs or declines in serum PSA levels. Target modulation was consistently observed in prostate tumors, bone marrow, and PBMCs following daratumumab treatment, but not with edicotinib. Agents targeting immunosuppressive molecular pathways within the TME such as daratumumab alone are insufficient to generate clinically meaningful anti-tumor responses in localized prostate cancer. Combinations with therapies that promote T cell infiltration are likely to be needed to improve clinical outcomes.

A007: Immune monitoring in patients with malignant peritoneal mesothelioma treated with adjuvant dendritic cell-based immunotherapy after cytoreductive surgery and hyperthermic intraperitoneal chemotherapy

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Background: Malignant peritoneal mesothelioma (MPM) is a highly aggressive neoplasm with a poor life-expectancy of only 12 months even after (palliative) surgery and/or chemotherapy. Cytoreductive surgery (CRS) and hyperthermic intraperitoneal chemotherapy (HIPEC) have resulted in improved median survival. Nonetheless, even after CRS-HIPEC, recurrence rates remain high. Dendritic cell-based immunotherapy (DCBI) has shown promising results in murine models with peritoneal mesothelioma and in clinical phase I/II studies for patients with pleural mesothelioma, indicating the potential of DCBI after CRS-HIPEC as therapeutic option for MPM.

Methods: Comprehensive immune cell profiling by multicolor flow cytometry was performed on prior to treatment and on treatment (after 1 and 3 vaccinations) peripheral blood samples of 14 MPM treated with DCBI after CRS-HIPEC as part of the ongoing MESOPEC trial (Dutch Trial Registry number NTR7060) in the Erasmus Medical Center, Rotterdam, the Netherlands. Data were analyzed by unsupervised clustering with FlowSOM.

Results: Treatment with DCBI was associated with an increased proliferation of circulating lymphocyte subsets, especially NK cells and CD4+ T-helper cells. In addition, effector memory (Tem) and central memory (Tcm) CD4+ T cells were more abundant after dendritic cell vaccination and frequencies of terminally differentiated effector memory (Temra) CD8+ T cells positively correlated with progression-free survival (PFS), suggesting that dendritic cell vaccination induces memory T cell differentiation. Co-stimulatory molecules, including ICOS, HLA-DR and CD28 were upregulated on CD4+ T-helper and CD8+ T cells, after treatment, specifically on memory and proliferating cells ($p<0.05$). Moreover, co-expression of ICOS and Ki67 on CD8+ T cells positively correlated with progression-free survival (PFS). This effect was

counterbalanced by significant upregulation of co-inhibitory molecules such as CTLA-4, PD-1 and CD39, on both the CD4+ T-helper and CD8+ T cell compartment.

Conclusion: Using comprehensive immune monitoring of peripheral blood of pre-treated MPM patients, dendritic cell vaccination has a diffuse immune modulatory effect on lymphoid cells. Overall the effects, such as the induction of proliferation and the expression of co-stimulatory molecules seemed to be more pronounced in patients showing a high PFS. This data shed light on immune modulatory effects of dendritic cell vaccination and provide a platform for future combination treatment strategies.

A008: Real-world comparator study: MVX-ONCO-1, a cell-based immunotherapy currently in Phase II, shows prolonged OS and PFS for patients with recurrent/metastatic Head & Neck squamous cell carcinoma (R/M HNSCC)

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BACKGROUND: MVX-ONCO-1 is an active-personalized cancer vaccine composed of irradiated autologous tumour cells and genetically modified encapsulated cells delivering standardized, sustained, stable level of the potent adjuvant granulocyte-macrophage colony stimulating factor (GM-CSF) at the vaccine site. Currently a multicentre Phase IIa trial evaluating its safety, feasibility and efficacy in R/M HNSCC patients (pts) in ≥ second line systemic therapy is ongoing (SAKK 11/16). This real-world study was designed to contextualize the phase 2 single-arm trial results by creating an external comparator arm.

METHODS: All 16 R/M HNSCC pts treated with MVX-ONCO-1 by April 1st 2022 are included in this analysis (14 from the ongoing SAKK11/16 trial and 2 from the Phase I trial). Eligible pts had R/M HNSCC progressing after at least one line of systemic therapy, including anti-PD-1 checkpoint inhibitor (CPI) in 13 pts. The vaccine therapy consists of 6 administrations weekly for 4 weeks followed by 2 boosters 2 weeks apart without maintenance. One administration consists of sub-cutaneous (sc) implantation of 2 capsules containing GM-CSF secreting cells and a sc injection of irradiated autologous tumour cell suspension.

A real-world comparator cohort was built with pts from two clinical sites from IQVIA's Oncology Evidence Network: University Hospital Frankfurt, Germany and Portuguese Oncology Institute of Porto, Portugal. Real-world adapted inclusion/exclusion (I/E) criteria from the SAKK 11/16 trial were applied resulting in a cohort of 62 pts which were treated according to the respective local guidelines (chemotherapy, cetuximab, CPI).

To improve comparability, the two cohorts were balanced at line of therapy (LOT) level along pre-defined prognostic factors (age, gender, year of advanced HNSCC diagnosis, time from initial to advanced stage diagnosis, tumour site, WHO score, Human Papilloma Virus (HPV) status, previous radio-chemotherapy or immunotherapy, and LOT number). Balance between the weighted cohorts was assessed. Balancing on LOT level rather than on pt level increases the sample size in the real-world comparator, as one eligible pt can have multiple eligible LOTs.

The primary objective was to evaluate the efficacy of MVX-ONCO-1 versus anti-cancer therapies in real-world practice using overall survival (OS), survival rate at 26 weeks, time to next therapy (TTNT) and progression-free survival (PFS). Results were verified with several sensitivity and subgroup analyses.

RESULTS: The 16 trial and 62 comparator pts were balanced for key prognostic variables (listed above). Median OS in the trial population was not reached (95% confidence interval (CI): 6.8 months, NA) vs 7.6 months (6.0, 9.2) in the comparator cohort. OS at 26 weeks was 87.1% (73.8, 100.0) versus 60.2% (49.5, 73.1) respectively, and therefore there was an absolute risk reduction (ARR) of death at 26 weeks due to MVX-ONCO-1 of 26.9% (8.4, 35.4). Median TTNT was 4.1 months (1.8, NA) in the trial cohort versus 8.7 months (7.3, 14.9) and median PFS was 4.1 months (1.8, NA) in the trial cohort and 2.7 months (2.1, 3.5) in the comparator cohort. Prolonged survival was observed in both HPV pos and HPV neg R/M HNSCC pts. No systemic adverse reaction related to the personalized immunotherapy was reported.

CONCLUSIONS:

The data from this Real-World Comparator Study shows that in heavily pre-treated R/M HNSCC pts, cell-based immunotherapy with MVX-ONCO-1 improved both OS and PFS. Indeed, we observed a clinically meaningful ARR of death at 26 weeks of 26.9%. Detailed analysis will be presented at the meeting. MVX-ONCO-1 treatment is feasible, safe and well tolerated. Pts in both cohorts had received post-study treatment, which may indicate a potential benefit to combine the MVX-ONCO-1 vaccine with other therapies to achieve further clinically relevant survival benefits.

A009: Androgen shapes sexual dimorphism of skin immunity by regulating skin-resident innate lymphoid cells

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Sex dimorphism in physiology is important in medicine because it can lead to sex differences in the susceptibilities to diseases and finally cause different clinical manifestations. The skin immune system mediates the skin health status, but the sex differences of the skin immune system are still unclear. This study aims to investigate the sex differences in the skin immune system and decipher the underlying regulatory factors. We found that female mice have a

significantly higher baseline level of skin resident innate lymphoid cells (ILCs) and CD4+ T cells than males. Females also have a higher magnitude of adaptive immune responses during commensal bacteria association and pathogen infection. These observed 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. Dendritic cells (DCs) play a fundamental role in adaptive immune responses that they can collect antigens from commensals and pathogens, and then migrate to lymph nodes to trigger immune responses. We found that female mice have a significantly higher baseline level of skin resident DCs, including $CD103^{+}$ cDC1, $CD11b^{low}$ cDC2 and Langerhans cells (LCs), which play a fundamental role in adaptive immune responses. Sex-related differences of DCs are also regulated by male sex hormones, that castration significantly increased the level of those DCs in males, but testosterone injection decreased the skin DC level in females. Our single-cell RNA-seq (scRNA-seq) data reveals that skin DCs from females have a more activating gene expression signature than ILCs from males. However, androgen receptor is not expressed in skin DCs, which suggests that androgen regulates DCs by an indirect manner. Interestingly, we found that skin ILCs, one of the dominant lymphocyte populations in the skin, express a dramatically higher androgen receptor (AR) than other lymphocytes based on the scRNA-seq data, which suggest ILCs could be the dominant androgen response immune cells. ILCs from females also have a more activating gene expression signature than ILCs from males. In addition, ILC2 are the dominant population of ILCs in the skin, and females have a significantly higher level of skin ILC2 than male mice. Cytokine production in ILC2 is also higher in females than in males. Moreover, our data suggest that skin DC homeostasis is tightly regulated by skin ILC2 that skin DC configuration is largely distorted in $Rag2^{-/-}\gamma c^{-/-}$ mice, but not in $Rag1^{-/-}$ mice. We also demonstrated that sex-related differences of skin DCs are dependent on the AR signaling in skin ILC2 that these sex differences of DCs are not observed in $Ar^{Flox}Il7r^{Cre}$ mice, in which the *Ar* gene is depleted in ILC2. In addition, our data reveal that IL-13 plays a critical role to maintain the level of skin DCs, that IL-13 deficient mice have a remarkable decrease of skin DCs compared with wild-type mice, and the sex differences of DCs are also largely impaired. Therefore, based on these findings, we suggest that AR signaling negatively regulates the level of skin resident ILC2s and DCs, thereby shaping sex-specific skin immunity to commensals, pathogens, and/or other stimuli. Our current results support the idea that sex hormones set up a more sensitive immune background in females, which could make females more sensitive to further inflammatory stimulation and thus more susceptible to inflammatory diseases but more resistant to infectious diseases or cancers.

A010: Identification and characterization of vaccine-induced IDH1R132H-specific T cell receptors in glioma patients

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More than 80% of World Health Organisation (WHO) grade II/III cases of glioma harbour the recurrent R132H point mutation in isocitrate dehydrogenase type 1 (IDH1). The shared clonal

neoepitope was shown to be presented on major histocompability complex II (MHC II) molecules and induces mutation-specific T helper cell responses. The NOA16 first-in-human phase 1 clinical trial proved safety and immunogenicity of an IDH1R132H long peptide vaccine in patients with gliomas.

AMPLIFY-NEOVAC (NOA21) is a randomized phase 1 “window-of-opportunity” clinical trial to demonstrate safety, tolerability and intratumoral immunogenicity of a long IDH1R132H peptide vaccine in combination with the programmed death-ligand 1 (PD-L1) targeting immune checkpoint inhibitor Avelumab in patients with resectable recurrent IDH1R132H-mutated gliomas, allowing for the analysis of post-treatment tumor tissue in all patients.

Using peptide-based expansion assays of PBMCs from seven NOA21 patients with varying human leucocyte antigen (HLA)-alleles, T cell receptors (TCRs) were selected and cloned for testing their reactivity against mutant IDH1. Of 146 tested TCRs, 104 (71%) were reactive. The anti-IDH1 immune response was tracked over time in both blood and tissue (pre- and post vaccination), and gene signatures of reactive TCRs from tissue were defined using single-cell sequencing of tumor-infiltrating lymphocytes (TILs). HLA-restrictions of reactive TCRs were analysed using CRISPR-Cas knockout of MHC II alleles in autologous lymphoblastoid B cell lines.

Identification of IDH1-reactive TCRs and analysis of their HLA-restriction may be used to build a TCR warehouse to offer patients with IDH1-mutant gliomas off the shelf adaptive T cell therapies.

A012: Gasdermin E antagonizes immune-driven tumor mutagenesis by limiting apoptotic recovery

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Cancer cells that recover from Caspase activation under chemical insults have been proposed to increase tumorigenesis, mutagenesis, and aggression. In the absence of therapeutic intervention, the primary mode of Cytolytic T Lymphocyte (CTL) and NK cell cytotoxicity within the tumor microenvironment remains apoptosis. Here, we utilized Gasdermin E (GsdmE), a pore forming executioner protein downstream of Caspase-3, to determine whether CTLs and NK cells induce sublethal cytotoxic stress to drive mutagenesis. We found that immune surveillance hindered the growth of GsdmE expressing tumors, with limited immune memory induction. Rather, GsdmE expression truncated the apoptotic process and diminished the recovery of cells undergoing apoptosis. GsdmE expression enhanced the effective per capital kill rate of CTLs by limiting the outgrowth of mutant clones. Using published human TCGA datasets, we found that globally, tumor GsdmE expression inversely correlated with tumor mutational burden and rates of insertion/deletion, in a manner dependent on tumor immune cell presence. Furthermore, the strength of the inverse correlation between GsdmE expression and genome instability can be further graded by expression of Caspase activated DNase, the DNA fragmentation factor downstream of Caspase-3 activation, which has been shown to induce mutagenesis in cells that recover from chemically induced apoptotic stress. Finally, in a mouse model of metastatic colon carcinoma, we found that cytolytic cells enforce the loss of expression of endogenous GsdmE, a

finding that provides mechanism to explain the long standing observation of GsdmE silencing by promoter hypermethylation in tumor tissues. From these data, we propose that GsdmE functions to truncate the apoptosis process under immune surveillance during tumorigenesis, consequently limiting immune-driven tumor mutagenesis. We further propose, that GsdmE silencing in cancer cells may be an immune-evasion strategy to allow for enhances apoptotic survival and mutagenesis.

A013: Tissue-specific interferon-gamma drives regulatory T cells to restrain DC1-mediated priming of cytotoxic T cells against lung cancer

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Although failure to respond to checkpoint blockade immunotherapies is frequently associated with a lack of T cell infiltration into the tumor, clinical data suggests that in patients with lung cancer, T cell-inflamed tumors can also be resistant to therapy. Recent work by our group identified that checkpoint blockade immunotherapy resistance in a T cell-inflamed pre-clinical mouse model of lung cancer is driven by a lung cancer-specific $CD8^+$ T cell dysfunctional program, characterized by reduced cytolytic capacity and established during priming in the tumor-draining mediastinal lymph nodes (mLN).[1] In this study, we sought to uncover lung-specific mechanisms that blunt priming of anti-tumor cytotoxic T cell responses.

To study T cell responses against lung cancer, we implanted a syngeneic lung cancer cell line (KP) orthotopically in the lungs or subcutaneously in the flanks of C57BL/6 mice. Although expansion and accumulation of tumor-reactive T cells was comparable between both inguinal (iLN) and mLN, $CD8^+$ T cells primed in response to lung tumors in the mLN failed to upregulate key markers of effector $CD8^+$ T cell differentiation, namely CD25 and Granzyme B.

To mechanistically interrogate the induction of $CD8^+$ T cell dysfunction in response to lung cancer, we first affirmed that conventional type-1 dendritic cells (DC1) were required for T cell activation against both flank and lung tumors, as tumor-reactive T cell responses were significantly diminished in *Batf3*$^{-/-}$ mice. Comparing DC1 from tumor-draining mLN and iLN revealed equivalent antigen load, but reduced expression of CD80, CD86 and IL-12 on DC1 from the mLN. However, this blunted stimulatory capacity of mLN-derived DC1 was lost *ex vivo*. Since regulatory T cells (Treg) have been described to suppress DC, we depleted Treg cells and observed rescue of both stimulatory molecule expression on DC1 and cytotoxic T cell priming in the tumor-draining mLN. Thus, lung cancer-specific $CD8^+$ T cell dysfunction required the local presence of Tregs during priming. *Ex vivo* co-cultures of antigen-specific $CD8^+$ T cells with DC1 and Tregs sorted from mLN fully recapitulated the *in vivo* observation, indicating that DC1 and Treg were both required and sufficient to induce dysfunctional $CD8^+$ T

cells. Immunofluorescence studies revealed that the suppression was spatially coordinated within tissue-specific LN microniches and required antigen-specific contact between DC1 and Tregs. Abrogating MHCII-dependent Treg:DC1 interactions restored DC1 capacity to prime lung cancer-specific cytotoxic T cell responses.

Next, we investigated the tissue-specific activation of suppressive Tregs in the mLN. *Ex vivo* co-culture assays revealed that mLN Tregs suppressed DC1 stimulatory capacity more effectively than iLN Tregs. Transcriptional analysis and T cell receptor sequencing of Treg cells revealed that the lung-specific suppression was associated with clonally expanded $CXCR3^+$ T_H1-like effector Tregs, which were induced upon interferon sensing in the mLN. Interferon-gamma neutralization early during tumor induction could prevent Treg suppression effects and restore cytotoxic T cell priming in the mLN. Similarly, in cancer patients, interferon-sensing $CXCR3^+$ Tregs but not $CD8^+$/Treg ratios correlated with poor responses to checkpoint blockade immunotherapy.

Thus, we found a novel immunoregulatory mechanism that limits induction of cytotoxic T cell responses against cancer. Our work suggests that the functional quality of Tregs, specifically the interferon-induced $CXCR3^+$ T_H1-like effector state, rather than the quantity of Tregs, is instrumental in restraining tumor-reactive T cell responses and this represents a critical barrier to productive anti-tumor immunity.

[1] Horton, B.L. *et al.* (2021). Lack of CD8(+) T cell effector differentiation during priming mediates checkpoint blockade resistance in non-small cell lung cancer. Sci Immunol 6, eabi8800.

A016: Cancer induced CD8 T cell dysfunction: Beyond inhibitory receptors.

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Although immune checkpoint blockade (ICB) such as using anti-PD-1 has represented a turning point in cancer care, clinical responses are not observed in the majority of cancer patients. The mechanisms underlying this lack of responsiveness are still poorly understood and finding additional signals that regulate $CD8^+$ T cell anti-tumor functions has become a major priority. While most of the experimental strategies actually focus on the identification of additional inhibitory receptors restraining anti-tumor reactivity of $CD8^+$ T cells, the importance of activating receptors with regards to anti-tumor $CD8^+$ T cell functions and ICB efficacy need to be better understood.

Interestingly, we discovered that loss of the activating receptor CD226 is a critical mechanism altering LFA-1 functions and tumor infiltrating CD8+ T cell responsiveness. We combined complementary sets of experiments involving human samples and mouse tumor models to demonstrate that the tumor microenvironment promotes the accumulation of a subset of CD8+ T cells that loose CD226 through a mechanism involving EOMES transcription factor. More importantly, we found that CD226-negative tumor infiltrating lymphocytes have altered TCR

signaling, reduced anti-tumor functions and fail to respond to immunotherapy. Thus, our results revealed a yet unappreciated mechanism, whereby the loss of the activating receptor CD226 restrains CD8+ T cell functions and the therapeutic efficacy of cancer immunotherapy (*Weulersse et al, Immunity. 2020*).

More recently, we focused on CD137 (4-1BB) activating receptor, an enigmatic yet, promising target for immunotherapy. We made the rather unexpected observation that CD137 agonists stimulated the expansion of exhausted T cell (Tex) characterized by the expression of multiple immune checkpoints (PD1, Tim3, Tigit) and reduced effector functions (*Pichler et al, immunity. submitted*). We found that a TCR-independent, T cell-intrinsic CD137 signaling, involving the RelA and cRel NF-kB subunits, induced CD8+ T cell chromatin remodeling and expression of Tex cell specific genes. We explored the physio-pathological significance of our findings using T cell-specific CD137 deficient mice and different tumor models. We found that CD137 absence limited the accumulation of tumor infiltrating lymphocytes (TILs) with a Tex phenotype, revealing a key role for CD137 in cancer-associated T cell exhaustion. Yet, CD137-deficient CD8+ T cells failed to persist in tumors, resulting in higher tumor burden in T cell-specific CD137-deficient mice. Understanding the cellular process that drive T cell dysfunction has crucial implications for the treatment of cancer and infectious diseases. Our study through epigenomic, single cell transcriptomic and functional approaches unravel a new paradigm in which TCR independent signaling induced by CD137 promotes T cell exhaustion. These results also underline the importance of CD137 and T cell exhaustion program in cancer for TIL maintenance and limiting tumor growth.

A017: ACSS2 and ACLY cooperatively regulate histone acetylation and CD8 T cell exhaustion

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Upon acute infection or vaccination, naïve T cells differentiate into functional effector cells (T_{EFF}) and a subset of these cells develop in to memory cells (T_{MEM}) after antigen clearance. However, during chronic infection and cancer, persistent antigen stimulation drives $CD8^+$ T cells to progressively lose their functionality and become exhausted (T_{EX}). This is accomplished through the upregulation of inhibitory receptors, or checkpoints, that dampen the production of cytotoxic molecules and cytokines. Therapeutically targeting these inhibitory receptors with immune checkpoint blockade restores effector functions to T_{EX} cells, and has led to significant clinical benefit in the treatment of many cancer types. Recent studies have identified T_{EX} cells as a heterogeneous population of three major subpopulations with distinct characteristics. Importantly, the progenitor exhausted subpopulation (Pro_T_{EX}), defined as PD-1^{int}, $SLAMF6^+$ and $TCF7^+$, mediate the proliferative burst and long-term immunity in response to immune checkpoint blockade in patients (Ref) by replenishing the more terminally differentiated $CD8^+$ T cells, including effector exhausted cells (Effector_T_{EX}) and terminally exhausted cells (Ter_T_{EX}). An increased ratio of Pro_T_{EX} to Ter_T_{EX} cells correlates with clinical success to

immunotherapy in melanoma patients, emphasizing the need to develop therapies that enrich for Pro_T_{EX} cells to augment the efficacy of current immune checkpoint inhibitors.

Epigenetic reprogramming of $CD8^+$ T cells plays a critical role in initiating and maintaining the fate commitment in T_{EX} cells. Epigenetic profiling of $CD8^+$ T_{EX} cell subpopulations during chronic infection revealed distinct chromatin accessibility and histone 3 lysine 27 acetylation (H3K27ac) landscapes between Pro_T_{EX} and Ter_T_{EX} cells. However, the underlying molecular mechanisms that precede chromatin remodeling in Pro_T_{EX} and Ter_T_{EX} differentiation have remained elusive. Intriguingly, histone acetylation is sensitive to availability of the metabolite, acetyl-CoA, which is generated by various metabolic pathways and alteration of nuclear acetyl-CoA pools affect histone acetylation. Because $CD8^+$ T cells also undergo massive metabolic rewiring during exhaustion, we hypothesized that epigenetic remodeling during exhaustion is dependent on the metabolic state adopted by different T_{EX} subpopulations.

Here we identify a requisite role for the metabolic enzymes ACSS2 and ACLY, as critical metabolic sensors, in regulating the histone acetylation that drives fate determination of Pro_T_{EX} and Ter_T_{EX} cells. During chronic infection and tumorigenesis, in the absence of ACSS2, CD8 T cells had impaired nuclear acetyl-CoA levels, decreased histone acetylation and impaired Pro_T_{EX} formation. By contrast, in the absence of ACLY, CD8 T cells exhibited enhanced Pro_T_{EX} development, along with increased histone acetylation and enhanced cytokine production. Consequently, ectopic overexpression of nuclear ACSS2 in CD8 T cells leads to effective tumor control mediated by enhanced histone acetylation, increased Pro_T_{EX}formation and cytokine production. Furthermore, pharmacological inhibition of ACLY improved PD-1 responses to tumors. Our data suggest that ACSS2 and ACLY are critical metabolic coordinators in regulating epigenetic states and directing fate decisions of T_{EX} cells and highlight the importance of nuclear metabolic enzymes as novel therapeutic targets.

A018: Phosphatidylserine as a nonclassical inhibitory molecule on exhausted CD8 T cells

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CD8 T cells eliminate infected and malignant cells. However, during chronic infection and cancer, CD8 T cells are constantly stimulated, leading to exhaustion and loss of killing potential. Inhibitory receptors on CD8 T cells such as PD1 and Tim3 contribute to this dysfunctional state, and as a result, have become immunotherapeutic prospects to reinvigorate exhausted CD8 T cells in cancer. Nevertheless, some patients remain unresponsive, highlighting the need to identify novel inhibitory molecules. We asked if phosphatidylserine (PS), a lipid metabolite, can function as a metabolic inhibitory checkpoint on exhausted CD8 T cells. Homeostatically, PS localizes to the inner plasma membrane but can be externalized during apoptosis where it has potent immunosuppressive actions. However, less understood is the exposure and signaling function of externalized PS on live cells *in vivo*. Using an LCMV chronic infection model and three different mouse tumor models we find that antigen specific exhausted CD8 T cells expose PS, independent of cell death. RNA-seq and metabolomics suggests an upregulated PS metabolic circuit within exhausted CD8 T cells, potentially contributing to its exposure. Therapeutically,

blocking exposed PS *in vivo* with aPS antibodies during chronic infection reinvigorated the exhausted CD8 T cell response and worked synergistically with current cancer treatments such as aPDL1. RNA-seq of CD8 T cells isolated from aPS treated mice suggest that blocking PS induced an increased proliferative state of the exhausted CD8 T cells. Lastly, CD8 T cells from human renal cell carcinoma and non-small cell lung carcinoma also expose PS and upregulate a PS metabolic circuit., highlighting the translational potential of targeting exposed PS. Overall, we demonstrate that live CD8 T cells externalize PS as a potential 'non-classical' inhibitory molecule in mice and humans and begin to uncover an interesting aspect of exhausted CD8 T cell biology.

A019: Costimulatory domains direct distinct fates of CAR T cell dysfunction

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While CAR T cell therapy can be curative in some patients, clinical data suggest that T cell dysfunction after infusion is a leading cause of therapeutic failure. We sought to elucidate the regulatory pathways responsible for driving T cells to dysfunctional states and determine if CAR costimulatory domains, the primary structures differentiating approved CAR T cell products, influence dysfunction-promoting programs.

We established an *in vitro* model that recreates prolonged anti-CD19 CAR stimulation and performed integrated functional and genomic profiling of CD28 or 41BB-based (19/28 or 19/BB) CAR T cells over time. Both cell types were initially able to control high quantities of tumor but became dysfunctional after ~14 days of chronic stimulation. This dysfunction manifested as an inability to kill target cells, secrete cytokines, expand and persist, mirroring defects in function seen in patients who do not experience durable responses. RNA sequencing revealed that 19/28 and 19/BB had very similar transcriptional profiles at rest and during activation but diverge as they lose function. Dysfunctional 19/28 cells bore the hallmarks of classical T cell exhaustion, expressing high levels of *PDCD1, CTLA4, TOX* and *NR4A*. In contrast, dysfunctional 19/BB cells preferentially expressed class II MHC genes as well as genes associated with T cell memory, such as *LEF1, TCF7* and *IL7R*. Evaluation of chromatin accessibility using ATAC sequencing revealed a similar trend, with opening of loci associated with exhaustion in 19/28 cells and not 19/BB.

To further clarify the transcriptional identity of dysfunctional 19/BB cells we performed single cell RNA sequencing of both cell types at rest, during activation and after onset of dysfunction. These data revealed that dysfunctional 19/BB cells were a heterogenous population, wherein some cells expressed class II MHC genes but the majority (70%) belonged to a unique cluster defined by expression of *GNLY, CCL5, KLRK1, GZMA* and *ID2*. Single cell RNA sequencing of CAR+ T cells from a patient with diffuse large B cell lymphoma who received Kymriah, a 19/BB-based CAR T cell product, demonstrated consistent transcriptional evolution. This patient had a partial response one month after treatment with disease progression at three months. We evaluated circulating CAR+ T cells at days 14 and 100 after treatment and observed that day 100 cells were similarly defined by high expression of *GNLY, CCL5, KLRK1* and *GZMA*.

We next sought to identify the transcriptional regulators that led to 19/BB dysfunction. Using our ATACseq data we performed motif analysis to identify which transcription factor binding sites were becoming more accessible as each cell type progressed from rest to dysfunction. As has been previously shown, 19/28 cells demonstrated opening of sites for AP-1 factors Jun and Fos. 19/BB cells, instead, had increased accessibility at binding sites for forkhead box-O (FOXO) proteins. Consistent with these data, we observed increased expression of FOXO3 target genes in dysfunctional 19/BB cells, confirming not only increased accessibility but also increased FOXO3 activity in dysfunctional 19/BB cells. To determine if FOXO3 was responsible for failure of 19/BB cells we disrupted the *FOXO3* locus in T cells expressing either 19/28 or 19/BB and subjected these cells to our chronic stimulation cultures. Intriguingly, disruption of *FOXO3* consistently delayed the onset of dysfunction for 19/BB but not 19/28 cells.

Collectively, these data suggest that costimulatory domains play a central role in CAR-driven T cell dysfunction that leads to therapeutic failure. While CD28-based CARs promote classical exhaustion programs, 41BB-based CARs drive a dysfunctional trajectory driven by re-activation of FOXO proteins. Development of strategies that can bypass both forms of failure will be critical to improving the efficacy of this platform.

A020: Secondary resistance to immunotherapy is associated with death and deactivation of activated T cells

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Background: Immunotherapies have transformed the care of patients with multiple tumor types, but the majority who respond achieve partial and not complete tumor shrinkage. Partially controlled tumors invariably progress after a period of stabilization, but the reasons for this are not well known. We set out to explore mechanisms of immunotherapy failure in this setting, using a murine model of melanoma treated with a regulatory T cell (Treg)-depleting antibody combined with a cancer cell vaccine.
Methods: C57BL/6 mice were injected subcutaneously with B16 melanoma cells. Treatment with a mouse IgG2a depleting αCD25 antibody was given on day 5. A genetically modified, GM-CSF secreting B16 whole tumor vaccine (GVAX) was given intradermally on days 6, 9, and 12. Tumors were harvested for single-cell RNA sequencing (scRNA-seq) or multiparameter flow cytometry using panels designed to interrogate the activation and differentiation landscape of infiltrating T cells.
Results: GVAX and αCD25 combination therapy resulted in three different clinical response patterns – no response, partial response, and secondary resistance, with characteristic immune phenotypes. Around 90% of partially responsive tumors relapsed after day 35 post tumor inoculation. Reasoning that loss of immune control precedes clinical progression, we characterized evolution of the immune landscape in pre-relapse tumors. We collected stable, partially responding tumors on days 28, 35, and 47 for flow cytometry analysis. Over time, we found a decrease in the abundance of 4-1BB+TIM-3+TCF7- CD8+ effector memory T cells and cycling (Ki67+) CD4 effector cells (Teffs). In parallel, non-activated TCF7+ T cells rose in abundance towards levels seen in untreated tumors. Treg abundance also recovered over time.

ScRNA-seq and scTCR-seq analyses of pre-relapse and relapse tumors revealed that non-activated CD4 Teffs accumulating at relapse were transcriptionally equivalent to their activated counterparts at pre-relapse except for expression of activation related genes. Overlaps were found in CDR3 usage between CD4 activated and non-activated populations at both pre-relapse and relapse, suggesting that the accumulating non-activated CD4 cells had been deactivated. In contrast, little overlap in CDR3 usage was found between CD8 activated and non-activated populations at relapse, indicating that the accumulating non-activated CD8 cells had been replaced by new, non-reactive clones. Additionally, we observed that in pre-relapse tumors, the percentage of Fas+ cells in activated Teffs is higher than that in non-activated Teffs. Blocking Fas/Fasl interactions with an αFasl antibody synergized with αCD25 on stable tumors to prevent relapse and improve survival. Thus, strategies to limit death of activated effectors could be clinically effective in prolonging survival amongst patients with partially controlled tumors. Conclusion: Combined Treg depletion/whole tumor vaccine therapy is effective in a poorly infiltrated B16 melanoma model. A proportion of mice achieve partial response which invariably relapses, mimicking what is often seen in human disease. Amongst partially responsive tumors, treatment effects on the immune landscape were observed to decay over time, with loss of abundance of activated Teffs and a rise in non-activated populations and Tregs, suggesting a return to pre-treatment immune equilibrium. ScRNA-seq and scTCR-seq analyses of pre-relapse and relapse tumors suggested that progression may be associated with a loss of immune fitness characterized by deactivation and death of activated infiltrating Teffs.

A021: Strength of CD28 costimulation directs self-renewal and differentiation of TCF-1+ PD-1+ CD8 T cells through metabolic regulation

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In chronic infections and cancer, CD8 T cells that recognize pathogens/tumors differentiate into a hypofunctional exhausted state to survive. Characterized by high expression of the inhibitory receptor Programmed Cell Death (PD)-1, exhausted CD8 T cells are a heterogeneous population. T cell factor (TCF)-1^{+}PD-1^{+} progenitor exhausted cells (Tpex) maintain the antigen-specific T cell pool by self-renewal and differentiation into effector-like TCF-1^{neg}PD-1^{+} CD8 T cells. Tpex are responsible for the proliferative burst following PD-1 blockade therapy, and the presence of TCF-1^{+} CD8 T cells in tumor has been associated with clinical responses to immunotherapy. However, the cellular and molecular mechanisms that support Tpex self-renewal or differentiation are not well understood. Tpex have high CD28 expression, and we have shown that during PD-1 targeted therapies, CD28 costimulation is required for the reinvigoration of CD8 T cell responses. In this study, we sought to better understand the role of sustained CD28 signaling for self-renewal and differentiation of Tpex. In mice chronically infected with lymphocytic choriomeningitis virus (LCMV), we abrogated CD28 signaling by blockade of B7 ligands and observed a sharp reduction of virus-specific CD8 T cells (both TCF-1^{+} and TCF-1^{neg} subsets). To better understand the role of CD28 on Tpex, we evaluated how reduction or

abrogation of CD28 signaling impacted virus-specific CD8 T cells. During established chronic infection, deletion of both *Cd28* alleles resulted in reduction of both Tpex and TCF-1^{neg} subsets. In contrast, when CD28 signaling was reduced (deletion of one allele), TCF-1^{neg} PD-1^{+} CD8 T cells decreased, but the number of Tpex was not affected. RNA sequencing and metabolic assays revealed that sustained CD28 signaling during persistent antigen stimulation is required to maintain mitochondrial fitness of Tpex. Whereas low CD28 signaling was sufficient for Tpex self-renewal, stronger levels of CD28 signaling were necessary for differentiation. Mechanistically, we uncovered that stronger CD28 signaling on Tpex was associated with increased glycolysis and differentiation into more effector-like exhausted CD8 T cells. Our work supports the novel hypothesis that CD28 signaling strength modulates Tpex fate decision through metabolic regulation. Our data show that continuous CD28 signaling is required for long-term maintenance of antigen-specific PD-1+ CD8 T cells and enhanced CD28 signaling may determine effectiveness of T cell responses. These findings have important implications regarding cells interactions of Tpex and response to checkpoint therapy in cancer.

A022: Distinct regulation of CD4+ T-cell function in cancer and autoimmunity by canonical NF-kappaB subunits

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CD4+ effector T cells (Teff cells) play pleiotropic roles in immune responses. Whereas they are critical to limit tumor progression, uncontrolled Teff cell activation can also lead to autoimmunity. Thereby, it is of utmost interest to decipher the signal transduction pathways and transcription factors orchestrating their activation and function. While NF-kappaB (NF-kB) has been largely implicated in inflammation and adaptive immune responses, the specific roles of each of the 5 subunits that compose this family of transcription factors remain unknown. Here we hypothesized that distinct NF-kB subunits were required for the function of Teff cells in cancer and autoimmunity.

Using mice carrying conditional ablation of RelA and c-Rel, two subunits of the canonical NF-kB pathway, in Teff cells, we found that RelA, rather than c-Rel, shaped the transcriptome of Teff cells in the steady-state and their ability to polarize toward the TH17 lineage. Similar conclusions could be reached using CRISPR/Cas9-edited primary human Teff cells. Mechanistically, ChIP-Seq analyses revealed that RelA controlled gene expression both through direct binding to target genes, and through the regulation of other transcription factors. Consistently, RelA-deficient mice were fully protected against neuro-inflammation in a murine model of multiple sclerosis. scRNA-seq analyses revealed a failure in the transition from naïve T cells to pathogenic TH17 cells in the central nervous system (CNS) of RelA-deficient mice. Intriguingly, we also observed an accumulation of “exhausted” T cells in the CNS of cRel-deficient animals, although clinical severity was unaltered. This led us to test whether this mechanism could impair tumor immunity. Strikingly, Teff-restricted ablation of c-Rel, but not

RelA, impaired their accumulation and function in the microenvironment of transplanted tumors, resulting in enhanced cancer burden.

Together, our data highlight novel, context-dependent, regulators of Teff cell function. Moreover, we demonstrate a division of labor between the different subunits of the NF-kB pathway, paving the way to subunit-targeted immunotherapies in autoimmunity and cancer.

A023: PD-L1 checkpoint blockade promotes regulatory T-cell activity which underlies therapy resistance

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Immune checkpoint blockade (ICB), especially inhibition of PD-1 or its ligand PD-L1, has provided a paradigm shift in cancer treatment by unleashing anti-tumor immunity. Although ICB has shown superiority compared to more conventional therapies for multiple types of cancer, the majority of treated patients do not or only temporarily respond for reasons incompletely understood. Regulatory T cells (Tregs) are an important suppressive immunocyte and are able to express high levels of PD-1 and could therefore be involved in dictating response to treatment with anti-PD-1/PD-L1. 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 to eventually improve 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 the AE17-OVA mesothelioma murine tumor model, which were validated in the MC38- and B16F10 model using multicolor flowcytometry. We further validated the effect on Tregs by performing RNA sequencing and *in vitro* suppression assays. Furthermore, Tregs were depleted using anti-CD25 to investigate the functional involvement in treatment resistance. For clinical importance, single-cell RNA sequencing (scRNA seq) data obtained from Tregs isolated from human skin cancer patients (n=11) prior to and after anti-PD-1 treatment were reanalyzed (Yost et al., Nat Med, 2019). In addition, peripheral blood obtained from lung cancer (n=28) and mesothelioma patients (n=15) was assessed for the activation of Tregs following ICB.

We found that Tregs were primarily activated in treatment resistant murine tumor models compared to CD8 T cells and CD4-Th cells. This was reversed in treatment responsive 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, we revealed that Tregs increase their immunosuppressive capacity on protein, gene and functional level following treatment in the treatment resistant AE17-OVA model. Treg depletion by using a Fc-optimized anti-CD25 antibody sensitized anti-PD-L1 resistant AE17-OVA and B16F10 tumors by delaying tumor

growth and prolonging survival compared to both monotherapies. This coincided with strong upregulation of proliferation (assessed by Ki67) and activation by CD8 T cells and CD4-Th cells in peripheral blood. Finally, we also showed the clinical importance of Tregs in resistance to ICB as scRNA seq data revealed upregulation of a suppressive transcriptional gene program post ICB treatment, which was associated with lack of treatment response. In addition, anti-PD-1/PD-L1 ICB induced PD-1^+ Treg activation in peripheral blood of lung cancer and mesothelioma patients which was especially pronounced in non-responders.

In conclusion, these data reveal a yet unappreciated role for Tregs underlying anti-PD-1/PD-L1 treatment resistance, thereby providing guidance for identification of novel therapeutic targets aimed at rewiring Tregs to improve anti-PD-1/PD-L1 efficacy.

A024: Genome-wide CRISPR screen in tumor cells identifies the transcription factor Zfx as a key regulator of T cell-mediated target cell killing

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A large fraction of cancer patients exhibit an intrinsic or adaptive resistance to immune therapies that aim at boosting T cell responses against tumor cells. To better understand susceptible and resistant mechanisms in tumor cells of T-mediated killing, we used a genetic screening approach to identify relevant genes in tumor cells. CRISPR-Cas9 whole-genome editing was utilized to generate gene knockouts in tumor cells that were then co-cultured with antigen-specific cytotoxic T cells. Use of the murine colon carcinoma cell line MC38 that is sensitive to *in vitro* T cell-mediated killing as well as to *in vivo* immunotherapy treatment, allowed us to screen for tumor cells that are resistant to CD8 T cell-mediated killing and to identify underlying genes. Among the top hits of the screen are several well-known genes involved in apoptosis and TNFa-signaling such as Casp8, Fadd and Tnfrsf1a. One novel hit was the transcription factor Zfx (zinc-finger protein X-linked). We showed that Zfx knockout MC38 tumor cells were resistant to CD8 T cell-mediated killing *in vitro* as well as *in vivo*. We further investigated the role of Zfx in killing resistance by gene expression profiling. Zfx regulates expression of genes in several apoptosis pathways. We identified Caspase-3 as one of the central components regulated by Zfx. Interestingly, low expression of Zfx in certain human cancer patients correlated with decreased overall survival. Our results demonstrate that Zfx regulates tumor cells sensitivity to T cell-mediated killing and suggest a potential novel resistance mechanism to immunotherapy.

A025: Insight to molecular mechanisms of TOX-mediated programming of CD8 T cell exhaustion via domain-level mutagenesis

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Immune checkpoint blockade (ICB) and cellular immunotherapies have demonstrated substantial therapeutic utility in the treatment of select, often refractory solid and hematologic malignancies. A major barrier to improving the clinical efficacy or durability of such therapies, however, is the sustained cellular dysfunction that characteristically arises in exhausted CD8 T cells ("Tex"), whose development is initiated via chronic stimulation by persistent antigen. There remains great

need to more completely elucidate the Tex-intrinsic mechanisms driving cellular phenotypes of exhaustion and the underlying epigenetic programming of this state.

The purpose of our study is to initiate structure-function analyses of the transcription factor TOX in order to accelerate the identification of which TOX-dependent molecular transactions on chromatin direct Tex differentiation during the initiation of exhaustion. TOX is essential for this initiation of Tex development, repressing effector differentiation and potentiating epigenetic commitment to the Tex lineage. However, the molecular mechanisms by which this occurs remain completely unknown, as do the structures and individual functions of the regions of TOX protein N- and C-terminal to its HMG-box DNA binding domain. We hypothesized that these N- and C-terminal domains ("NTD," "CTD") offer important and useful insight to the Tex-specific function of TOX. First, they together comprise ~84% of the TOX protein. Second, there remains little evidence to support a sequence-specific DNA binding activity of the TOX HMG domain, suggesting a role for other factors in determining the genomic localization of TOX. Third, determining NTD or CTD function may enable opportunities to therapeutically modulate TOX activity via selective mutagenesis of these regions. Although TOX-deficient CD8 T cells are, at least initially, phenotypically enriched for Teff-specific features and attenuated of Tex features, they characteristically exhibit time- and differentiation-dependent deficits in survival or proliferation that may limit the therapeutic utility of complete TOX deletion.

To first test whether the NTD and CTD are required for known TOX activities *in vitro*, we queried surface expression of multiple IRs in wild-type CD8 T cells following retroviral overexpression of either full-length ("FL") TOX, the individual HMG, NTD, or CTD domains, or domain deletion mutants lacking either the NTD or CTD ("ΔNTD," "ΔCTD"). In this context, only FL TOX was sufficient to increase PD-1 expression. We then tested this requirement *in vivo* using the murine lymphocytic choriomeningitis virus model of chronic infection coupled with adoptive co-transfer of transgenic, antigen-specific *Tox*$^{-/-}$ and wild-type CD8 T cells each overexpressing each separate aforementioned construct. All regions of the TOX protein were again required to "rescue" intact PD-1, CD39, and 2B4 expression among transferred *Tox*$^{-/-}$ cells, as was also the case for population of KLRG1+ CD127- effector cells and subsets of the Tex lineage defined by surface Ly108 and CX3CR1 expression. Lastly, among transferred cells, the ratio of wild-type to "rescued" *Tox*$^{-/-}$ cells achieved via ΔNTD or ΔCTD overexpression approached that seen with rescue by FL to a greater extent than with the HMG domain alone.

Collectively, these results demonstrate that all regions of the TOX protein contribute structurally or functionally to its *in vivo* activity during the initiation of exhaustion, and that Tex proliferation capacity may be more amenable to NTD- or CTD-specific perturbation than to total TOX deletion. Our ongoing efforts are focused on identifying more specific critical regions within the NTD or CTD, and on identifying which interactions with other proteins these regions may mediate. These studies will have important implications for the design of future strategies to therapeutically target TOX.

A026: Optimally Stimulated Functional Effector CD8 T cells in Solid Tumors

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Cytotoxic T cell function is a critical mediator of anti-tumor immunity in cancer. In solid tumors,

CD8 T cells typically get activated in the tumor draining lymph node and rapidly undergo a differentiation towards exhaustion upon entry into the tumor. The dominant path to exhaustion is increasingly well understood—characterized by a transition from a $TCF7^{hi}Tox^{lo}$ progenitor phenotype through intermediate steps to terminally exhausted $TCF7^{lo}Tox^{hi}$ cells. Although chronic activation is considered a major driver of T cell dysfunction in the tumor microenvironment (TME), there is a virtual absence of methodologies to discern acute vs. chronic stimulation states in T cells. We address this critical knowledge gap by using a novel genetic marking system—a T cell reporter to delineate stimulation history, which illuminates an elusive CD8 T cell subset within the exhausted milieu with exemplary functional capability.

Repeated stimulation drives the downregulation of early activation gene transcripts in CD8 T cells, including that of *Cd69*, whose surface expression, on the other hand, indicates current activation. We thus generated and used a novel *Cd69* transcriptional reporter mouse, where the fluorophore TFP (Teal Fluorescent Protein) is knocked in to the 5' end of the gene and dissociated from 3' UTR-mediated post-transcriptional regulation which limits CD69 protein expression. Using *in vitro* repeated stimulation assays and probing CD8 T cell states under homeostasis, we established that TCR stimulation history is inversely imprinted on the baseline TFP levels. This transcriptional readout TFP, in conjunction with surface CD69 expression was thus used to demarcate chronic vs. acute stimulation in CD8 T cells. Using flow cytometry in vivo, ex vivo tumor slice culture system and subsequently single cell RNA sequencing, we mapped these activation states onto intratumoral CD8 T cells, revealing a trajectory of differentiation from poised progenitors (TFP^{hi}CD69-) to acutely activated effectors ($TFP^{hi}CD69^{hi}$) and eventually chronically stimulated terminal exhaustion (TFP^{lo}). An inflection point along this path, where acutely stimulated cells veer towards chronic stimulation-driven dysfunction, is marked by the expression of the tetraspannin CD81. Phenotypically, this $CD81^{hi}$ subset of functionally superior cytotoxic CD8 T cells comprises of activated progenitor and KLR (Killer Cell Lectin-like Receptor)-expressing exhausted cells and is enriched in mice during T cell-mediated tumor control. Likewise, human T cell specific *CD81* expression as well as an ensemble gene signature to identify this pool in bulk tumor transcriptome both emerge as robust and significant correlates of favorable patient outcomes in tumors with CD8 T cell prominence.

Using a novel reporter mouse, we report the discovery of a functional subpopulation of effector CD8 T cells in the post exhaustion TME. Based on this work, future research dissecting these elusive CD8 T cells and their niche within the exhausted milieu will uncover molecular programs that enable T cells to resist chronic stimulation-driven dysfunction.

A027: PI3K: a key driver of effector differentiation under conditions of T cell exhaustion

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Upon T cell receptor ligation, naive $CD8^+$ T cells differentiate into effector cells that help eliminate virus-infected cells and tumors. When infection persists, chronic antigen stimulation drives a state of T cell hyporesponsivness, called exhaustion. Although exhaustion is associated with T cell dysfunction, it can also prevent excessive T cell activation and immunopathology. However, exhausted T (Tex) cells are a heterogeneous population. Notably, a small subset of progenitor Tex cells (pTex), is required to maintain T cells during exhaustion; this cell population is also critical for successful responses to immune checkpoint blockade therapy in cancer. pTex cells are defined by and require the expression of the transcription factor TCF-1. Although our understanding of molecular mechanisms and signaling pathways associated with T cell exhaustion is advancing, further studies are necessary to identify pathways for therapeutic applications.

Class IA phosphatidylinositol 3-kinases (PI3Ks) are lipid kinases that are important for T cell signaling, differentiation, survival, and metabolism; p110δ is the predominant catalytic isoform in leukocytes. We have recently shown that TCF-1 expression is repressed in $CD8^+$ T cells from patients with Activated PI3K Delta (PI3Kδ) Syndrome (APDS) and in a mouse model of activated PI3Kδ syndrome (*Pik3cd*$^{E1020K/+}$ mice) expressing activated p110d. These mice displayed an increased effector $CD8^+$ T cell expansion and the inability to generate central memory responses in acute viral infections. How activated PI3Kδ affects Tex cell development and function is unknown. To dissect the role of PI3Kδ in Tex cell survival, differentiation, and function during exhaustion, we developed a high-dimensional, 37 color spectral flow cytometry panel and used this to evaluate T cell exhaustion *in a well described chronic infection model, lymphocytic choriomeningitis virus (LCMV) clone 13* in *Pik3cd*$^{E1020K/+}$ mice.

We found that more than half of *Pik3cd*$^{E1020K/+}$ mice rapidly died post-infection with LCMV cl13. Nonetheless, the few *Pik3cd*$^{E1020K/+}$ mice that survived recovered more rapidly than WT mice. In depth T cell analyses revealed that *Pik3cd*$^{E1020K/+}$ mice showed a large expansion of $KLRG1^+$ effector-like cells, with increased effector functions, but repressed Tex development, associated with decreased numbers and percentages of $TCF\text{-}1^+$ pTex cells. These observations suggest that hyperactivated Pi3Kd might lead to increased effector cells inducing early immunopathology yet increased viral clearance. Surprisingly, LCMV cl13 infected *Pik3cd*$^{E1020K/+}$ mice also showed increased percentages of terminal $TCF\text{-}1^-$ Tex cells at day 8 of chronic infection. However, contrary to terminal Tex cells from WT mice, *Pik3cd*$^{E1020K/+}$ Tex cells showed decreased levels of inhibitory receptors such as Lag-3, CD39 and Tim-3 and produced more effector cytokines such as TNF-a and IFN-g, suggestive of increased effector function. These "terminal Tex" cells from *Pik3cd*$^{E1020K/+}$ mice also downregulated key transcription factors of terminal exhaustion, such as Eomes and Tox, a key factor for epigenetic remodeling in exhaustion. Cell transfer experiments indicated that these "terminal Tex" cells induced death of recipient mice when transferred into infection matched animals. Our results suggest that PI3Kδ is a key regulator of the balance of effector vs Tex cell differentiation and highlight that a carefully balanced activation of PI3Kδ is required to maintain T cells under exhaustion, while permitting acquisition of effector cell function. Our findings may provide new insights into therapeutic strategies to reinvigorate effector functions in terminal exhausted T cells.

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A028: 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 of 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 functions thereby limiting the efficacy of ICB in pre-clinical models. In melanoma patients the frequency of $TCF1^+$ CD8 T cells correlates with positive response to ICB and progression free survival but does not reliably stratify responders versus non-responders. These findings indicate that the dependance 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. Strikingly, 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 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. Conversely, in MC38OVA tumors TCF1 cKO T cells showed reduced activation at baseline which could be rescued by one dose of ICB. In vitro, we further showed defective proliferation, reduced PD-1 and CD28 up-regulation and reduced phosphorylation of key signaling molecules (LAT, ZAP70, ERK and AKT) downstream the T cell receptor pathway when TCF1 cKO T cells were stimulated with low doses of antigens but not when stimulated with strong TCR signals. These data indicate that TCF1 poises T cells for optimal antigen responsiveness. Indeed, transcriptional profiling of T cells in the TDLN further revealed the accumulation of a subset of tumor-specific naïve T cells poised to give rise to short-lived effectors in TCF1 cKO mice and thus is less suited to sustain anti-tumor responses in poorly immunogenic tumors where expansion of T cells retaining stem-like potential is required for durable anti-tumor responses. In tumors, single-cell RNA sequencing and immunophenotyping indicated that highly and poorly immunogenic tumors differentially instruct the differentiation trajectory of CD8 T cells. In the MC38OVA model, both WT and TCF1 cKO mice expanded a population of CD8 cells sharing a transcriptional signature with highly cytotoxic transitory effector cells that mediate ICB efficacy in chronic viral infection models. These cells likely accounted for the strong anti-tumor response observed in both WT and TCF1 cKO mice. Conversely, B16-OVA tumors retained higher frequency of stem-like T cells, failed to expand transitory effector cells and preferentially accumulated Tox+ CD8 T cells, which shared a transcriptional signature with T cells expanded in patients that fail to respond to ICB. In these tumors, TCF1 expression was required for the maintenance and proliferation of stem-like precursors, which are required to seed durable effector responses. Furthermore, loss of TCF1 was associated with reduced expression of the transcription factor Tox which was required for the

survival of late effector cells in the tumor microenvironment thus contributing to the failure of TCF1 cKO mice to sustain effective ICB responses. Overall, our study highlights a role for TCF1 in the early stages of the CD8 T cell response with important implications for guiding the choice of the optimal therapeutic intervention in tumors expressing low neoantigen levels.

A029: Targeting immunotherapy persister tumor cells

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Introduction: Persister tumor cells are the discrete and typically undetectable cells that survive therapy and are thought to give rise to tumor relapses, representing a major barrier to successful cancer treatment. Persister tumor cells reside in a 'dormant' non-dividing cell state. However, when the patient is no longer receiving cancer therapy these 'dormant' persister cells reawaken and resume their growth making complete tumor eradication very difficult. Reduced apoptotic sensitivity in tumor cells is a well-known mechanism of resistance to conventional chemotherapies. However, whether reduced apoptotic sensitivity is also a shared mechanism of resistance in tumor cells that persist after immunotherapy or drug treatment remains unknown. Therefore, our timely study aims to determine if immunotherapy persister cells (IPCs) and drug tolerant persister tumor cells (DTPs) possess shared or distinct mechanisms of resistance across multiple cancer types. And if development of this persister cell phenotype after one particular drug or cellular therapy can also produce a resistant phenotype that is translated into a pan-resistant phenotype against other drugs, cellular therapies and radiotherapy. Finally, we will also determine if pharmacological inhibition of programmed cell death pathways might increase the sensitivity of IPCs and DTPs to apoptosis.

Methods: IPCs were generated by chronically co-culturing tumor cell lines (murine B16-OVA melanoma cells or human HeLa-EGFR cervical tumor cells) with T cells (OVA-T-1 murine T cells or human EGFR-CAR T cells) for 3, 7 and 14 days at an effector:target ratio of 0.1 and 0.3. Ten different DTP cell lines were generated by chronically treating tumor cells for 3, 7 and 14 days with a high dose of drug that killed approximately 90% of the cells after 3 days using 5-FU, oxaliplatin, docetaxel, etoposide, temozolomide, dinaciclib, birinapant, ABT-199, Panobinostat and an EZH2 inhibitor (EPZ005697). Firstly, we investigated if IPCs and DTPs possess a pan-resistant phenotype which would have important implications for the rationale design of drug schedules. Using a CellTiter-glo assay, we determined if IPCs were sensitive to anti-cancer pharmacological drugs and radiotherapy. Conversely, we also assessed if DTPs were sensitive to T cell killing, radiotherapy and other anti-cancer pharmacological agents. To garner a more functional understanding of the potential mechanisms of resistance that might be contributing to resistance in IPCs and DTPs, a microscopy-based BH3 profiling approach was employed which functionally measures how close a cell is to the mitochondrial apoptosis threshold. Using this approach, the apoptotic sensitivity of IPCs and DTPs was assessed and compared to untreated parental tumor cell lines. Chemical vulnerabilities were also investigated using microscopy-based BH3 profiling to determine if IPCs or DTPs rely on specific anti-apoptotic proteins for their survival to reveal novel therapeutic targets in persister cells. Subsequently, we tested if

pharmacologically inhibiting these identified anti-apoptotic dependencies in IPCs and DTPs using BH3 mimetics could enhance the sensitivity of IPCs and DTPs to apoptosis to identify rational therapies for eradicating IPCs and DTPs.

Results: DTPs and IPCs acquired a pan-resistant phenotype demonstrated by reduced sensitivity to other drug classes, T cell killing and radiotherapy treatment via a CellTiter-glo assay. DTPs also displayed a reduced apoptotic sensitivity and inhibition of specific anti-apoptotic dependencies increased apoptotic sensitivity of persister cells.

Conclusion: We identified that reduced apoptotic sensitivity is a shared mechanism of resistance across IPCs and DTPs. IPCs and DTPs have distinct anti-apoptotic dependencies compared with their untreated parental cell lines wherein pharmacological inhibition of such dependencies enhanced the apoptotic sensitivity of IPCs and DTPs.

A030: Activated TCF1+ CD4 T cells act as precursors to effector CD4 subsets in the tumor response

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CD4 T cells play an important role in coordinating immune responses by differentiating into effector subsets that mediate specific functions. The importance of CD4 T cells in cancer has been well described, however, how cancer affects both the subset differentiation and continued function of CD4 T cells is currently unknown. In this work we aim to understand the differentiation trajectory of CD4 T cells in response to tumor antigens and their function in the anti-tumor response.

To characterize the heterogeneity of CD4 T cells in cancer, we first performed single cell 10X RNAseq of activated PD1+ CD45RA- CD4 T cells infiltrating human kidney tumors. We found three main activated CD4 T cell populations. Two effector clusters consisting of Th1-like (EOMES+) and Treg cells, and a third cluster expressing TCF1, and genes associated with stemness and survival that did not fit defined CD4 effector lineages. Transcriptional and flow cytometry analysis revealed that the TCF1+ PD1+ CD4 population had a distinct phenotype when compared to other well defined CD4 helper subsets, with little to no expression of lineage defining transcription factors BCL6, TBET, FOXP3 and EOMES. To examine the function of these cells, we sorted cell trace violet labeled tumor activated (PD1+) TCF1+ and effector (Th1-like and Tregs) CD4 T cells and cultured them *in vitro* under various polarizing conditions. The TCF1+ CD4 populations underwent extensive proliferation and, most interestingly could differentiate into various effector lineages according to the stimulation conditions provided. In striking contrast, the Th1-like and Treg cells underwent no proliferation or phenotype changes. These *in vitro* results suggest that TCF1+ CD4 T cells are an activated but undifferentiated population that can act as a precursor cell to various effector lineages in the tumor.

To further test this hypothesis *in vivo,* we adoptively transferred tumor specific (SMARTA) CD4 T cells followed by TRAMPC1-GP tumor inoculation. Transferred SMARTAs activated and first acquired a TCF1+ phenotype in the TDLN. Activated SMARTAs failed to acquire a TH1 or TFH program throughout the tumor response (5 weeks) and instead predominantly acquired a

Treg phenotype as early as 1-week post transfer. We next asked whether CD4 T cells primed in a tumor model are in a dysfunctional state and are thus not able to acquire TH1 and TFH effector programs. To test this, we sorted 5-week activated (CD44+ PD1+) SMARTAs with a TCF1+ phenotype from the TDLN of TRAMPC1-GP bearing tumor mice into naïve mice that were immediately infected with LCMV Armstrong. We found that TCF1+ CD4 T cells were able to differentiate into TH1 and TFH cells in response to the virus, with minimal differentiation towards a Treg phenotype, similar to the endogenous virus specific (GP66+) CD4 T cells. These results suggest that these cells are functional and capable of giving rise to different effector populations.

Overall, this work shows that CD4 T cells in cancer remain in a stable activated TCF1+ phenotype that may serve as a precursor population, with the capacity to support effector differentiation throughout the tumor response.

A032: Longitudinal single cell transcriptional and epigenetic mapping of effector, memory, and exhausted CD8 T cells reveals shared biological circuits across distinct cell fates

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During cancer and chronic infection, persistence stimulation drives CD8 T cells to differentiate into a distinct cell state known as T cell exhaustion. T cell exhaustion is characterized by altered effector functions and represents a major barrier to disease control. Immunotherapies, such as anti-PD1, can transiently reinvigorate exhausted CD8 T cells (T_{EX}), but this treatment does not reprogram these cells into more functional effector CD8 T cells (T_{EFF}) or memory CD8 T cells (T_{MEM}). This limited cellular effect may explain why most patients do not achieve a durable clinical response. The developmental pathways from naïve CD8 T cells towards T_{EFF}, T_{MEM}, T_{EX} populations are associated with distinct transcriptional and epigenetic changes that endow cells with different functional capacities and therefore therapeutic potential. However, there are key gaps in our understanding of these developmental relationships, the molecular circuitry underlying these cell states, and extent of population heterogeneity. Therefore, our ability to manipulate T cell differentiation trajectories to a specific outcome or to perturb/enhance specific effector functions to improve immunotherapies remains limited.

We used the lymphocytic choriomeningitis virus model of acutely-resolved or chronic viral infection to generate longitudinal single cell RNA-seq and single cell ATAC-seq of T_{EFF}, T_{MEM}, and T_{EX}. We also performed PD1 blockade and analyzed the effect on T_{EX} differentiation by single cell ATAC-seq. These data defined population heterogeneity and identified gene expression and epigenetic patterns associated with major branches of CD8 T cell differentiation. These analyses uncovered new subpopulations of T_{EFF}, T_{MEM}, and T_{EX}, including a T_{EX} subset expressing NK cell-associated genes that required the TF Zeb2 for differentiation. This Zeb2 circuitry was shared with cytotoxic subsets of T_{EFF} and T_{MEM} following acute infection despite distinct epigenetic landscapes. In addition, we identified multiple epigenetically distinct populations of TCF1+ antigen-experienced CD8 T cells. T_{EX} precursor cells found early in chronic infection were distinct from T_{EX} progenitors at later timepoints, and both of these TCF1+

populations were different from T_{MEM} precursors and mature T_{MEM} generated after acute infection. The transition from early T_{EX} precursors to committed T_{EX} progenitors in chronic infection was accompanied by dampening many cellular processes, including metabolism and translation. This relative return to quiescence may be a key event that allows the T_{EX} population to persist despite strong ongoing antigenic stimulation. Indeed, we identified the cell stress response gene, B cell translocation gene (BTG)/TOB family member (BTG1) as a novel regulator for establishing the T_{EX} population. Disentangling closely related but distinct CD8 T cell populations such as T_{EX} precursors and progenitors could have key relevance for understanding immune responses after treatment and for identifying clinical biomarkers. Altogether, this transcriptional and chromatin accessibility map highlighted how the same biological circuits such as cytotoxicity or stem/progenitor pathways can be used by CD8 T cells with highly divergent underlying chromatin landscapes and may help identify specific targets or pathways for future therapeutic manipulation to improve anti-tumor T cell function.

A033: Fc–IL-4 enhances longevity of terminally exhausted CD8+ T cells by promoting glycolysis to potentiate ACT immunotherapy

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Induction of type I immunity is the main strategy in current cancer immunotherapies, including immune checkpoint blockade therapy (ICB) and adoptive T cell transfer therapy (ACT). However, durable responses in patients with solid tumors remain limited in part due to T cell exhaustion associated dysfunction. Tumor antigen-specific $CD8^+$ T cells progressively differentiate into terminally exhausted $CD8^+$ T cells ($CD8^+$ T_{TE}) under chronic antigen stimulation in the tumor microenvironment (TME). $CD8^+$ T_{TE} cells are highly tumor-cytolytic but fail to sustain an adequate population and effector functions to achieve durable tumor growth control. Moreover, $CD8^+$ T_{TE} cells do not respond to most existing immunotherapies including ICB. Recently, it has been discovered that T helper 2 (Th2) cell-mediated tissue-level cancer treatment may be a potential alternative to type I immunity-mediated cancer cell killing. However, whether and how type I and II immunity can be well-coordinated to achieve more effective ACT immunotherapy remains unknown. Interleukin-4 (IL-4) is a prototypical Th2 cytokine and discovered as a growth and survival factor that can significantly promote lymphocyte survival. Tumor cells engineered to secrete IL-4 are highly immunogenic and can evoke durable antitumor immune responses. Here, for the first time, we showed that exogenous administration of half-life-extended IL-4 fusion protein (Fc-IL-4) dramatically augmented the antitumor immunity of ACT by enhancing the longevity and effector function of $CD8^+$ T_{TE} cells in tumor. Specifically, Fc-IL-4 enriched $CD8^+$ T_{TE} cells by recusing them from necroptosis instead of promoting their proliferation or transformation from progenitor exhausted $CD8^+$ T cells. The enriched $CD8^+$ T_{TE} cells were highly polyfunctional and contributed to long-lasting complete tumor regression induced by the combination therapy of Fc-IL-4 and ACT in multiple solid tumor models. In addition, we found that Fc-IL-4 promoted longevity of $CD8^+$ T_{TE} cells by enhancing their glycolytic metabolism in an LDHA-dependent manner. Our findings provided preclinical evidence that Fc-IL-4, as a type II cytokine, appears to be an unexpected but potent cancer immunotherapy, which synergizes with ACT therapy to potentially enhance patients' response rate in the clinic.

A034: Enforcement of exhausted T cell epigenetic fate by HMG-box transcription factor TOX

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T cell exhaustion is one of the major barriers limiting efficacious anti-tumor therapy. Exhausted CD8 T cells (T_{EX}) develop following persistent antigen stimulation and are characterized by a unique epigenetic state, expression of PD-1 and other inhibitory receptors, dampened effector function, and limited capacity to control disease. Though checkpoint blockade temporarily improves T_{EX} function, the underlying epigenetic landscape of T_{EX} remains largely unchanged and these "reinvigorated" T_{EX} revert to less effective antitumor T cells. Thus, a better understanding of the epigenetic determinants and flexibility of T_{EX} fate commitment should reveal novel therapeutic opportunities for tuning T cell differentiation in cancer and other diseases. Our lab and others recently identified the HMG-box transcription factor TOX as an essential transcriptional and epigenetic initiator of T_{EX} lineage differentiation. Here, we investigated whether sustained TOX expression was required to maintain the T_{EX} epigenetic identity and fate inflexibility. Induced TOX ablation in committed T_{EX} reduced T_{EX} numbers and impaired expression of PD-1 and other canonical T_{EX} inhibitory receptors. This effect of TOX loss appeared to be driven by altered T_{EX} proliferation and survival. Accordingly, Bim knockdown numerically rescued TOX-deficient T_{EX}; yet, PD-1 loss and other key T_{EX} phenotypic differences were retained. This incomplete rescue alluded to a global role for TOX in enforcing T_{EX} differentiation, beyond directly regulating T_{EX} survival. Indeed, single-cell RNAseq and ATACseq revealed that TOX was required to maintain transcriptional modules of mitochondrial function, protein synthesis, terminal differentiation, and epigenetic factor expression. These gene expression differences correlated with loss in chromatin accessibility. Furthermore, removal of TOX-deficient T_{EX} from a chronic environment enabled their partial reprogramming into the more functional effector lineage, thus identifying TOX as one of the epigenetic barriers that constrain the fate flexibility of T_{EX}. Together, these findings suggest that TOX transcriptionally and epigenetically enforces critical components of the T_{EX} program, and that TOX manipulation provides an avenue for rewiring T_{EX} identity. By improving molecular understanding of the role of TOX in enforcing T_{EX} identity and in constraining T_{EX} fate reprogramming, this study will inform future immunotherapies that seek to re-engineer T_{EX} into customized differentiation states with amplified potential for tumor control.

A035: Distant antimetastatic effect of enterotropic colon cancer-derived alpha4beta7 CD8 T cells

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Despite the high prognostic value of immune infiltrates in colorectal cancer (CRC), metastatic disease remains resistant to immunotherapy by immune checkpoint blockade (ICB). Thus,

metastatic (m)CRC represents a pivotal challenge for therapy improvement. Our team focuses on tumor immunity, including immune response in metastases, in clinical and preclinical settings.

We established a preclinical mCRC model by implanting tumor cells orthotopically, in mCRC tumor sites (ie intra-colon and intra-hepatic). Using this multi-tumor model, we demonstrated, that primary colon tumors can spontaneously exert a colon-specific and immune-dependent antimetastatic effect on distant hepatic lesions. We established, by flow cytometry, an immune signature, integrating antitumor CD8 T cells, dendritic cells and protumor macrophages, that we called MicroEnvironment (ME)-score, and that was predictive of the antimetastatic effect. ScRNA-Seq and phenotypic analyses revealed that enterotropic α4β7 integrin-expressing tumor neoantigen-specific CD8 T cells were key components of the systemic immune response responsible for the antimetastatic effect. We observed that enterotropic $\alpha4\beta7^+$ CD8 T cells were able to migrate from colon tumors toward hepatic metastases. Accordingly, the presence of concomitant colon tumors improved the efficacy of proof-of-concept ICB on liver lesions and generated a protective memory immune response whereas antibody-mediated partial depletion of $\alpha4\beta7^+$ cells or absence of these cells in knock-out mice, abrogated the control of metastatic disease by the primary tumor. Finally, in a metastatic CRC patient cohort, we show increased expression of genes encoding α4β7 integrin in ICB responsive metastases concomitant with increased proportions of circulating $\alpha4\beta7^+$ CD8 T cells. Our work shows, in multi-tumor mouse models and in mCRC patients, that enterotropic tumor-specific $\alpha4\beta7^+$ CD8 effector T cells impact both the fate of distant extra-intestinal metastases and their response to ICB.

Our findings identify a systemic cancer immunosurveillance role for gut-primed tumor-specific alpha 4 beta 7^+ CD8 T cells.

A036: Immunological evaluation of immunostimulatory gene therapy targeting CD40/4-1BB in advanced cancer patients (LOKON002)

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LOAd703 (delolimogene mupadenorepvec) is an immunostimulatory gene therapy based on an oncolytic adenovirus, which is modified to express two transgenes (trimerized membrane-bound CD40L (TMZ-CD40L) and 4-1BBL), vital for the induction of an anti-tumor immune response. Herein, we present the initial results of the single arm, open-label phase I/II clinical trial LOKON002 (NCT03225989) evaluating the safety and effect of LOAd703 in patients with advanced solid cancers. This interim report encompasses the first 15 patients with a focus on the immunological effects. In Phase I (n=9), a dose escalation with three dose levels ($5x10^{10}$ viral particles (VP), $1x10^{11}$ VP, $5x10^{11}$ VP) was performed and in Phase II, patients were treated with the maximum tolerated dose ($5x10^{11}$ VP). LOAd703 was administered intratumorally and given as an add-on to standard of care chemotherapy tailored to the indication, or immune-conditioning gemcitabine chemotherapy if established treatments had been given (i.e last line). Patients received a maximum of eight LOAd703 treatments, which were given every second week while chemotherapy was given according to standard protocol. Patients had advanced disease,

and most were refractory to standard treatment. The primary tumor was pancreatic cancer (n=6), colorectal cancer (n=5), ovarian cancer (n=3) and biliary cancer (n=1). LOAd703 treatment in combination with chemotherapy was tolerable and stabilization of disease was observed for at least two months in 5 patients and partial response was achieved in 2 patients. All patients had anti-adenoviral antibodies in their serum at baseline and the levels increased after LOAd703 treatment. There was no apparent correlation between antibody levels and dose level or treatment outcome. Gene expression analysis with nCounter® PanCancer Immune Profiling Panel from NanoString of matched tumor biopsies from the injected tumor lesion, taken at baseline and after six LOAd703 treatments (7 patients), revealed a strong induction of inflammatory responses. This included upregulation of viral-response genes (Toll-like receptor 2 and downstream signaling proteins, IRAK 1 and 4), genes connected to dendritic cell (DC) activation (MHC class I and II, CD80/86 and CD40) and T-cell responses (IFNγ, Granzyme A and B). Furthermore, all except one patient evaluated displayed an overall increase in gene profiles that can predict responses to immune checkpoint inhibition. Interestingly, chemokines implicated in immunosuppressive cell recruitment and adhesion molecules connected with tumor progression were downregulated in responding patients and these gene signatures may serve as a biomarker for response to LOAd703 therapy. We also noted systemic treatment effects as seen by an upregulation of pro-inflammatory cytokines (IFNγ and IL-15) and chemokines (CXCL10, CCL2, IL-8, IL-6), as well as a reduction of regulatory T cells (CD4+CD25+CD127low/-), immunosuppressive myeloid cells (CD11b+CD163+ and CD11b+CD14+CD15-HLA-DRlow/-) and NK cells (CD14-CD3-CD56+CD16+) in the blood. T cells and in particular CD8+ effector memory T cells (CD45RA-CCR7-) appeared enriched in patient´s blood after treatment. In conclusion, LOAd703 in combination with chemotherapy mediated inflammation even in otherwise immunologically "cold" tumors and may be an ideal candidate to sensitize resistant tumors to immunotherapies such as immune checkpoint inhibitors, or in vitro expanded T- or NK cells, including CAR T cells.

A037: TILT-123, an oncolytic adenovirus encoding tumor necrosis factor alpha (TNFa) and interleukin (IL-2), for immunotherapy of solid tumors – Experience from phase I clinical trials

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Background: Immunotherapies modulating the function of T-cells, such as adoptive T cell therapy or immune checkpoint inhibitors, have emerged as a pillar of cancer treatment in the past few decades. Such immunotherapies can lead to durable response rates in solid tumors, however, a large fraction of patients remains unresponsive to therapy, highlighting the need for improved therapeutic options. Oncolytic viruses can contribute to closing the gap due to their ability to cause direct tumor cell lysis and induce expression of immune-stimulatory payloads, resulting in

immunologically cold tumors becoming hot. Following observations from patients treated with earlier versions of the oncolytic adenovirus candidate, we constructed a chimeric serotype 5/3 oncolytic adenovirus encoding tumor necrosis factor alpha and interleukin-2 (Ad5/3-E2F-D24-TNFa-IRES-IL2; TILT-123), specifically aimed at revamping anti-tumor T-cell activity. Preclinical data indicated these arming devices as optimal for recruiting and activating T-cells. Laboratory work showed that TILT-123 is capable of safely inducing antitumor efficacy as monotherapy or in combination with a wide range of therapies, including immune checkpoint inhibitors (ICI), tumor-infiltrating lymphocyte (TIL) therapy and chimeric antigen receptor T-cell therapies.

Methods: We designed two open-label, 3+3 dose-escalation, phase I clinical trials to evaluate the safety of TILT-123 in humans: TILT-T115 (NCT04695327; TUNIMO) and TILT-T215 (NCT04217473; TUNINTIL). In TILT-T115, patients with injectable advanced refractory and recurrent solid tumors that cannot be treated with curative intent are being administered with multiple injections of TILT-123 monotherapy, at the Helsinki University Hospital and Docrates Cancer Center in Helsinki, Finland. In TILT-T215, patients with injectable advanced refractory and recurrent melanoma that cannot be treated with curative intent who are receiving TIL therapy without pre- or postconditioning are being administered with multiple injections of TILT-123, at the National Center for Cancer Immune Therapy, Herlev, Denmark and CHU Nantes, Nantes, France. The primary endpoint is safety of TILT-123 based on the occurrence of adverse events, severe adverse events, vital signs, electrocardiogram, and safety laboratory results; Secondary endpoints are aimed at determining safety of combination therapy (only for TILT-T215) throughout the treatment period, efficacy of the therapies, biodistribution of the virus, deciphering the mechanism of action of the virus, among others.

Results: Interim safety data from the patients treated in the first 3 TILT-123 dose-levels (=9 patients) of each trial show that trial treatments did not cause severe adverse events and that none of the TILT-123-related adverse events were considered dose-limiting. TILT-123-related adverse events, most commonly (per patient) including fever, nausea, chills and fatigue, were typically low-grade, according to the Common Terminology Criteria for Adverse Events (CTCAE) v5.0, and the safety profile was consistent with other virus-derived immunotherapies. Preliminary data from both trials at the mentioned dose levels demonstrated signs of systemic antitumor efficacy, according to Response Evaluation Criteria in Solid Tumors (RECIST) 1.1/immune (i)RECIST/Positron emission tomography (PET) criteria. In addition, data from biospecimens acquired from patients indicate intratumoral T-cell inflammatory response and acceptable biosafety.

Conclusions: Based on the preliminary data obtained from TILT-T115 and TILT-T215 clinical trials, TILT-123 is considered safe and capable of inducing antitumor activity both as single-agent or in combination with TIL therapy. These preliminary results pave the way for continued dose-escalation in the abovementioned trials and platform development in clinical trials with other T-cell-based immunotherapies, including ICI (NCT05271318 and NCT05222932).

Reference: Patent US20150232880

A038: Loss of PNKP potentiates type-I IFN response via ROS-induced damage to DNA

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DNA damage has been implicated in the activation of the type-I interferon (T1IFN) response, a pathway that is critical for the activation and homing of cytotoxic T lymphocytes to tumors exposed to ionizing radiation (IR). This opens the possibility of using DNA repair inhibitors to augment the activation of the immune system. Supporting this are new lines of evidence suggesting that downregulation of DNA repair proteins enhances the T1IFN response. In this study, we aim to unravel a new role for the DNA repair protein, polynucleotide kinase phosphatase (PNKP) in the T1IFN response.

PNKP is a DNA end-processing enzyme that possesses both 5′-kinase and 3′-phosphatase activities, and its downregulation has been shown to sensitize cancer cells to IR and Hydrogen peroxide. We, therefore, hypothesized that loss of PNKP will increase IR- and reactive oxygen species (ROS)-induced damage to DNA and consequently potentiate the T1IFN response.

To determine the effects of PNKP depletion, cells were transiently depleted of PNKP using small interfering RNA (siRNA) prior to exposure to radiation, reactive oxygen species (ROS) scavengers, or drug inhibitors. Western blotting, immunofluorescence, real time quantitative polymerase chain reaction (RT-qPCR) and ROS and cell proliferation/viability kits were used to analyze changes in protein or gene expression levels, ROS production, cell proliferation and viability following PNKP depletion.

Our preliminary data showed that knockdown of PNKP in MCF7 breast cancer cells causes a robust phosphorylation of signal transducer and activator of transcription 1 (STAT1), upregulation of interferon (IFN)-stimulated genes (ISGs), as well as accumulation of cytosolic DNA. We confirmed an elevation of STAT1 phosphorylation in multiple cancer cell lines, including the pancreatic cancer cell line PANC-1 and the prostate cancer cell line PC-3, as well as the non-tumorigenic immortalized human mammary epithelial cell line, MCF10A, which suggests that the T1IFN response following loss of PNKP is not specific to cancer cell lines. We provided further evidence that this T1IFN response following depletion of PNKP is IR-independent as treatment of PNKP-depleted MCF7 cells with 8 Gy of IR did not further increase STAT1 phosphorylation nor induce the ISGs. Using pharmacological inhibition and/or RNA interference, we found that STAT1 activation and the consequent induction of pro-inflammatory genes following knockdown of PNKP was independent of the DNA sensor cyclic guanosine-adenosine monophosphate synthase (cGAS), but dependent on other downstream proteins, including stimulator of interferon genes (STING), tank-binding kinase 1 (TBK1) and interferon-regulatory factor 3 (IRF3). Lastly, we examined the possibility that ROS and damage to mitochondrial DNA (mtDNA) may be responsible for the increased STAT1 phosphorylation and downstream ISG induction following PNKP knock-down. Depletion of mtDNA, using 2',3' dideoxycytidine (ddC) or a low dose of ethidium bromide, indicated that leakage of mtDNA into the cytosol was indeed required for the observed STAT1 phosphorylation following loss of PNKP. Moreover, we validated our results using reagents such as the voltage-dependent anion channel 1 inhibitor (VBIT-4) and cyclosporin A that are known to block mitochondrial permeability transition pore opening. Exposure of PNKP-depleted cells to these inhibitors

dramatically reduced STAT1 activation and impaired induction of the ISGs, indicating that loss of PNKP causes damage to mtDNA that eventually leaks into the cytosol to promote induction of pro-inflammatory genes.

The data above provide evidence that PNKP inhibition might help to potentiate the T1IFN response leading to enhanced immunogenic targeting of tumors. Results from this study will afford further support for the development of an inhibitor against PNKP for clinical applications possibly in combination therapy with IR and immune checkpoint inhibitors against tumors.

A039: Reprogramming hypoxia-recruited tumor-associated macrophages with poly(I:C) containing hypoxia-responsive nanocomplex

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Tumor-associated macrophages (TAMs), which dampen the therapeutic efficacy of cancer immunotherapy, are the key players in the immunosuppressive tumor microenvironment (TME). Therefore, reprogramming TAMs into tumoricidal M1 macrophages possesses considerable potential as a novel immunotherapy. Still, the low bioavailability of polarization agents and limited accumulation in TAMs restrict their anti-tumor efficacy. Herein, we focused on tumor hypoxia condition, where TAMs differentiated and accumulated, and designed the hypoxia-responsive nanocomplex to efficiently target TAMs and overcome immunosuppressive TME.

First, we synthesized a hypoxia-cleavable polymer, poly(ethylene glycol)-azo-poly(L-lysine) (PEG-azo-PLL), and formulated a nanocomplex by simple mixing PEG-azo-PLL and polarization agent, polyinosinic-polycytidylic acid (poly(I:C)). Under *in vitro* hypoxia mimicking conditions, PEGazoPLL/poly(I:C) could increase the cellular internalization by converting its surface charge and enhance the delivery efficiency of poly(I:C) to TAMs. PEGazoPLL/poly(I:C) protected poly(I:C) from degradation, increasing the bioavailability of polarization agent. Furthermore, immunomodulation effect of PEGazoPLL/poly(I:C) was investigated by *in vivo* B16F10 tumor-bearing mouse model. Immunofluorescence image and flow cytometry data presented that PEGazoPLL/poly(I:C) could successfully reduce the population of M2-like TAMs in hypoxic region and promote infiltration of $CD8^+$ T cells *in vivo*. The reprogramming of TAMs in hypoxia resulted in the favorable conversion of immunosuppressive TME. Finally, PEGazoPLL/poly(I:C) could elicit a significant anti-tumor effect in B16F10 tumor-bearing mice and elongate survival time, demonstrating that hypoxia-responsive nanocomplex PEGazoPLL/poly(I:C) would be a promising approach for TAM reprogramming immunotherapy for solid tumors.

A040: Late CTLA-4 Ig treatment improves antitumor efficacy of immunotherapy

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The promising approach of combining immune checkpoint therapies such as anti-CTLA-4 and anti-PD-1 increases antitumor response and overall survival rate relative to single treatments. However, it also increases the frequency and severity of immune-related adverse events (irAEs),

such as cardiotoxicity. Our previous results showed that CTLA-4 Ig (abatacept), an inhibitor of T cell costimulation through CD28, can reverse those irAEs in patients with cancer, however, its effect on the antitumor response remains unclear. In the B16F10 melanoma model, we injected mice with CTLA-4 Ig antibody when: 1) mice were first treated with anti-CTLA-4, anti-PD-1, or combination (early time point) and 2) the immunotherapy was finished (late time point). We demonstrated that CTLA-4 Ig at the early time point compromised the antitumor efficacy of the immunotherapy. In contrast, the antitumor efficacy of the immunotherapy was improved if mice were treated with CTLA-4 Ig at the late time point. We also found that the antitumor response induced by CTLA-4 Ig (late) was dependent on CD80/86. Interestingly, the frequency of ICOS+ Foxp3+ Tregs was significantly reduced by CTLA-4 Ig at the late time point. Thus, we treated B16F10 tumors in Foxp3DTR mice with anti-CTLA-4 and then with CTLA-4 Ig (late). The antitumor tumor efficacy was similar between mice treated with or without Diphtheria toxin (DT) at the late time point. This suggests that the improved antitumor efficacy of CTLA-4 Ig at the late time point was Treg dependent. Collectively, we show that CTLA-4 Ig treatment has a differential role throughout the time course of immunotherapy treatments, and this work suggests a potential combination treatment strategy with checkpoint blockade and late CTLA-4 Ig treatment.

A041: Targeting Mitochondrial Stress Response Mediator LONP1 in Tumor Infiltrating Myeloid Cells

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The limited availability of effective therapies targeting immunosuppressive tumor-infiltrating myeloid cells (TIMC) represents a critical barrier to successful and persistent antitumor immunity and treatments, especially immunotherapy. The results presented herein explore the promising therapeutic avenue of addressing TIMCs to augment current cancer treatment regimens towards lasting tumor regression. Upon entering the tumor microenvironment (TME), myeloid cells survive by overcoming and maintaining stress response systems but consequently gain suppressive functionality that impairs antitumor immunity (and therefore become myeloid-derived suppressor cells, MDSC). Our understanding of the contribution of ER stress responses in TIMCs is established but unknown is the role of the mitochondrial stress responses in TIMC immunosuppression. Therefore, our experimental approach focused on the multifunctional mitochondrial protease LONP1 and its role in TIMC mitochondrial stress and suppressive functionality using human $CD33^+$ MDSC and mouse bone marrow MDSCs along with tumor challenges in mouse myeloid conditional knockout strains of LonP1. We show that LONP1 is upregulated in many human cancers and specifically in human $CD33^+$ TIMC as compared to blood circulating $CD33^+$ myeloid cells in patient and healthy controls. Further, inhibition of LONP1 activity with the synthetic triterpenoid derivative CDDO-Me impairs the immunosuppressive functions of human and murine MDSCs. To precisely interrogate the role of LonP1 in myeloid actions, we show in our mouse models that LonP1 is i) upregulated in TIMCs or under tumor explant conditioning and ii) CDDO-Me inhibition of LonP1 ablates immunosuppressive abilities. Using our genetic approach with myeloid conditional knockouts, we show that selective ablation of LonP1 expression improves antitumor responses and significantly lowers tumor volume. This drastic control of tumor burden is closely tied to the reprogramming of myeloid cells into strongly effective tumor associated macrophages with an

M1-like profile pattern and increased $CD4^+$ tumor infiltration. These ongoing research efforts suggests an integral role of LONP1 in myeloid cell pro-tumor immunosuppressive activities that is ideal for therapeutic targeting to redirect the tumor myeloid compartment into an anti-tumor promoting immune milieu.

A042: Empowering GITR-targeted agonistic antibody-based immunotherapy by Fc engineering

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Alongside the clinical success, immune checkpoint blockade (ICB) therapy has notable limitations: they elicit differential responses between individuals and tumor types. Therefore, additional therapeutic approaches to boost anti-tumor immune responses alone or in combination with ICB are being developed to improve outcomes. One such promising approach is targeting the immuno-stimulatory receptor Glucocorticoid-induced tumor necrosis factor receptor [TNFR] family-related protein (GITR) by using agonistic antibodies. This study elucidates the role of human Fc-gamma receptors (FcγRs) in the activity of GITR monoclonal antibodies (mAbs). We applied Fc-engineering and glyco-engineering approaches to modify the FcγR interactions of GITR mAb and characterized the anti-tumor immune response in-vivo of these Fc-variants. By utilizing a mouse model that recapitulates human FcγRs expression, we showed that the engagement of different human FcγRs by GITR mAb significantly dictates their anti-tumor activity. While the engagement of the inhibitory hFcγRIIB compromises the efficacy of GITR mAb, engagement of the activating hFcγRIIA and/or hFcγRIIIA enhances the anti-tumor activity of this mAb. Our study identified an Fc-optimized human IgG scaffold of GITR agonistic mAb that results in enhanced therapeutic effect by increasing the CD8/T regulatory (Treg) ratio within the tumor microenvironment (TME). This enhanced activity is mediated by multiple mechanisms, including GITR agonism, Fc-dependent depletion of Tregs, and engagement of beneficial FcγR-signaling pathway on myeloid cells, all contributing to the enhanced effect by the Fc-optimized mAbs compared to that of the wild-type IgG1. Altogether, the data suggest that the anti-tumor activity of GITR mAbs can be increased when engaging the activating FcγR pathways and provides a rationale for Fc-engineering GITR human mAbs to increase their therapeutic activity by harnessing FcγR pathways.

A043: Combination radio-immunotherapy regimens and epigenetic modifier inhibitors to improve response in a cold murine neuroblastoma

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Neuroblastoma (NBL) is the most common extracranial solid tumor in children, with ~50% of patients presenting with high-risk (HR) NBL. Despite advances in care for HR-NBL, ~60% of patients still die. Frequently, these tumors are immunologically "cold," a classification characterized by a low tumor mutation burden, MYCN amplification, poor immune cell infiltration, and low MHCI expression. In 9464D-GD2, a murine model of HR-NBL, we recently described an effective radio-immunotherapy regimen (combination adaptive and innate immunotherapy regimen [CAIR]) which includes radiation, anti-CD40, CpG

oligodeoxynucleotides, hu14.18-IL2 immunocytokine (anti-GD2 mAb linked to IL-2), and anti-CTLA4. With a non-MHCI inducible line (9464D-GD2-NI) and an MHCI inducible line (9464D-GD2-I), via stimulation with Interferon Gamma, we showed that 9464D-GD2-I tumors are less responsive to CAIR as compared to 9464D-GD2-NI tumors. We hypothesized that T cells are involved in the anti-tumor response against 9464D-GD2-I tumors, yet the relative failure to completely respond to CAIR may be due to exhaustion of these intratumoral T cells as the tumors begin to evade the immune response. Thus, we investigated whether PD-L1, a major regulator for T cell exhaustion, is upregulated in the tumor following CAIR and if the addition of anti-PD-L1 antibody to CAIR could improve 9464D-GD2-I responses to therapy. Here, we show that both tumor cells ($p=0.001$) and infiltrating immune cells ($p=0.04$) from 9464D-GD2-I tumors upregulate expression of PD-L1 following CAIR, relative to untreated controls. We additionally show that mice bearing small ($50mm^3$) 9464D-GD2-I tumors have increased overall survival ($p<0.0001$) and improved response rates ($p=0.001$) when treated with CAIR plus anti-PD-L1 compared to CAIR alone.

CAIR alone can cure ~40% of small 9464D-GD2-NI tumors, but cured mice fail to reject tumors upon rechallenge, suggesting a lack of functional immunological memory. Since CD8 T cells are engaged via interactions with MHCI, it is likely that the lack of MHCI inducibility of these tumors contributes to the poor memory response. MHCI expression by tumor cells can be regulated by several mechanisms, including epigenetic modifications, and several epigenetic modifiers (EMs) have been found to be dysregulated in NBLs. With the aim of rescuing MHCI expression in 9464D-GD2-NI tumors, we investigated the ability of EM inhibitors (EMis), including guadecitabine (inhibitor of DNA methyltransferases) and entinostat (inhibitor of histone deacetylases), to restore MHCI expression on NBL cells. Here, we show that MHCI expression on 9464D-GD2-NI is restored by exposure to guadecitabine. Accordingly, we observed increased transcription of genes involved in the antigen presenting machinery for MHCI, including Tap1 and PSMB9, in this cell line following guadecitabine treatment. Intriguingly, in several human NBL cell lines, we observed an increase in both MHCI and MHCII expression following treatment with guadecitabine and entinostat together.

The data presented here suggests that anti-PD-L1 treatment may be a beneficial addition to our previously described CAIR therapy to reinvigorate T cells to allow persistent anti-tumor efficacy in the 9464D-GD2-I model. Furthermore, in the 9464D-GD2-NI model that responds to CAIR but lacks functional immunological memory, the addition of EMis to CAIR therapy has the potential to restore MHCI and MHCII, which might enhance not only response rates but the formation of immune memory as well. Future studies are aimed to investigate the implications of EMis *in vivo* on the memory response in the 9464D-GD2-NI model and on the augmented response to adding anti-PD-L1 in the 9464D-GD2-I model.

A044: Novel Bispecific Immune Synapse Engagers harness potent T cell anti-tumor immunity

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Immunotherapy has revolutionized the treatment of cancer over the past several years. Approved checkpoint inhibitors, dominant by anti-PD-1 monoclonal antibodies, are primarily aimed at

blocking inhibitory signaling by immune cells and constitute a front-line treatment for many cancer types. However, most patients do not respond to these inhibitors, while others rapidly become resistant. Recent studies suggest that T cell/dendritic cells (DC) crosstalk is essential for the efficacy of anti-PD-1 immunotherapy. Here, we show that conventional type 1 DC (cDC1s), a rare population of DCs within the tumor microenvironment (TME) specialized in cross-presentation and priming of T cells, are essential for effective tumor elimination by PD-1 immunotherapy. Temporary depletion of cDC1 at the active phase of anti-PD-1 administration abrogates the expected anti-tumor response. Based on this observation, we hypothesize that the low cDC1 frequency in the TME limits PD1 efficacy. We postulate that a reagent that will facilitate specific physical engagement of T cell-DC crosstalk in the TME in combination with blocking PD-1 checkpoint signaling may greatly enhance the potency of current immunotherapy treatments, which only attempt to block different T cell checkpoints. Therefore, we developed a novel family of cDC1-targeted, PD-1 blocking bispecific antibodies that facilitate T cell-DC immune synapse formation in the TME, while blocking the PD-1/L1 axis, to enhance treatment potency. We term these new reagents Bispecific Immune Synapse Engagers (BiSE). We generated several versions of BiSE, engaging T-DC synapses through different targets, affinities and valencies, and characterized their biochemical properties, their biophysical ability to facilitate T-DC immune synapses, and their PD-1 blocking activity. BiSE treatment results in potent T-cell mediated anti-tumor activity, characterized by increased T effector/T regulatory ratio in the TME and increased cDC1 migration and formation of DC-T synapses in the tumor-draining lymph node, in several pre-clinical models as compared to traditional anti-PD-1 treatment. Moreover, BiSE treatment controls tumor growth in B16 melanoma model that shows minimal PD-1 response. We suggest BiSE as a novel and highly effective cancer therapy that effectively sensitizes tumors to PD-1 inhibition. Moreover, BiSE represents a general approach that can lead to the development of a family of new reagents that engage immune synapses in order to enhance the activity of various immune checkpoints targets currently limited in their pre-clinical and clinical efficacy.

A045: Fc glyco-engineered PD-L1 antibody harnesses FcγRs for increased antitumor efficacy

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FDA approved anti-PD-L1 antibodies bear the IgG1 isotype, either in wild type format, or in an Fc-mutated version eliminating Fcγ receptor (FcγR) engagement. Here, we employed humanized FcγR mice to study the contribution of human FcγRs to the anti-tumor activity of human anti-PD-L1 antibodies. We observed similar antitumor efficacy, and comparable effect on tumor immunity utilizing these two IgG scaffolds. However, in-vivo antitumor activity of anti-PD-L1 IgG1 was enhanced by combining it with FcgRIIB blocking antibody to overcome FcgRIIB suppressive function in the tumor microenvironment (TME), or by Fc glyco-engineering of the antibody to enhance its binding to activating FcgRIIIA. Treatment with Fc-afucosylated version of the PD-L1 mAb Avelumab induced stronger antitumor responses compared to the parental IgG, associated with decreased frequencies of PD-L1^{+} myeloid cells and increased infiltration of T cells in the TME . Our data reveal that the current design of FDA-approved anti-PD-L1 do not optimally harness FcγR pathways, and suggest strategies to enhance FcγR engagement to optimize anti-PD-L1 immunotherapy.

A046: Using a cancer-on-a-chip approach to study the pancreatic cancer tumor microenvironment

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Pancreatic ductal adenocarcinoma (PDAC) is a lethal malignancy that is overwhelmingly resistant to therapy. PDAC tumors are characterized by an immunosuppressive fibroinflammatory stroma and can be broadly classified into those with an abundance of T cells or a paucity of T cells. Our lab has previously identified several tumor cell intrinsic factors that regulate the degree of T cell infiltration and response to immunotherapy, but it remains unknown how these factors influence T cell trafficking through the vasculature to the tumor. We used *in vivo* models and a cancer-on-a-chip platform to examine immune-vascular crosstalk in the pancreatic tumor microenvironment (TME). We first sought to characterize the vascular landscape in PDAC. Following up from previous work in the lab, we used bulk RNA sequencing appraoches to identify a subset of hyper-vascular murine PDAC lines. Using an *in vivo* dextran assay, we determined that endothelial-high tumors have increased vascular perfusion. We next utilized an *in vitro* tube formation assay to assess if the defects seen in tumor vasculature are from tumor-cell derived factors. Endothelial cells cultured in conditioned media from endothelial-low tumors had defects in tubule formation. We further identified endothelial-low tumors have a defect in pericyte coverage of the vessel. We next utilized organ-on-a-chip devices to assess angiogenesis in PDAC. Endothelial cells and fibroblasts self-assemble into a 3D vascular network when incorporated into the microdevices. In the absence of tumor cells, mature vascular networks formed on day 4 of culture. Interestingly, vascular networks formed faster in the presence of PDAC tumor cells, suggesting a tumor derived factor enhanced angiogenesis. The cancer-on-a-chip devices are perfusable and allow for the incorporation of other TME components. Future directions, include incorporation of myeloid cells and perfusing fluorescently labeled T cells into the vascular networks and using live imaging to monitor T cell trafficking in different PDAC TME settings. Further, we will use immune checkpoint blockade and CAR T cells to identify methods to enhance tumoral T cell infiltration.

A047: Disruption of tumor-promoting desmoplasia by adoptive transfer of fibroblast activation protein targeted chimeric antigen receptor (CAR) T cells enhances anti-tumor immunity and immunotherapy

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The efficacy of chimeric antigen receptor (CAR) T-cell therapy in solid tumors is limited by inefficient T-cell infiltration and hypofunctionality within the immunosuppressive tumor microenvironment (TME). Fibroblast activation protein (FAP) is highly expressed in almost all epithelial carcinoma, particularly in pancreatic ductal adenocarcinoma (PDAC), which is associated with highly abundant pro-tumorigenic FAP^+ cancer associated fibroblasts (FAP^+-CAFs). Importantly, FAP^+-CAFs and extracellular matrix (ECM) are major deterrents of immune cell infiltration and potent mediators of immunosuppression in the TME. By using real-

time tumor fragment-based 2-photon microscopy, multiparametric flow cytometry and multiplexed immunofluorescence staining, we showed that FAP targeted CAR T cells efficiently traffic into tumors compared with tumor-antigen (mesothelin) targeted CAR (Meso-CAR) T cells which were trapped in the stroma-rich and matrix-dense areas. Depletion of FAP^+-CAFs reprogrammed the fibrillar collagen network surrounding tumor nests, advancing the infiltration of FAP-CAR T cells into tumor nests. Strikingly, depletion of FAP^+ cells and disruption of the matrix following FAP-CAR T administration rendered tumors permissive to meso-CAR T cells allowing their infiltration and function within tumor. Moreover, ablation of FAP^+-CAFs enhanced tumor infiltration of endogenous T cells, altered the immune and stromal landscapes, which further enhanced anti-tumor immunity and immunotherapy in PDAC models. Thus, our date establishes that FAP-CAR T cell-mediated ablation of immunosuppressive FAP^+-CAFs and disruption of the desmoplastic stroma they generate, can enhance accumulation and functionality of CAR-T cell therapy in the context of highly desmoplastic solid tumors.

A048: Modulation of the breast tumor microenvironment by targeting CD40 leads to tumor clearance and long-term immune memory

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While immunotherapy has revolutionized the treatment of several cancers, the majority of patients with triple negative breast cancer (TNBC) do not respond to this approach. Thus, new ways to mobilize the immune system to fight TNBC are needed. In this study we aim to develop and characterize CD40-targeting antibodies (Abs), enhance our understanding of breast cancer immunobiology and support the translation of CD40-targeting Abs to the clinic for the treatment of patients with TNBC.

CD40 is a tumor necrosis family receptor superfamily member that when engaged by its ligand, CD40 ligand (CD154), trimerizes to stimulate downstream signaling and activation of antigen presenting cells (APCs). Antigen presentation, in particular by dendritic cells (DCs), is required to stimulate T-cell responses against tumor cells. Prior anti-CD40 Abs tested in the clinic showed little activity and were accompanied by toxicity when delivered systemically (thrombocytopenia and transaminitis). A partial reason for the limited activity can be explained by insufficient binding of the Fc (Fragment Crystallizable) portion of the anti-CD40 Ab to the inhibitory Fc-Receptor (FcR) FcRIIB, and thus – the Abs were unable to initiate the necessary cross-linking needed for optimal CD40 signaling and APC activation.

Our hypothesis is that CD40 is expressed on DCs within the breast TME and in the tumor draining lymph node (TDLN). Engagement of CD40 by the Fc-enhanced agonistic anti-CD40 Ab will lead to immune activation of APCs and recruitment of cytotoxic CD8 T-cells, which will result in tumor elimination and development of a long-term immune memory.

We engineered a human IgG1 anti-hCD40 agonistic Ab (clone 2141) by introducing several point mutations to its Fc portion (termed herein: V11) in order to selectively increase its binding affinity to the receptor FcRIIB. We have also developed murine model systems - TNBC cell lines (E0771 and 4T1) with/without human tumor antigens (such as MUC1) that can be inoculated orthotopically to the mammary fat pad to establish primary tumors or intra-venously

to mimic metastatic disease. We have also 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).

The Fc-modified variant demonstrated superior anti-tumor activity in vivo, in hCD40/hFcR mice inoculated orthotopically with murine TNBC cells. Because of the dose limiting toxicities seen in clinical studies, we successfully demonstrated that in situ administration (injecting the anti-hCD40 Ab into tumors) leads to complete tumor clearance. Moreover, in a bi-lateral tumor challenge, we have shown that both tumors fully regress, despite administrating the Ab to only one of the tumors. This abscopal effect suggests a robust systemic immune activation. Mice that have successfully rejected the tumors upon anti-CD40 treatment were fully protected from a tumor re-challenge (inoculated with a 5x tumor cell dose), suggesting development of a long-term anti-tumor immune memory. Depletion studies and adoptive cell transfer experiments of specific immune sub-populations showed that this long-term memory and protection were mediated by CD4 and CD8 T-cells, but not B-cells. Finally, this new CD40-targeting antibody proved efficacious and safe not only in transgenic immunocompetent mouse models, but also in patients with TNBC treated in our Phase1 clinical trial (NCT04059588).

Overall, our findings characterize the mode of action of CD40-targeting antibodies, identify the immune sub-populations that mediate the anti-tumor activity and suggest that our in-situ administration approach may be beneficial for patients with TNBC. Using Abs with a human Fc that target human CD40 in our unique hCD40/hFcR transgenic mouse models, our results are readily translatable to human clinical trials.

A050: Priming the tumor microenvironment with 5'Triphosphate-RNA improves CAR T cell infiltration and promotes antigen spreading in a KPC-derived murine pancreatic cancer model

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The efficacy of chimeric antigen receptor (CAR) T cell therapy against solid tumors remains unsatisfactory due to lack of CAR T cell persistence, impaired trafficking into the tumor and the presence of immunosuppressive mechanisms, mediated for instance by myeloid suppressor cells (MDSC), which are known to inhibit T cell function. Moreover, initially responsive tumors tend to relapse due to antigen loss and lack of antigen spreading.

5'-triphosphate double-stranded RNA (3p-RNA) is recognized by the intracellular pattern recognition receptor retinoic acid-induced gene I (RIG-I). RIG-I activation by 3p-RNA mimics an antiviral immune response, ultimately leading to the expression of type I interferons (IFN), proinflammatory cytokines and chemokines, all of which enhance immune surveillance. In addition, 3p-RNA mediates immunogenic cell death (ICD) via RNase L. We hypothesized that priming the tumor microenvironment (TME) with 3p-RNA increases the overall efficacy of CAR T cell therapy in solid tumors while promoting ICD, subsequently leading to a *de novo* adaptive immune response with antigen spreading.

We used KPC-derived T110299 pancreatic tumor cells engineered to express murine epithelial cell adhesion molecule (EpCAM) as a CAR T cell target. Tumors were induced subcutaneously (s.c.) or orthotopically in C57BL/6J female mice and treated with intratumoral (i.t.) or intravenous (i.v.) injections of 3p-RNA, respectively, followed by i.v. injection of syngeneic murine T cells, retrovirally transduced to express anti-EpCAM CAR. Three, nine and fourteen days after CAR T cell injection, immune cell composition, CAR T cell infiltration into the tumor, and CAR T cells persistence in the blood were assessed by flow cytometry. Additionally, tumor growth and survival were monitored. Tumor antigen uptake in response to treatment cells was assessed with *in vitro* phagocytosis assays of tumor cells co-cultured with bone marrow-derived $CD103^+$ DC ($iCD103^+$ BMDC). *In vivo* antigen spreading and induction of a *de novo* immune response was assessed using mice with T110299 $EpCAM^+$ tumors expressing the model antigen OVA. The generation of tumor reactive T cells against OVA was tested by stimulating isolated blood cells with SIINFEKL peptide and measuring IFN-γ spot forming units (SFU) by ELISpot.

Injections of 3p-RNA into tumors reshaped the myeloid immune compartment in the TME with decreased numbers of polymorphonuclear (PMN)-MDSCs. This was paralleled by an increase in the infiltration of CAR T cells into tumors and prolonged persistence in the blood of combination therapy-treated mice. Interestingly, anti-EpCAM CAR T cells alone failed to control the tumor growth of T110299 $EpCAM^+$ tumors, monotherapy with 3p-RNA slightly delayed tumor growth, while the combination therapy lead to a clinical benefit with tumor regression in 50% of the treated mice in the s.c. tumor model and prolonged survival in the orthotopic model. Moreover, 3p-RNA treatment enhanced ICD marker expression in tumor cells, which was paralleled by improved antigen uptake, activation marker expression and antigen cross-presentation by $iCD103^+$ BMDC *in vitro*. Generation of a *de novo* immune response was indicated by higher numbers of IFN-γ SFU upon peptide stimulation of T cells from mice that received the combination therapy.

In conclusion, 3p-RNA therapy facilitates CAR T cell activation and infiltration into solid tumors while eliminating immunosuppressive PMN-MDSC resulting in enhanced tumor control in an immunologically "cold tumor" pancreatic cancer model. ICD-induced antigen spreading may facilitate *de novo* anti-tumor immune responses for long-term tumor control.

A051: ICOS co-stimulation in combination with CTLA4-blockade remodels intratumoral T cells to effector T cells, and macrophages to critical anti-tumor phenotype.

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We have previously demonstrated the synergy between the combination of ICOS co-stimulation (IVAX) and CTLA-4 blockade in anti-tumor therapy. In this study, we performed an unbiased protein and gene expression analysis of tumor-infiltrating cells by Cytof and single-cell RNA-seq after the combination therapy treatment. Our data show significant remodeling of both the lymphoid and myeloid compartments. Interestingly, compared to tumors treated with anti-CTLA-4 monotherapy, tumors treated with the combination therapy were enriched in Th1 CD4 T

cells, effector CD8 T cells, and macrophages with anti-tumor phenotypes. These M1-like tumor-associated macrophages (TAMs) were critical to the therapeutic efficacy of anti-CTLA-4 plus IVAX/anti-PD-1 combination therapy. Depletion of macrophages was accompanied by a significant reduction of CD8 and CD4 effector T cells in the tumor and weakened anti-tumor functions of these effector cells. The recruitment and polarization of M1-like TAMs required IFN-γ. Therefore, in this study, we show that there is a positive feedback loop between intratumoral effector T cells and the TAMs, in which the IFN-γ produced by the T cells polarizes the TAMs into M1-like phenotype, and the TAMs in turn reshape the tumor microenvironment to facilitate T cells infiltration and immune functions and tumor rejection.

A053: ON203: A new anti-oxMIF antibody exerts antitumorigenic activity and modulates the tumor microenvironment in patient-derived tumoroids

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The therapeutic response of solid cancers to immunotherapy is largely determined by the tumor microenvironment (TME). The macrophage migration inhibitory factor (MIF) has been described to be a key player in generating an immunosuppressive TME by inducing polarization of macrophages to the M2 subtype and cytotoxic T cell suppression, and to be associated with poor response to immune checkpoint therapy. Due to its ubiquitous nature, MIF is considered an unsuitable target for therapeutic intervention. In contrast, the disease-related structural isoform of MIF, termed oxMIF, is specifically present in solid tumor tissue including colorectal, lung, ovarian and pancreatic cancer. In this study, we determined the antitumorigenic and TME-modifying potential of the new oxMIF-specific antibody ON203.

ON203 is a fully human monoclonal anti-oxMIF antibody, bioengineered to (i) neutralize oxMIF and therefore to inhibit the tumorigenic activity attributed to MIF, (ii) exert enhanced Fc-mediated effector functions and (iii) show highly efficient tumor penetration and tumor retention. The effects of anti-oxMIF antibody ON203 on primary human colorectal adenocarcinoma (CRC) tumoroids from five CRC patients were assessed by treating freshly isolated 3D tumoroids showing an intact TME with ON203 or isotype control. Tumor cell death was assessed by high-content 3D computational bioimaging and changes in tumor-associated immune cells were analyzed by flow cytometry. The secretome was analyzed by ELISA and multiplex cytometric bead assays for changes in cytokine secretion.

Tumor penetration and retention as well as tumor-suppressive activity of ON203 were demonstrated in mouse xenograft models and revealed reduced angiogenesis, tumor cell proliferation and intravasation leading to impaired tumor growth. ON203 induced tumor cell killing in tumoroids isolated from four out of five CRC patients. Analysis of the immune contexture revealed that NK and NKT cells of the responders were activated and the degranulation markers Granzyme B and CD107a were elevated upon ON203 treatment. The fraction of immunosupportive M1 macrophages was increased in the ON203-treated, but not in the isotype control-treated CRC tumoroids, which correlated with reduced IL-10 levels in the secretome of ON203 responding-tumoroids. Analysis of the secretome implied that ON203 modified the TME in patient-derived tumoroids towards an enhanced therapeutic response.

Targeting oxMIF with ON203 has a high potential to become a new treatment option for patients with solid tumors (e.g. colorectal, lung or ovarian cancer) as a standalone therapy or in combination with checkpoint inhibitors, chemotherapeutics, anti-angiogenic drugs, or kinase inhibitors. Our mouse xenograft and human tumoroid study results support our planned first-in-human, Phase 1, open-label, dose-escalation study to assess safety, tolerability, pharmacokinetics, and pharmacodynamics of ON203 in patients with malignant solid tumors.

A054: Identifying regulators of tumor adjuvanticity using a high-throughput cancer cell – dendritic cell co-culture model

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The efficacy of anti-tumor immune responses is determined by tumor immunogenicity. Tumor immunogenicity requires antigenicity (presence of neoepitopes presented on MHC class I, recognized by cytotoxic T cells (CTLs)); and adjuvanticity (presence of signals which activate antigen presenting cells such as dendritic cells (DCs)). DCs can be activated by endogenous danger/damage-associated molecular patterns (DAMPs), which include primary signals such as cytosolic or extracellular DNA, release of ATP or HMGB1; and secondary signals, such as production of type I interferons. The pathways leading to the generation of DAMPs by cancer cells have great potential as immunotherapy targets, but they are not well understood. We seek to understand how the production of adjuvants is regulated in cancer cells.

We have developed a DC – cancer cell in vitro co-culture system, where we monitor DC activation (as upregulation of surface maturation markers) to detect the production of adjuvants by cancer cells. We have used our co-culture system in a series of high-throughput screens to identify pathways involved in cancer cell adjuvant production; and to understand how these adjuvants are sensed in DCs.

We first tested the potential for our co-culture system to detect the presence of DAMPs using a secretome library – a collection of proteins known (or predicted) to be secreted to the extracellular environment. We detected several potential novel DAMPs, which we will be studying further.

We then performed a whole genome arrayed CRISPR-Cas9 screen in our co-culture system to find regulators of tumor adjuvanticity in cancer cells. We carried out CRISPR-Cas9 editing of cancer cells for more than 20,000 individual genes and identified 360 hits. In a secondary screen we confirm the effect of more than 40 hits. We selected the top 12 hits for further assays, and finally prioritized four genes for validation.

In order to characterize the adjuvant production in our hit knockouts, we analyzed the transcriptomes of our cancer cell knockouts. Additionally, to understand how DCs sense the adjuvants produced, we performed a high-throughput pooled CRISPR Cas9 screen in the DCs in

co-culture with knockout cancer cells. Finally, we are currently developing in vivo models using inducible knockouts to test the potential of our hits to evoke an anti-tumor immune response.

In conclusion, we have developed a high-throughput cancer cell – dendritic cell co-culture model to detect changes in tumor adjuvanticity and used it to identify novel regulators of tumor adjuvanticity, which we are currently characterizing further.

A055: Longitudinal noninvasive in vivo bioluminescence imaging (BLI) of an endogenous immune cell related increased increase in NFκB activity within the tumor micro environment as a novel innovative tool to predict the efficacy of cancer immunotherapy

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Introduction

Since decades, cancer research is highly engaged in understanding the underlying mechanisms of tumor cell immune escape. We have recently established a combined cancer immunotherapy (COMBO) consisting of an initial 2 Gy whole body radiation, tumor-antigen (TA) specific IFN-y producing $CD4^+$ T cells (Th1) as well as immune checkpoint blockade (ICB: anti-PD-L1 and anti-LAG-3 mAbs) and have experimentally proven that an enhancement of glucose metabolism within the bone marrow predicts an efficient anti-cancer immunotherapy. Here we aimed to visualize and monitor the sites of immune cell activation by noninvasive *in vivo* visualization of NFκB activation within the tumors as well as the bone marrow in adenocarcinoma and melanoma bearing NFκB-luciferase (NFκB-luc) reporter mice.

Methods

We s.c. inoculated ovalbumin expressing (OVA)-MC38 adenocarcinoma or (OVA)-B16 melanoma tumor cells in NFκB-luc reporter mice which express luciferase (luc) exclusively upon NFκB activation to follow the effect of our COMBO-treatment approach on the tumor-micro-environment (TME) and the bone marrow in an ICB sensitive and non-sensitive tumor model. Treatment efficacy was determined by daily measurements of the tumor volume using a caliper. Our COMBO-treatment consisting of an initial 2 Gy total body radiation, one OVA-Th1 cell administration (OT-II) and three ICB injections was initiated four days after tumor cell inoculation. Longitudinal noninvasive *in vivo* BLI was performed daily to monitor NFκB-activation within the tumors and bone marrow until the end of the experiment. For *ex vivo* characterization of the TME, we additionally performed histopathology (H&E) and

immunohistochemistry (IHC; $CD3^+$ and $B220^+$) of tumors and multicolor flow cytometry (MFC) analysis of the immune cell infiltrate within the tumors as well as the primary and secondary lymphatic organs ($CD4^+$, $CD8^+$, $CD19^+$ etc.) and the immune cell activation markers CD69.

Results/Discussion

COMBO treatment significantly decreased the relative tumor growth (p=0.0289) of OVA-MC38 tumor-bearing NFκB-luc reporter mice (n=4-5) whereas the relative tumor growth of OVA-B16 melanomas was not significantly affected when compared to Sham-treated mice. Exclusively the endogenous immune cells within COMBO-sensitive OVA-MC38 adenocarcinomas exhibited an enhanced NFκB activity-related signal intensity (SI: 5015 p/s/cm^2/sr) when compared to the tumors of Sham-treated mice (SI: 3432 p/s/cm^2/sr) indicating a COMBO-induced immune cell activation within the TME (p=0.0005). No COMBO-related differences in immune cell-derived NFκB activation was determined within OVA-B16 melanomas when compared to the Sham-treatment group. In this context it is important to mention that our therapeutic OVA-Th1 cells do not express the NFκB-luc reporter. MFC analysis revealed a significant increase in B cells within the TME of OVA-MC38 tumors and the tumor draining lymph nodes of COMBO-treated mice when compared to Sham-treated mice ($p<0.05$). Furthermore, the number of CD69 expressing activated $CD4^+$ T cells within the spleen and bone marrow was more pronounced in COMBO-treated OVA-MC38 tumor bearing mice but not in OVA-B16 bearing mice. Moreover, we observed a significantly pronounced infiltration of $CD4^+$ (p=0.0484) and $CD8^+$ T cells (0.0251) in the not COMBO-responsive OVA-B16 tumors but without an enhancement of CD69 expression probably indicating a lack of T cell activation.

Conclusion

COMBO treatment of OVA-MC38 and OVA-B16 bearing NFκB-luc reporter mice effectively inhibited tumor growth of OVA-MC38 adenocarcinomas but not of OVA-B16 melanomas. Treatment efficacy was associated with an increased NFκB activity of endogenous immune cell within the MC38 tumors and the presence of activated $CD4^+$ T cells within the TME. Thus, longitudinal noninvasive *in vivo* monitoring of immune cell derived NFκB activation within the TME might exhibit a novel innovative tool to identify immune therapy response or failure.

A057: Effect of adipose mesenchymal stromal cells on mouse mammary and lung metastatic tumor formation

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BACKGROUND: Breast cancer (BC) is the most prevalent cancer in females and the leading cause of death with over 2 million new cases worldwide per annum. The major disadvantage of cancer therapies such as chemotherapy and radiotherapy is that they not only target tumor cells but also healthy cells. The ultimate aim is to develop therapeutic strategies that target tumor cells directly without compromising healthy cells. Mesenchymal stromal cells (MSCs) are reported to

"home" to tumor sites where they modify the immune response. Currently, there is no consensus on the impact of MSCs on the tumor micro-environment as both anti- or pro-tumorigenic properties have been reported. These contradictory findings are likely to be due to experimental design, and in particular, the use of xenogeneic models that do not recapitulate what happens in humans. This study used an isogenic mouse model of spontaneous BC development to investigate the effect of murine MSCs on BC progression. METHODS: MSCs isolated from FVB/N mouse adipose tissue (mASCs) were administered intravenously at 2 million cells per mouse on days 30, 37 and 44 in heterozygous FVB/N-Tg(MMTV-PyVT)634Mul/J female mice that develop palpable mammary tumors (MT), while control mice received saline. Tumor volume, mass, histopathological necrosis and gene expression analyses of MT and lung metastatic lesions (LT) were performed. RESULTS: No change in MT mass or volume was observed between mASC treated and control mice. mASC treatment led to increased necrosis in LT but this was lower in MT. Additionally, immunohistochemistry showed CD163 positive anti-inflammatory macrophages were higher in MT but lower in LT. Downregulation of *tgf-β3*, *vegfr1* and *cd105* was observed in both MT and LT treated mice. Additionally, *cd36* and *mtdh* were downregulated in MT and LT respectively. Downregulation of these genes in MT and LT suggests an anti-tumorigenic effect. However, this effect only led to a decrease in anti-inflammatory activity in LT as reflected by the lower number of CD163 positive cells and an increase in necrosis. The anti-tumor effect of mASCs in LT could be due to the presence of mASCs trapped in the lung. CONCLUSION: mASCs increased necrosis in LT. The mASC-induced changes in gene regulation are suggestive of antitumor activity.

A058: Intratumoral adoptive transfer of inflammatory macrophages enhanced by co-activation of TLR and STING signaling pathways showed robust antitumor activity

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Despite the success of chimeric antigen receptor T cells against hematologic malignancies, there is still no effective adoptive cell therapy (ACT) against solid cancers because of anti-inflammatory tumor microenvironment (TME) and antigen heterogeneity of solid tumors. In this study, we developed an enhanced inflammatory macrophage-based ACT for the treatment of anti-inflammatory solid tumors. Inflammatory macrophages are well known to have various antitumor activities, such as modulating the surrounding environment into an inflammatory environment by secreting pro-inflammatory cytokines, presenting tumor antigens to T cells, expressing co-stimulatory molecules and killing tumor cells in antigen recognition-independent manner. These properties make them a promising candidate for ACT for the treatment of solid cancers. However, inflammatory macrophages polarized only by treatment of interferon-γ (IFN-γ), showed insufficient therapeutic efficacy in past clinical studies. Recent studies have shown that the antitumor activities of the inflammatory macrophages can be enhanced by activation of pattern recognition receptor (PRR) signaling pathways such as, toll-like receptor (TLR) and stimulator of interferon genes (STING) signaling pathways. However, little is known about how the simultaneous activation of these PRR signaling pathways enhances the antitumor activities of the inflammatory macrophages. Here, we investigated the effects of the simultaneous activation of TLR and STING signaling pathways on the antitumor activities of the inflammatory macrophages. We treated TLR4 ligand and STING ligand alone or simultaneously to mouse bone marrow-derived macrophages and investigated antitumor activities *in vitro* and *in vivo*.

Secretion of pro-inflammatory cytokines, expression level of co-stimulatory molecules and ability to kill tumor cells were synergistically and complementarily enhanced by co-activation of TLR4 and STING signaling pathways. The co-activated inflammatory macrophages maintained the inflammatory phenotype after adoptively transferred into the anti-inflammatory TME whereas conventional inflammatory macrophages polarized only by IFN-γ were repolarized to anti-inflammatory phenotype. Intratumoral adoptive transfer of the macrophages increased activated $CD8^+$ T cells in TME and tumor antigen-specific $CD8^+$ T cells in blood, and showed robust tumor growth inhibition in the MO5 mouse melanoma model, well known to have anti-inflammatory TME. In conclusion, the co-activation of TLR and STING signaling pathways robustly enhances the antitumor activities of the inflammatory macrophages, and intratumoral adoptive transfer of the enhanced macrophages has the potential as an effective ACT for the treatment of solid cancers.

A059: A robust B cell-dependent anti-tumor immunity that hampers pancreatic cancer progression is triggered by combination of ENO1 vaccination and PI3Kgamma inhibition

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Background: Pancreatic ductal adenocarcinoma (PDA) is the fourth leading cause of cancer mortality, with one of the poorest prognoses among all cancers. Phosphoinositide-3- kinase gamma (PI3Kg) plays a critical role in PDA by driving the recruitment of myeloid derived suppressor cells (MDSC) into tumor tissues, supporting tumor growth and metastasis. This may also impair the efficacy of immunotherapy, such as vaccination against alpha-enolase (ENO1). In this study, we assessed the hypothesis that the targeting of MDSC, via pharmacological or genetic inhibition of PI3Kg, synergizes with ENO1 DNA vaccination in countering PDA growth.

Methods: PDA mouse models genetically devoid of PI3Kg or pharmacologically treated with PI3Kg inhibitor were vaccinated with ENO1 DNA plasmid. Anti-tumor immunity induced by the different treatments were investigated by histology and immunohistochemistry, quantitative real time polymerase chain reaction, enzyme-linked immunosorbent assays, serologic proteome analysis, flow cytometry, enzyme-linked immunosorbent spot and cytotoxicity assays. Tumor size or overall survival were also analyzed. The apparent diffusion coefficient (MRI-ADF) and vessel permeability were evaluated by mean of Magnetic Resonance Imaging.

Results: Mice received ENO1 vaccination followed by PI3Kg inhibitor therapy showed a significant decrease in tumor size in comparison to those treated with ENO1 alone and the control group that correlated with the i) increased circulating anti-ENO1 specific IgG1and IFNg secretion by T cells; ii) increased of tumor infiltration of CD8+ T cells and M1-like macrophages as well as the up-modulation of T cell activation and M1-like related transcripts; iii) decreased infiltration of T reg FoxP3+ T cells, endothelial cells and pericytes and a down-modulation of stromal compartment and T cell exhaustion gene transcription; iv) reduced mean of the fibrosis

related-marker MRI-ADF and vessels permeability; v) increase of IgG recognition of a greater number of tumor associated antigens. Of interest, PDA mouse models genetically devoid of PI3Kg showed an increased survival, a sustained antibody response and a pattern of transcripts into the tumor area similar to that of combination-treated PI3Kg-proficient mice. Notably, tumor growth reduction was abrogated by B cells depletion in PDA orthotopically-injected mice in which ENO1 vaccine and PI3Kg inhibitor was administered.

Conclusions: These data highlight a novel role of the PI3Kg in restraining B cell activation suggesting its depletion as a strategy to unleash the humoral anti-tumor response elicited by ENO1 DNA vaccine.

A060: Therapeutic modulation of tumor-infiltrating T cell function in fibrolamellar carcinoma

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Introduction

Fibrolamellar carcinoma (FLC), a rare liver cancer primarily affecting young patients without cirrhosis, presents an opportunity for novel immunotherapeutic treatments given its high rate of recurrence and metastasis, suboptimal response to conventional therapies, and unique immune landscape. Current evidence implicates an immunosuppressive tumor microenvironment (TME), including sequestration of tumor infiltrating lymphocytes (TILs) away from the carcinoma compartment and impaired cytotoxic T cell activity, as among the key drivers of aggressive tumor biology in FLC. We hypothesized that precisely targeting the TME could enable immunotherapy for this challenging disease.

Procedures

We performed multiplex immunohistochemistry (mIHC) on formalin-fixed paraffin-embedded (FFPE) tissue to delineate the nature and abundance of immune cell types in the carcinoma compartment, stroma, non-tumor liver (NTL), and interface between NTL and tumor. We created tumor slice cultures (TSC) from fresh human FLC tumors and treated with drugs aimed at overcoming potential immunosuppressive mechanisms (e.g., C-X-C chemokine receptor type 4 (CXCR4) and its ligand (CXCL12), programmed cell death receptor (PD-1) and its ligand (PD-L1), and IL-10). We employed live microscopy and single color IHC to visualize T cell infiltration and apoptosis after drug treatment, staining for epithelial cell adhesion molecule (EpCAM, a marker for carcinoma cells), CD8, and cleaved caspase-3 (cC3, a marker for apoptosis).

Data

There were significantly higher densities of $CD4^+$ and $CD8^+$ T cells per mm^2 in the interface compartment compared to the tumor itself ($p=0.005$ and $p=0.03$, respectively), and in the stroma

compared to the carcinoma compartment (p=0.006 and p=0.03, respectively). Two patients without known recurrence at 5 years trended toward relatively higher densities of T cells in the carcinoma compartment compared to two with recurrence. Drug treatment in TSC models altered T cell infiltration and tumor apoptosis based on live microscopy and IHC: PD-1 blockade effected a trend toward a greater percentage of $EpCAM^+$ carcinoma cells with CD8+ T cells in close proximity (<20 μm) compared to IgG monoclonal antibody (mAb) control (25% vs. 14%, p>0.05), suggestive of increased T cell infiltration; antibody blockade of IL-10 or PD-1 each increased cC3 expression compared to IgG control (52% and 33%, respectively, vs. 13%, p<0.001), suggestive of enhanced tumor apoptosis; and combination PD-1 and CXCR4 blockade increased T cell infiltration (40% vs 14%, p=0.01) and tumor apoptosis (40% vs. 30%, p=0.06) compared to IgG mAb control.

Conclusion

TILs are sequestered outside the carcinoma compartment in FLC, contributing to an immunosuppressive TME that has thus far rendered most systemic treatments of FLC ineffective. TSC models indicate that modulation of the TME with blockade of IL-10 or CXCR4 and PD-1 has the potential to reactivate endogenous anti-tumor immunity. Future work includes use of mIHC to further characterize the spatial distribution of TILs after CXCR4, PD-1, and IL-10 blockade. These findings may have important clinical implications with respect to FLC recurrence, prognosis, and development of novel immunotherapies.

A061: Modulation of the gut microbiota potentiates immune checkpoint blockade in multiple myeloma by restraining expansion of pro-tumoral Th17 cells

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Whereas T cell exhaustion in the bone marrow of patients affected by multiple myeloma (MM) and PD-L1 expression on neoplastic plasma cells represent a strong rationale for the use of immune checkpoint blockade (ICB) to treat MM, results of related clinical trials have so far been disappointing. The gut microbiota is a strong modulator of the immune response and, together with ICB, can contribute to the expansion of pro-inflammatory Th17 cells that eventually propel MM aggressiveness. We hypothesized that modulation of the gut microbiota at the time of treatment with anti-PD-L1 antibodies would have increased the therapeutic efficacy of ICB in Vk*MYC mice developing *de novo* MM. We previously reported in Vk*MYC mice that the human commensal *Prevotella heparinolytica* (*P.h.*) induces Th17 cells in the gut that migrate to the bone marrow where they sustain the survival and proliferation of neoplastic plasma cells. At odds, *P. melaninogenica* (*P.m.*) restrains MM progression by limiting the expansion of Th17 cells. Thus, two strains belonging to the same genus may exert opposing effects on the immune system. We combined here oral administration of *P.m.* with anti-PD-L1 antibodies in mice affected either by asymptomatic MM (Early-MM) or full-blown disease (Late-MM). The two treatments showed synergy against neoplastic plasma cells in both disease phases and, remarkably, delayed the progression from Early-MM to Late-MM. These findings are

particularly relevant because MM is a treatable but incurable disease and therapeutic options for patients affected by asymptomatic MM (i.e., Smoldering MM; SMM) are still lacking. Mechanistically, *P.m.*, while preventing the generation of gut-borne Th17 cells, did not restrain expansion of ICB-driven cytotoxic T lymphocytes (CTLs) accumulating in the bone marrow of MM mice and favorable CTL/Treg ratios. We also found that dendritic cells (DCs) in the bone marrow of MM-bearing mice treated with *P.m.* produced lower levels of IL-6 and IL-1β than DCs from *P.h.*-treated mice. Both cytokines are relevant as they induce Th17 polarization. Indeed, *in vitro* stimulation of human and mouse DCs with *P.m.* or its conditioned medium resulted in lower polarization of naïve T cells to Th17 cells when compared to *P.h.* or its conditioned medium. Taken together, our data support the use of anti-PD-L1 antibodies in combination with microbiota-based immunotherapies to treat full-blown MM and to prevent progression of patients affected by SMM to MM.

A062: Boosting innate and adaptive immune response against osteosarcoma by intralesional administration of TLR9 agonists

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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 tumors at diagnosis. For metastatic disease, however, the prognosis is dismal, making urgent the identification of new, more efficient and possibly less toxic, theraputic strategies.

The concept of "abscopal effect", i.e. the regression of a tumor lesion outside the treatment field or of a distant metastasis, has been reported mainly for local radiotherapy, but it could similarly be applied also to other therapeutic approaches, including immuno-modulatory agents, administered intralesionally.

In immune competent OS mouse models we tested the efficacy of intralesional administration of a TLR9 agonist in inhibiting the growth of both treated and untreated controlateral lesions, obtained injecting tumor cells on both flanks of the same animal. The TLR9 agonist effectively halted the growth of the locally-treated tumor and largely suppressed the contralateral lesion. Multiparametric flow cytometry analysis showed a reduced infiltration of CD206+ M2-like macrophages, paralleled by an increased infiltration of activated CD8 T cells in both lesions. Experiments in immune-deficient mice indicated the need of CD8 T cells for inducing an efficient abscopal effect, but not for inhibiting the treated tumors. Additional experiments were performed by surgically removing the treated primary tumor before a challenge with live tumor cells into the controlateral flank to confirm the induction of systemic immunity and memory response. These immunization-challenge experiments showed a complete protection from the take of a second tumor and the expansion of CD8 T cells with a memory phenotype in the lymph node draining the challenging site. T cell receptor sequencing of tumor infiltrating CD8 T cells indicated the expansion of specific TCR clones only in mice whose tumors received the TLR9

agonist. Interestingly, some of the expanded clonotypes were identical in treated and untreated lesions.

Overall these data indicate that the TLR9 agonist acts as local anti-tumor vaccine, activating an innate immune response sufficient to suppress local tumor growth, while inducing a systemic adaptive immunity able to inhibit the growth of a controlateral tumor lesion and to protect from a secondary tumor challenge.

A063: Inhibitory receptor PIRB modulates immune cell recruitment in pancreatic cancer and delays its progression

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Paired immunoglobulin-like receptor B (PIRB) is a murine inhibitory receptor expressed on the surface of many immune cells including macrophages, granulocytes, dendritic cells and B lymphocytes. The presence of 3 intracellular Immunoreceptor Tyrosine-based Inhibitory Motives enable the recruitment of Src homology 2 domain-containing protein tyrosine phosphatases (SHP-1/2), which switch off the kinase activating signaling pathways. Therefore, when activated by contacting its principal ligand, namely major histocompatibility complex class I molecules, PIRB acts as an immune checkpoint. PIRB loss has been previously associated with 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. Because of these premises, we aimed to characterize PIRB role in modulating the anti-tumoral immune response in mouse models of pancreatic ductal adenocarcinoma (PDA): mice lacking PIRB gene (*Pirb*$^{-/-}$) were crossed with genetically engineered mice (GEM) spontaneously developing PDA. We found that PIRB deletion significantly improved overall survival of GEM compared to the *Pirb* proficient counterpart, and this correlated with their significantly reduced percentage of transformed ducts in both early- and late-stage of disease. Preliminary immunohistochemical analyses of pancreatic tissues demonstrated increased frequency in tumor infiltrating CD8^{+} and CD4^{+} T lymphocytes, as well as in 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, *Pirb*$^{-/-}$ mice immunized with OVA-overexpressing tumor cells showed a reduced growth of living tumor cells, together with an enhanced number of IFNg-secreting T cells and higher levels of OVA-specific IgG. 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.

A064: Induced pluripotent stem cells display a unique set of MHC I-associated peptides shared by human cancers

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The development of cancer immunotherapies is limited by the lack of well-defined tumor-specific antigens. Previous reports showed that mouse vaccination with pluripotent stem cells (PSCs) induces durable anti-tumor immune responses via T-cell recognition of some elusive oncofetal epitopes. We characterized the MHC I-associated peptide (MAP) repertoire of human induced PSCs (iPSCs) using proteogenomics. Our analyses revealed a set of 46 pluripotency-associated MAPs (paMAPs) absent from the transcriptome of normal tissues and adult stem cells but expressed in PSCs and multiple adult cancers. These paMAPs derived from coding and allegedly non-coding (48%) transcripts involved in pluripotency maintenance, and their expression in TCGA samples correlated with source gene hypomethylation and genomic aberrations common across cancer types. We found that several of these paMAPs were immunogenic. However, paMAP expression in tumors coincides with activation of pathways instrumental in immune evasion (WNT, TGF-β, and CDK4/6). We propose that currently available inhibitors of these pathways could synergize with immune targeting of paMAPs for the treatment of poorly differentiated cancers.

A065: Targeting the BAF-nucleosome remodeling complex in tumor-associated macrophages to boost response to immunotherapy

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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. Targeting signaling pathways that re-program TAMs from an immunosuppressive “tumor-promoting” to a pro-inflammatory “tumor-fighting” phenotype have shown promise for treating cancer in pre-clinical models. However, in order to find the best method to reprogram macrophages, we need a better understanding of the molecular pathways that control tumor-associated macrophages. 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 are using myeloid-specific Cre-drivers to delete BAF complex subunits and examine the effects on macrophage gene expression, tumor growth, and immune function. We are utilizing a combination of flow cytometry, functional genomics and epigenomic techniques in both in vivo and in vitro models. We hypothesize that manipulation of BAF-complex function in myeloid cells could be a potential method to increase response to existing immunotherapies.

A066: Immunity and immune resistance in MSI-H tumors

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Patients with Lynch Syndrome, an inherited mismatch repair deficiency, have an increased risk for developing microsatellite unstable (MSI-H) cancers. Our team recently identified shared immunogenic frameshift peptides in patient tumors that can be targets of T cell surveillance and preventative MSI-H cancer vaccines. We hypothesize that in Lynch Syndrome some pre-malignant lesions are capable of evading immune surveillance from cytotoxic T lymphocytes due to immune checkpoint expression and immunosuppression from tumor, myeloid and stromal cell populations. Immune checkpoint blockade (ICB) showed promising results in the treatment of MSI-H tumors and are being evaluated in the adjuvant setting of patients with Lynch syndrome post surgical resection. However, a significant percentage of advanced MSI-H tumors resist ICB suggesting evolving mechanisms of immune resistance. We will use a MSI-H in vivo mouse model and additionally develop 3D spheroids cocultured with immune cells to characterize the underlying immune resistance mechanisms. The MSI-H mouse model will first be applied to identify shared frameshifts in MSI-H tumors by WES to develop vaccination approaches preventing tumor growth. Then, we will quantify MSI-H tumor growth and immune infiltration in the MSI-H mouse model, with or without ICB. We will analyze the presence of immunosuppressive pathways and myeloid subsets in high growth resisting tumors by immunohistochemistry, scRNAseq, spatial transcriptomics and flow cytometry. In addition, we will perform short-term in vitro spheroid-splenic cell co-cultures to better characterize the early immune resistance mechanisms. We will also perform these spheroid-immune cell co-cultures using the immune cells taken from mice after vaccination or ICB to identify the early myeloid immune resistance mechanisms. Overall, the identification of shared immune frameshifts in MSI-H tumors will allow developing peptide vaccination approaches to prevent tumor growth. Further, the identification of myeloid resistance pathways will guide therapeutic strategies against tumor resisting to ICB and/or vaccination.

A067: BT7480, a synthetic Bicycle tumor-targeted immune cell agonist® (Bicycle TICA™) induces reprogramming of the tumor immune microenvironment through tumor localized CD137 agonism

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Bicycles are fully synthetic constrained peptides with antibody-like affinities that target selectively, readily penetrate tumor tissue, have relatively short half-lives, and can be chemically linked together to generate multifunctional molecules. BT7480 is a novel, first-in-class, Nectin-4/CD137 *Bicycle* tumor-targeted immune cell agonist® (*Bicycle* TICA™) that was designed to activate CD137 through co-ligation of CD137 on immune cells and Nectin-4 on tumor cells. Nectin-4 is reported to be highly expressed in wide range of solid tumors including bladder, pancreas, breast, ovary, esophagus, head and neck, stomach, and lung cancers (1, 2). In addition,

Nectin-4 and CD137 are co-expressed in many of these tumor types (3, 4) and thus may benefit from Nectin-4 targeted CD137 agonism.

We used a suite of in vitro and in vivo assays to characterize BT7480 pharmacology and mechanism of action. These include primary human PBMC/tumor cell co-culture assays and efficacy and transcriptional profiling studies in a syngeneic mouse tumor model.

BT7480 elicited potent Nectin-4-dependent CD137 agonist activity in vitro as measured by increase in interferon gamma and interleukin-2 production from stimulated PMBCs in the presence of Nectin-4 expressing tumor cells. In contrast, BT7480 did not trigger cytokine release in human whole blood. Treatment of immunocompetent mice bearing Nectin-4-expressing tumors with BT7480 led to a profound reprogramming of the tumor immune microenvironment including an early increase in several T-cell chemotactic cytokines that preceded T cell infiltration and upregulation of cytotoxicity-related genes. Using various BT7480 exposure profiles in mice, we were able to demonstrate that the observed anti-tumor activity with complete tumor regressions was not dependent on continuous circulating drug levels, but that circulating drug exposure for approximately two days per weekly cycle was sufficient for optimal anti-tumor activity. In rat and non-human primate safety studies BT7480 appears well tolerated at doses that are far greater than those expected to be clinically relevant.

We have now demonstrated that BT7480 is potent, specific, effective, and well tolerated in preclinical species and are therefore uniquely positioned to test the hypothesis in humans that intermittent CD137 agonism may benefit cancer patients. BT7480 entered first-in-human clinical trial at the end of 2021 (NCT05163041).

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A068: Overcoming the suppressive tumor microenvironment with a live bacterial immunotherapy

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Recent advances in cancer immunotherapies have produced remarkable results in some cancer types. Unfortunately, efficacy is limited to particular subpopulations in certain indications. While the underlying reasons are many and not fully understood, tumor-infiltrating myeloid cells, a major immunosuppressive population, are one of the culprits. Within the tumor microenvironment (TME), they supress anti-tumor immunity through direct and indirect inhibitory mechanisms. However, inherent myeloid plasticity offers the opportunity to

reprogram these cells, reversing their pro-tumorigenic phenotype. Novel therapeutics that leverage myeloid plasticity have potential to revolutionize immunotherapy treatment for many types of cancers by complementing therapies that target other aspects of tumor immunology. One class of agents with this potential are bacteria and their products, which act locally on suppressive cell populations and have also been shown to induce long-lasting systemic reprogramming of myeloid cells through a process termed trained immunity. Prokarium is leveraging *Salmonella*'s unique biology, developing a live attenuated *Salmonella enterica* serovar Typhi strain (ZH9) to be the next cancer immunotherapy and sought to establish whether live attenuated *Salmonella* reprograms myeloid cells and enhances anti-tumor immune function.

Firstly, we examined the ability of *Salmonella* to modulate the existing phenotype of suppressive myeloid cells. Indeed, *in vitro Salmonella* treatment repolarized human monocyte-derived M2 macrophages towards an anti-tumor phenotype, including upregulating costimulatory molecules (CD40, CD80 and CD86), increasing secretion of pro-inflammatory cytokines and chemokines (IL12, IL18, CXCL10), and relieving suppression of co-cultured T cells. Through a series of *in vitro* and *in vivo* experiments, we also show *Salmonella* treatment reprogrammed unpolarised myeloid cells and that this may translate to an improved tumor control. Oral treatment of healthy mice with *Salmonella* induced long-term phenotypic and functional changes, including upregulation of co-stimulatory molecules (CD80, CD86) and MHC class II on systemic dendritic cells, monocytes and macrophages, and increased responsiveness of CD11c+ splenocytes to secondary stimuli, indicative of trained immunity. Further, oral treatment with *Salmonella* as a monotherapy was able to suppress tumour growth in subcutaneous and experimental metastasis models, suggesting the *Salmonella*-induced myeloid phenotype may translate to effective changes in the myeloid compartment of the TME. Finally, using human *in vitro* and mouse *in vivo* models we investigated whether *Salmonella* effects could complement other cancer therapies. *In vitro*, *Salmonella*-trained human monocytes overcame the suppressive phenotype induced by subsequent culture in M2-polarizing conditions to synergize with checkpoint inhibitors in driving T-cell proliferation. *In vivo*, oral *Salmonella* treatment synergized with anti-PD-L1 checkpoint therapy in supressing growth of subcutaneously implanted murine colon tumor cells.

Taken together, these data show that *Salmonella* immunotherapy can both reverse established suppressive myeloid phenotypes and systemically prime myeloid cells, likely rendering them resistant to immunosuppression in the TME, thereby elucidating an important part of the mechanism of action leveraged by *Salmonella* to drive anti-tumor responses.

A069: Tailoring vascular phenotype through AAV-LIGHT therapy promotes anti-tumor immunity and prolongs survival in glioma

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Glioblastomas (GBMs) are aggressive brain tumors which are resistant to immunotherapy. This is partly due to the immunosuppressive tumor microenvironment (TME) and the dysfunctional tumor vasculature, which hinder T cell infiltration. Lymphoneogenic cytokines such as LIGHT/TNFSF14 can stimulate formation of high endothelial venules (HEV) and tertiary lymphoid structures (TLS), suggesting that their therapeutic expression in the TME may promote recruitment and priming of tumor-reactive T cells. Here, we used an adeno-associated viral vector targeted to brain endothelial cells to express LIGHT in the glioma vasculature (AAV-LIGHT). Systemic AAV-LIGHT treatment resulted in the formation of intratumoral HEVs and T cell-rich TLS, and successfully prolonged survival in anti-PD-1 resistant murine glioma. AAV-LIGHT treatment reduced T cell exhaustion and promoted TCF1/7^{+} stem-like CD8^{+} T cells, which were present within the TLS and in intratumoral immune cell niches. Tumor regression upon AAV-LIGHT therapy correlated with the induction of tumor-specific cytotoxic and memory CD8^{+} T cells. Our work reveals that altering vascular phenotype through vessel-targeted expression of LIGHT using AAV promotes efficient anti-tumor T cell responses and prolongs survival in glioma. This has broader implications for the treatment of other immunotherapy-resistant cancers.

A070: SYN101, a first in class, immune cell-targeted TGFbeta therapy that restores immune function and drives tumor clearance in vivo

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TGFb is a pleiotropic cytokine that is essential in maintaining tissue homeostasis, particularly in the heart, lungs and bone. TGFb is also a validated cancer promoting and immunosuppressive pathway overexpressed in the majority of solid tumors. Cancer patients with elevated TGFb levels present with more metastatic cancers and have a worse prognosis. Although TGFb inhibitors have long been of interest as cancer therapies, systemic TGFb therapies have significant host toxicity and have fallen far short in efficacy. Although TGFb can impact most cells in the tumor microenvironment, its' dominant role is immune suppression. In preclinical studies, genetic blockade of TGFb signaling only in T cells is sufficient to drive tumor clearance. Moreover, systemic TGFb inhibitors are ineffective in immune deficient mice, indicating that systemic TGFb inhibitors require immune function to drive tumor clearance. In cancer patients, elevated levels of TGFb limit T cell activation and can preclude other immunotherapies from being effective. Not surprisingly, TGFb causes resistance to immune checkpoint inhibitors in solid tumors, such as colorectal, bladder, skin, liver and prostate. Currently, there are no FDA-approved TGFb therapies for cancer patients. Novel, targeted TGFb therapies are required to improve the therapeutic window and increase patient response rates.

To address the limited efficacy and bypass the toxicity of systemic TGFb inhibitors, Synthis is developing a first in class, cell targeted TGFb inhibitor platform based on antibody drug conjugate (ADC) technology for cancer patients. By integrating Synthis' deep expertise in ADCs, TGFb and immunotherapies, we are developing the next generation of cell selective TGFb therapies, with improved safety and efficacy for cancer patients. Our lead program, SYN101, is an immune cell targeted ADC, comprised of a proprietary, immune cell specific antibody linked to a potent TGFb receptor (ALK5) inhibitor payload that restores immune function in cancer patients. Using an engineered HEK cell line expressing both the target

receptor and a TGFb responsive luciferase reporter, we have demonstrated both the specificity and potency of SYN101 (IC50=18.1±7.1 nM*)* to inhibit TGFb signaling. In primary human immune cells, SYN101 reverses the suppressive effects of TGFb and restores expression of critical T cell functions that are required for tumor clearance, such as Granzyme B in CD8+ T cells, a marker of activated cytotoxic killer cells, IFNg levels and cell proliferation. In human PBMCs, combination of SYN101 plus antiPD1 checkpoint therapy cooperate effectively to increase IFNg expression by 4 fold, compared to only 2-fold induction with monotherapy treatment. More importantly, in preclinical mouse syngeneic tumor models, SYN101, in combination with checkpoint inhibition, drives tumor inhibition and regression in EMT6 breast tumors and 2 different colorectal tumor models. In the EMT6 model, SYN101 + antiPD1 combination led to >90% tumor regression, with 3/5 CRs and 1/5 PR. Mice that had complete responses to SYN101 combination therapy, were resistant to subsequent tumor rechallenge and once again rejected the EMT6 tumor (i.e., immunological memory). Using blood FACS analysis, SYN101 + checkpoint blockade significantly increased T cell activation and expansion, relative to checkpoint therapy alone, which correlated with a robust antitumor response and clearance. By developing a non-cytotoxic ADC therapeutic to inhibit TGFb induced immune suppression, 1) SYN101 is differentiated from current systemic TGFb therapies and 2) the only ADC in the TGFb space to drive tumor clearance in vivo. Current studies are dedicated to expanding the in vivo efficacy studies and performing non clinical safety studies. Safer, more effective TGFb therapies, like SYN101, will improve overall patient response rates and provide novel therapeutic options to the majority of cancer patients that have elevated TGFb levels.

A071: Investigate a novel fascin inhibitor in gynecological cancer

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Fascin is an actin-bundling protein and plays an important role in tumor migration and metastasis. Elevated fascin levels have been reported in many human cancers and correlated with clinical progression, prognosis and survival outcomes. Thus, it is a candidate biomarker for multiple cancer types and a potential therapeutic target. Previously, our lab identified a small molecule fascin inhibitor, which has been shown to repress tumor migration, invasion, and metastasis, as well as prolong the overall survival of mice across a variety of cancer types.

In recent years, multiple groups performed single-cell RNA sequencing(scRNA-seq) from cancer patient tumor tissue samples and reported a subset of "fascin-high" expression dendritic cells(DCs) in Tumor Microenvironment(TME), which indicated its potential role in antitumor immunity. Prior research also revealed that fascin inhibitor promoted DC antigen uptake as well as T cell activation. The fascin inhibitor likely acts on such “fascin-high” DCs to invigorate anti-cancer immune response.

My research project uncovered a new function of this fascin inhibitor in repressing cancer cell growth and proliferation, most significantly in gynecological cancer cells. Expanded nucleus and vacuole accumulation were observed under phase contrast microscope. Based on the phenotype observation, we proceeded to mechanistic study. First, we performed proteomic analysis by LC-MS/MS, filtering out differentially expressed genes(DEGs) with significance($p<0.05$), and performed KEGG and GO pathway enrichment analysis. Results showed Cell Cycle Pathway

was remarkably changed upon fascin inhibitor treatment. DEGs classified in the Cell Cycle pathway were: CCNB1, YWHAE, BUB1, TTK, CHEK1, CDC27, and ANAPC10. Among them, we picked up TTK and CHEK1 for further study. We co-treated cells with either TTK or CHEK1 inhibitor together with fascin inhibitor and found TTK/CHEK1 inhibitors both had synergistic effects with fascin inhibitor in terms of inhibiting cell growth and proliferation. TTK and CHEK1 inhibitors have been reported to suppress tumor growth by disrupting cell cycle and inducing chromosome missegregation. This inspired us to the hypothesis that fascin inhibitor might exert its effects in a similar manner. Following that, we performed cell cycle analysis by flow cytometry, separating cells into G1, S, G2, and M phases. It was revealed that cells in the M phase significantly($p<0.05$) decreased upon fascin inhibitor administration. Since enhanced cell cycle activity in cancer cells has been linked with suppression of antitumor immunity by several articles, it is reasonable to regard fascin inhibitor as a promising candidate for next-generation targeted therapy.

In conclusion, from a translational medicine perspective, our preliminary data revealed that this novel fascin inhibitor suppressed gynecological cancer growth by impeding cell cycle progression. Given that mounting evidence is associating cell cycle regulation with antitumor immunity modulation, our research provides new opportunities for cancer immunotherapy. From a basic research perspective, we uncovered a novel role of fascin in regulating cell cycle besides its classic well-known roles in forming cytoskeleton and facilitating tumor metastasis.

A072: Anti-tumor response of Mycobacterium indicus pranii (MIP) involves direct impact on cancer cells

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Cancer is a challenging disease which requires a multi-pronged approach for its treatment. Immunotherapy has now been recognised as one of the most effective treatment modality. Out of all the available immunotherapeutic strategies one of the most unique and widely accepted is use of BCG (*Bacillus Calmette Guerin*) in bladder cancer and it is the only approved bacterial immunotherapy. Use of Mycobacterium in cancer treatment provides better protection from tumors as it has impact on both immune activation as well as direct killing of tumor cells. Our group has been working with *Mycobacterium indicus pranii* (MIP) and showed that it has unique immunomodulatory properties. MIP isa non-pathogenic, saprophytic mycobacterium. Genome analysis of MIP revealed it shares a significant amount of antigenic epitopes with that of its pathogenic counterparts such as *M. leprae* and *M. tuberculosis* which made MIP a potent therapeutic agent for clearing these pathogenic infections by inducing strong Th1 type of immune response. Along with heightened immune reaction, MIP also gets clear from the body at a very high rate which further prevents the patients from damages of chronic inflammation. These unique properties of MIP prompted us to study its effect on cancers. For this, we have used B16 mouse melanoma as *in vivo* tumor model and B16F10 cells were used to perform *in vitro* studies. When treated with three weekly doses of MIP, the tumor bearing mice showed decrease in tumor volume and increased survival. All the past studies done by our group have shown that MIP induced strong immune response involving activation of innate (DCs and macrophages) and adaptive immune cells ($CD4^+$T cell, $CD8^+$ T cell and NK cell) which suggested it is the immune compartment which plays a major role in MIP mediated tumor

regression. But, the studies done on BCG suggest mycobacterium can also directly affect the cancer cell survival. For our group the impact of MIP directly on cancer cells remained unanswered till now. So, the direct effect of MIP on B16F10 cells was evaluated by treating these cells with MIP *in vitro* to rule out the effect of immune cells. Interestingly, there was significant decrease in viability, proliferation and cell cycle progression of these cells after MIP treatment. These promising results further prompted us to study the MIP-cancer cell interaction. We found that MIP interacts with cancer cells by getting internalised at the very first step. The internalization is majorly through macropinocytosis and 40.9 % (+ 5.5%) cancer cells uptake MIP. It has been reported that such internalisation events modulates the survival strategies acquired by cancer cells to promote its growth throughout which involves (a) elevated ROS levels; (b) upregulated autophagy which helps in meeting increased nutrient requirements and also help during epithelial to mesenchymal transition (EMT) and (c) upregulation of anti-inflammatory molecules to hide from immune system. Interestingly, ROS levels decreases after MIP treatment in cancer cells whereas there was no effect of MIP on oxidative status of non-cancerous cells (HFF-1 cells). Since, ROS acts as a major signalling molecule in autophagy induction, MIP treatment reduces ROS in cancer cells which in turn reduces autophagy flux. Autophagy is known to promote tumorigenesis by promoting cancer cell proliferation and EMT which involves CD44 as a key player. MIP treatment leads to decrease in levels of CD44 and other cancer progression markers on these cells which help the tumor to grow. MIP treatment also leads to the decrease in the expression of anti-inflammatory markers (PDL1, CTLA4) on cancer cells which might be a result of decreased autophagic levels. Overall this study suggests that direct effect of MIP on cancer cells could be one of the important mechanisms involved in its anti-tumor activity besides activation of immune response in the tumor microenvironment.

A073: Targeting mitochondrial vulnerabilities to drive intrinsic melanoma immunogenicity
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Immunotherapies, especially checkpoint inhibitors, have marked a turning point in the treatment of advanced melanoma, the most dangerous form of skin cancer. However, a large fraction of patients either do no response or develop acquired resistance to these therapies. On the other hand, studies have shown that an abundance of mitochondrial - but not nuclear - DNA from tumor cells is found in the cytosol of tumor-infiltrating dendritic cells, implicating the mitochondria as an important component of antitumor immunity.

Interestingly, our preliminary results indicate that specific deletion of mitochondrial complex I subunit NDUFS4 led to dramatic decreases in tumor growth in allograft melanoma models in immunocompetent settings but led to no effect in tumor growth in immunocompromised mice. These results indicated that the immune system is playing a critical role in modulating the antitumor effects of NDUFS4 deficiency. RAN-seq and Mass spectrometry proteomic analyses of tumor samples reveal that NDUFS4 depletion induces a strong expression of proteins involved in antigen presentation and components of MHC I. Immunoprofiling of tumor samples from immunocompetent mice shows NK cells and NKT cells are significantly upregulated in the tumors with NDUFS4 depletion. Metabolomic analyses of the same samples show a significant accumulation of metabolites in choline metabolism pathway, which might contribute to NDUFS4 depletion induced NK cells and NKT cells mediated anti-tumor immunity. Based on our

preliminary data, we hypothesize knockout of NDUFS4 causes choline-dimethylglycine accumulation and then results in the recruitment and activation of NK cells and NKT cells, which together with the upregulated MHC-I on tumor cells perform the anti-tumor immunity.

In the following, we will continue to determine the mechanisms whereby specific bioenergetic failures of complex I, through depletion of NDUFS4, cause the accumulation of choline-dimethylglycine and how the accumulation of choline-dimethylglycine changes the lipid profiling and active NK cells and NKT cells.

A074: Cancer-induced type I interferon response by astrocytes promotes myeloid cell infiltration and tumor growth in the brain

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In the brain, both astrocytes and microglia play a role in innate immune response. While microglia are the major brain-resident immune cell population, there are significantly more astrocytes that are in close contact with the blood-brain barrier. These astrocytes are the first to encounter invading cancer cells during early stages of metastatic colonization, triggering a neuroinflammatory cascade. This study aims to unravel how cancer-induced astrocyte activation contributes to neuroinflammation and change the unique brain microenvironment to support brain metastasis (BrM) progression.

After coculturing astrocytes with cancer cells, RNA sequencing and Ingenuity Pathway Analysis revealed that type I interferon (IFN) response genes were highly enriched in both cell types. To confirm the presence of type I IFN in BrM *in vivo*, we used syngeneic mouse models of breast cancer and melanoma BrM. Cancer cells stably transduced with a GFP-reporter downstream of *IFIT1* were injected into the mice. GFP^+ cells were observed in the brain using flow cytometry and intravital imaging, providing evidence that chronic type I IFN signaling is part of an innate immune response in the brain against invading cancer cells. The downstream effects of type I IFN activation in astrocytes were further investigated. Using GFAP-Cre transgenic mice, the type I interferon receptor (IFNAR1) was specifically knocked-out in reactive astrocytes. While the role of type I IFN in cancer is controversial, many suggest that it is detrimental to tumor growth. Surprisingly, bioluminescence imaging showed reduced tumor burden in GFAP-Cre^+ mice compared to GFAP-Cre^- mice. Therefore, our results suggest a pro-tumor effect of type I IFN signaling in the brain tumor microenvironment.

Since IFN signaling is associated with tumor-immune interactions, flow cytometry analysis was performed to screen for immune populations in BrM. Compared to naïve mice, there was a significant increase in the number of $CD45^{hi}$ infiltrating leukocytes. Notably, there was also an increase in $CD45^{lo}$ microglia. When compared to primary tumors, there was a significantly increased proportion of $CD11b^+/Ly6C^{hi}/Ly6G^-$ monocytes. These monocytes expressed high levels of *Nos2* and *Arg1*, suggesting that they are an immune-suppressive phenotype. Interestingly, tumors from GFAP-Cre^+ mice contained significantly reduced proportions of $Ly6C^{hi}$ monocytes. Mechanistically, it was determined that the recruitment of these myeloid cells was due to increased *CCL2* expression by astrocytes in response to type I IFN. Thus, we hypothesized that blocking the recruitment of these $Ly6C^{hi}$ M-MDSC could

inhibit tumor growth. Both CCR2 knock-out mice and pharmacological inhibitors of CCR2 resulted in significantly reduced infiltration of Ly6C^{hi} monocytes and decreased BrM burden.

In conclusion, a cascade of neuroinflammatory events reshape the brain immune microenvironment during metastatic colonization and growth. These events are partly mediated by astrocytes which secrete type I IFN in response to invading cancer cells. The type I IFN acts in an autocrine manner, inducing CCL2 production by the astrocytes. This ultimately results in the recruitment of Ly6C^{hi} monocytes to further support tumor growth. Targeting this myeloid population could potentially lead to discovery of new therapeutics against BrM.

A075: SNAI1-dependent upregulation of CD73 increases extracellular adenosine release to mediate immune suppression in TNBC

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Triple-negative subtype of breast cancer (TNBC) is hallmarked by frequent disease relapse and shows the highest mortality rate. Although immunotherapy based on anti-PD-1/PD-L1 immune checkpoint blockades has recently shown promising clinical benefits, the overall response rate remains largely insufficient. Hence, alternative therapeutic approaches are warranted. Given the immunosuppressive properties of CD73-mediated adenosine release, CD73 blocking approaches are emerging as attractive strategies in cancer immunotherapy. Understanding the precise mechanism regulating the expression of CD73 is required to develop effective anti-CD73-based therapy. Our previous observations demonstrate that the transcription factors driving epithelial-to-mesenchymal transition (EMT-TF) can regulate the expression of several inhibitory immune checkpoints including PD-L1 and CD47. Here we analyzed the role of the EMT-TF SNAI1 in the regulation of CD73 in TNBC cells. We found that doxycycline-driven SNAI1 expression in the epithelial TNBC cell line MDA-MB-468 results in CD73 upregulation by direct binding to the CD73 proximal promoter. SNAI1-dependent upregulation of CD73 leads to increased production and release of extracellular adenosine by TNBC cells and contributes to the enhancement of TNBC immunosuppressive properties. Our data are validated in TNBC samples by showing a positive correlation between the mRNA expression of CD73 and SNAI1. Overall, our results reveal a new CD73 regulation mechanism in TNBC that participates in TNBC-mediated immunosuppression and paves the way for developing new treatment opportunities for CD73-positive TNBC.

A076: Unconventional pH-sensitive phosphatase STS1 partners with Cbl-b to suppress T cell function in acidic environments

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T cells responses are inhibited in acidic environments. T-cell antigen receptor (TCR) signaling is transduced by cytoplasmic tyrosine kinases that phosphorylate downstream signaling molecules. To properly regulate the magnitude of the TCR signaling pathway, phosphorylated proteins are subjected to dephosphorylation and ubiquitination. Little is known about how the environmental pH affects intracellular signaling pathways. Here, we report that STS1, an unconventional phosphatase which relies on a pH-sensitive histidine in its catalytic core, inhibits T cell responses in a Cbl-b dependent manner. Upon TCR stimulation, STS1 inducibly associates with the Cbl-b ubiquitin ligase and dephosphorylates protein substrates bound to Cbl-b to suppress signaling. Deficiency of either STS1 or Cbl-b desensitizes T cells to the inhibitory effects of extracellular acidic pH. Moreover, T cells deficient in STS1 or Cbl-b infiltrate more in the tumor, show higher effector molecule expression and are more resistant to exhaustion. Deficiency in STS1 or Cbl-b in mice inhibits tumor growth and improves survival. These results reveal a unique role of a TCR-induced STS1-Cbl-b complex in sensing environmental acidity and tuning T cell responses.

A077: The role of EYA3 in formation of the pre-metastatic niche

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Metastatic disease is the leading cause of breast cancer-related deaths, with more than 42,000 patients predicted to die from breast cancer this year. Immunotherapy, which re-engages the immune system to fight cancer cells, has extended patient survival in many cancer types, but current treatment options have shown limited efficacy in metastatic breast cancer. A comprehensive understanding of the mechanisms utilized by metastatic breast cancer cells to evade the immune system is critical to the development of clinical strategies that specifically target metastatic cells. Previous data from our lab has demonstrated that the developmental factor EYA3 promotes primary tumor growth of breast cancer cells and immune evasion at the primary tumor site in a CD8+ T cell-dependent manner. Given the immune modulatory role of EYA3 in the primary tumor, we interrogated its effect on anti-tumor immune responses during metastasis. Utilizing knockdown approaches, we found that EYA3 expression in the primary tumor promotes spontaneous metastasis to the lungs. From the primary tumor, cancer cells secrete factors to prepare distant sites for metastasis. To determine whether EYA3 affects anti-tumor immune responses at future sites of metastasis, we analyzed the lungs of tumor-bearing, pre-metastatic mice by flow cytometry. We observed increased infiltration of inflammatory monocytes and interstitial macrophages, and decreased levels of cytotoxic Natural Killer (NK) cells in the pre-metastatic lungs of mice bearing EYA3-expressing primary tumors. To identify putative EYA3 targets that may mediate these effects, we interrogated cytokines known to alter innate immune cell recruitment to the pre-metastatic niche and observed decreased levels of the cytokine CCL2 in EYA3 knockdown cells. We further identified CCL2 as a target downstream of an EYA3-NFkB signaling axis, which may exert additional effects on pre-metastatic niche formation. Altogether, this study shows that EYA3 promotes spontaneous metastasis to the lung, associated with changes in the immune cell composition within the pre-metastatic niche. Importantly, this study identifies an unusual role for an embryonic protein, EYA3, in regulating immune cell recruitment to future sites of metastasis.

A078: TCR-independent upregulation of A2AR on CD8 T cells promotes tumor growth in B16 melanoma

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Purinergic receptors sense extracellular nucleotide DAMPs such as ATP and adenosine, which are present in high concentrations in the tumor microenvironment (TME). A2AR, an adenosine receptor that is expressed on both T cells and tumor cells, generally promotes immunosuppression. However, the impact of the TME on changes in purinergic receptor expression on CD8 T cells, as well as the overall dynamic between A2AR expression and tumor control, have not been clearly elucidated. Using in vitro co-culture experiments and in vivo murine tumor models, we found that A2AR is significantly upregulated on tumor-infiltrating CD8 T cells. This upregulation was partially dependent on CD8 T cell-tumor contact, and independent of cognate antigen recognition, which we identified via transwell co-cultures, as well as the combination of different transgenic lines of CD8 T cells and tumor cells. We confirmed this observation in vivo using the transfer of activated OTI cells into B16.OVA-bearing mice. Using CRISPR/Cas9-mediated knockout of A2AR on activated CD8 T cells, we found that tumor-bearing mice receiving A2AR knockout CD8 T cells had increased tumor control. Taken together, these results suggest that contact-dependent, but TCR-independent signals in the TME promotes upregulation of A2AR on CD8 T cells, leading to impairment of CD8 T cell-mediated tumor control. In future studies, we will test whether these signals are dependent on physical contact (e.g., via interaction with co-inhibitory receptors in CD8 T cells) or if they are a consequence of microenvironmental alterations in the CD8 T cell-tumor interface.

A079: Isolated human RCC tumor cells detail cancer-intrinsic effects on the immune landscape

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Immune checkpoint inhibitors are an important regimen in the control of renal cell carcinoma (RCC); however, it is not universally effective. One prognostic indicator that positively correlates with immunotherapy sensitivity is increased tumor CD8 T cell infiltration. Our group has defined a stem-like CD8 T cell population that serves as a reservoir for effector cells in RCC. These stem-like CD8 T reside in dense areas of antigen presenting cells to form ‘immune niches’ which resemble T cell zones of secondary lymphatic tissues. Tumors that fail to form these structures progress more rapidly suggesting that immune niche manipulation by cancer cells may be one mechanism of immune escape. Current studies use whole tumor biopsies to examine cancer-mediated immune evasion, and although useful, they don’t allow the analysis of cancer cell-specific effects given the cellular complexity of the tumor tissue. To more directly examine cancer cell mediated immune evasion in human RCC, we developed a strategy to isolate cancer cells from the heterogenous tumor. Using scRNA-seq of the whole tumor sample, we identified RCC cancer cell specific markers allowing us to FACS sort distinct populations. Whole genome sequencing (WGS) of our sorted populations revealed 100% VHL mutation allelic frequency, highlighting high tumor purity. Next, we isolated pure cancer cells from over 100 patients with immunologically hot or cold tumors and performed RNA-seq and WGS. RNA-seq analysis

revealed that cancer cells from cold tumors upregulate genes involved in the extracellular matrix, inflammatory responses, and oxidative phosphorylation, suggesting potential avenues of immune suppression. Moving forward using human-derived organoids and a mouse tumor CRISPR-Cas9 system we will test the role of these genes *in vivo*. Overall, this work uses a novel human cancer cell isolation approach to provide direct insight into the cancer cell-immune landscape relationship for future therapeutic interventions and improved immunotherapy in RCC.

A080: Understanding PERK signaling in tumor-associated dendritic cells

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Immunotherapeutic strategies aimed at exploiting the anti-cancer capacity of T cells have shown impressive effects for the treatment of some tumor types, however the response rate of ovarian cancer patients is only marginal. One possible reason for this may be the adverse environment of ovarian tumors, which inhibits the inherent protective function of immune cells. Indeed, in ovarian cancer, the harsh conditions of the tumor microenvironment can perturb the protein-folding ability of the endoplasmic reticulum (ER), thus inducing a cellular state of "ER stress" which is mitigated by the unfolded protein response (UPR). Sustained activation of the UPR negatively affects the activity of several intratumoral immune cell populations, including dendritic cells (DCs) and T cells. Here we aim to investigate whether chronic activation of PERK, an understudied ER stress sensor in the context of the tumor microenvironment, in DCs affects their function during ovarian cancer progression. Single-cell RNA sequencing on immune cells isolated from malignant ascites and metastatic omentum of tumor-bearing mice showed multiple DC clusters with different levels of general UPR activation, but experiencing the same level of PERK activation. In addition, we found that genetic and pharmacological inhibition of PERK *in vivo* may induce a rewiring of the same pathway. To bypass this critical compensatory mechanism, we will use mice that lack *Atf4*, a transcription factor downstream of PERK, in DCs and we will perform therapeutic interventions, including ATF4-deficient bone marrow-derived DC vaccination studies. Successful accomplishment of these steps will reveal new combination immunotherapies able to elicit protective and durable immunity against metastatic OvCa.

A081: Myeloid MHC class II-restricted antigen presentation is required to prevent dysfunction of cytotoxic T cells in brain tumors

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Cancer immunotherapy critically depends on the fitness of cytotoxic and helper T cell responses and dysfunctional cytotoxic T cell states in the tumor microenvironment (TME) are a major cause of failure of cancer immunosurveillance and resistance to immunotherapy. Therefore, maintenance and restauration of tumor-reactive T cell fitness remains the key challenge in immune-oncology. Tumor-infiltrating myeloid cells, particularly blood-borne myeloids (bbm), are key drivers of T cell dysfunction in the TME and are a hallmark of disease progression in brain tumors. Here, using transgenic mouse models, multi-parameter in situ hybridization assays and antigen-specific tumor models we provide evidence that bbm represent a critical source of intratumoral major histocompatibility complex (MHC) class II-restricted antigen presentation that is essential to control the growth of human and murine brain tumors. By employing longitudinal single-cell RNA and ATAC sequencing, a novel receptor-ligand interaction prediction algorithm and trajectory analysis we found that intratumoral MHC class II is required for sustained cytotoxic $CD8^+$ T cell responses. Loss of MHC class II drives a distinct dysfunctional transcriptional state in tumor reactive $CD8^+$ T cells through increased chromatin accessibility and expression of TOX, a critical regulator of T cell exhaustion. Mechanistically, intratumoral MHC class II-dependent activation of $CD4^+$ T cells restricts myeloid-derived osteopontin expression in murine and human brain tumors. Osteopontin in bbm MHC class II deficient tumors triggers chronic activation of *nuclear factor of activated T cells* (Nfat)2 in tumor-reactive $CD8^+$ T cells following antigen recognition leading to TOX-mediated dysfunction. In summary, we provide evidence that intratumoral MHC class II-restricted antigen presentation on bbm is a key mechanism in modulating the TME and directly maintains functional cytotoxic T cell states in brain tumors. These findings provide a basis for the rational design of $CD8^+$ T cell-targeting immunotherapies against brain tumors.

A083: Harnessing type 2 immunity at the epithelial interface to treat lung cancer

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Non-small cell lung carcinoma (NSCLC) is the leading cause of cancer death worldwide. While immunotherapies such as PD-1 blockade have revolutionized NSCLC treatment, only a minority of patients respond. This is in part due to the highly immunosuppressive tumor microenvironment (TME), which is enriched with diverse populations of myeloid cells that limit anti-tumor immunity. An understanding of the complex immune interactions within the TME and the signals that control them is essential for the design of more effective therapies.

We performed single-cell RNA-sequencing on over 500,000 tumor-infiltrating leukocytes from 35 NSCLC patients and in the *Kras^{G12D}TP53$^{-/-}$* murine lung adenocarcinoma model. In both species, we observed a remarkable Type 2 cytokine transcriptional signature within tumor-infiltrating myeloid cells. Type 2 immune responses - those characterized by the cytokines IL-4, IL-5, and IL-13 - evolved to defend against parasitic infection at barrier sites such as the lung,

skin, and intestines. While pathogenic Type 2 immunity has been well studied in diseases such as asthma, atopic dermatitis, and ulcerative colitis, the role of these processes in anti-tumor immunity remains largely unexplored. This is despite the fact that many cancers occur at barrier sites, which are primed to engage Type 2 immunity in response to epithelial damage.

We found that antibody blockade of IL-4 profoundly reduced lung tumor burden across several murine models. While IL-4 producing Th2 cells were surprisingly absent from the lung TME, further analyses revealed that basophils were the dominant source of IL-4 within tumors and that antibody-mediated depletion of basophils significantly reduced tumor growth *in vivo.* Using a panel of mice with conditional deletions in the IL-4Ra coupled with single-cell transcriptomics and mixed bone marrow chimeras, we identified monocyte-derived macrophages and neutrophils as the dominant cell types responding to IL-4/IL-13 signaling to promote lung tumor progression. We subsequently designed and opened a clinical trial in which patients with relapsed/refractory NSCLC receive IL-4Ra blockade (Dupilumab, Regeneron, Inc.) given in conjunction with PD-1 blockade following progression on standard chemoimmunotherapy combinations. In a subset of these patients, IL-4Ra blockade strongly reduced circulating monocytes and neutrophils. The clinical study is ongoing and we hope to have clinical outcome results by the time of the meeting. These results highlight a Type 2 cytokine-myeloid axis as a therapeutic target for combination immunotherapy in NSCLC.

A085: Intrahepatic IgA upregulates PD-L1 on cancer-associated fibroblasts in HCC

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Objectives:

Cancer associated fibroblast (CAF) is a group of activated fibroblasts and play a key role in the tumor microenvironment. Immunoglobulin A (IgA) usually neutralize pathogens against infections at the mucosal sites. In contrast to IgA's protective role in human immunity, IgA has been reported to contribute to inflammation or dismantling antitumor immunity in human liver. On inflamed livers and HCC, recent report demonstrated that IgA complex induces PD-L1 upregulation in intrahepatic monocytes/macrophages. In this study, we investigated the effects of IgA complex on CAFs in tumor microenvironment of HCC.

Materials and Methods: `

CAFs and non-tumor intrahepatic fibroblasts were isolated from 8 different HCC samples and corresponding non-tumor tissues. Tissues were homogenized and CAFs and non-tumor fibroblasts were isolated by density gradient centrifugation. The passage number of cultured fibroblast and CAF between 1 to 10 is used for experiments. CAF and fibroblast were treated with mock or IgA dimer for some experiments. CD71 and PD-L1 expression levels in CAF were analyzed by multi-color flow cytometry and real-time polymerase chain reaction (PCR).

Results:

Flow cytometry and real-time PCR analysis for fibroblast markers demonstrated that the relative mRNA level of α-smooth muscle actin, fibroblast activation protein in isolated CAF was significantly elevated compared with hepatoma cells. The transferrin receptor (CD71) is known as a receptor for IgA1, and CAFs and non-tumor fibroblasts expressed CD71 robustly. After treated with IgA complex, CAFs and non-tumor fibroblasts showed increased surface levels of IgA, suggesting that IgA complex attached to these cells. IgA dimer-treated CAFs showed increased PD-L1 mean fluorescence intensity (MFI) values compared with mock-treated CAF. Additionally, PD-L1 and Interleukin-6 mRNA levels of IgA dimer treated CAFs was upregulated compared to mock treated CAF.

Conclusions: Overall, intrahepatic IgA stimulates CAFs to upregulate PD-L1 in HCC. PD-L1high CAFs may serve new therapeutic targets in HCC. Ongoing study is now revealing the exact role of these IgA-stimulated CAFs in TME of HCC.

A086: Characterizing the role of a mRNA decay cofactor in melanoma immune interactions

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The development of immunotherapy has revolutionized the field of melanoma treatment by prolonging survival of patients. Nevertheless, a significant proportion of patients either do not respond to immunotherapy or relapse. To understand the mechanisms underlying resistance to immune checkpoint blockade (ICB) therapy, we aim to dissect intrinsic melanoma resistance pathways to identify new potential therapeutic targets and prognostic biomarkers.

In order to characterize melanomas in depth on a molecular level we generated a murine RiboTag-modified melanoma model. An adapted CRISPR/Cas9 approach was used to fuse endogenous ribosomal protein L8 (Rpl8) with a 3xHA tag. The incorporation of tagged Rpl8 into translating polyribosomes enabled the isolation ribosome-associated mRNAs by immunoprecipitation.

We used RiboTag melanoma cells in a C57BL/6 mouse model for ICB therapy and found the expression of a cofactor of mRNA decay significantly downregulated in ICB treated melanomas. In previous studies analyzing cutaneous melanoma, this cofactor has been described to harbor hotspot mutations located in its protein binding site.

To investigate the impact of these mutations on melanoma-immune interactions, we performed Gene Set Enrichment Analysis (GSEA) of a cutaneous melanoma dataset and observed an enrichment of immune activation and antigen presentation signatures in tumors bearing mutations. A knockdown of the cofactor in human melanoma cells induced similar expression patterns. To further investigate the functional effects of these mutations, we modelled the mutated protein structure and its interaction with potential partners, which revealed decreased binding capacities with different immune modulatory proteins.

The use of RiboTag in combination with an experimental model for ICB therapy facilitated a comprehensive characterization of melanoma-specific gene expression patterns during therapy.

Thus, our work can help to better understand melanoma immune interactions which furthermore gives the opportunity to improve tailored treatment for malignant melanomas.

A087: Hepatic stellate cells differentially modulate transendothelial T-cell-trafficking in a model of pancreatic liver metastis

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Introduction: Recent studies indicate that liver metastasis may lead to tumor immune escape (TIE) at distant tumor sites. We therefore sought to analyze how hepatic stellate cells (HSCs) may affect transendothelial migration (TEM) of T cells when interacting with pancreatic adenocarcinoma (PDAC) hence potentially contributing to this phenomenon.

Methods: To simulate interaction between stromal and tumor cell lines *in vitro,* we implemented a 3D-coculture model. Herein, tumor cell lines are cultured "hanging" on the basolateral side ("underside") and stromal (stellate) cells on the apical side of a non-cell-permissive transwell insert, hence allowing tight interaction without overgrowth. Adjunct, CD3/CD28-bead activated, untouched T-cells from healthy donors were left to interact with stellate cells for 3 days followed by MACS-sorting for $CD3^+$ cells. TEM of $CD3^+$ T-cells was then assessed using so called flow adhesion assays equipped with TNFα-activated HUVECs cultured in a microcapillary slide (µ-Slide®, Ibidi, Gräfeling, Germany). This live-cell imaging technique allows the visualization of the three major phases within the TEM-multi-step-cascade using phase contrast microscopy (Axio Observer®, Zeiss, Oberkochen, Germany): Adherence to the apical side *(Phase 1)*, the actual process of TEM on para- or transcellular routes *(Phase 2)*, finalization of TEM at the basolateral side/entering the interstitial space *(Phase 3)*. The perfused cells are stratified to one of these phases based on their light-refractivity and shape using a well-established protocol (Shetty S. et al. JOVE 2014 doi: 10.3791/51330). Additionally, T-cells were analyzed using flow cytometry upon coculture (i.e. Th_1/Th_2-polarization).

Results: To address the heterogeneity in PDAC, we employed two distinct cell lines, PANC-1 reflecting basal/dedifferentiated and Capan-1 reflecting mesenchymal-differentiated PDAC. The hepatoma cell line Huh-7 was used as a non-PDAC control. 3D-cocultures were equipped with the HSC-cell-line LX-2 or the pancreatic stellate-cell-line hPSC in the apical side of the insert. While culturing T-cells with hPSC resulted in impaired TEM as compared to matched control independently of the tumor cell line applied, we observed a more differential effect for LX-2. Similar to hPSC, culturing T-cells with LX-2 led to an impairment of the complete TEM-cascade relative to matched control when 3D-cocutrues were equipped with Capan-1 or Huh-7. However, when basal-like PANC-1 cells were used in combination with LX-2, T-cell-TEM was far less affected in *Phase 2* (LX-2/Huh-7: 44% vs. LX-2/Capan-1: 55%vs. LX-2/PANC-1: 75%) and *Phase 3* (34% vs. 42% vs. 45%). In *Phase 1,* T-cells deriving from LX-2/PANC-1 even showed a trend to out-performing the matched control resulting in a significant difference as compared to T-cells from LX-2/Capan-1 cocultures (102% vs. 54%, p=0.02, paired t-test; vs. LX-2/Huh-7:

55%, n.s.). Importantly, no such difference was observed when T-cells were cultured with either one of the stellate or tumor cell lines. Interestingly, we observed a similar but less pronounced effect when T-cells were cultured in the well of the culture-plate instead of the LX-2 containing insert indicating that this effect may not require cell-cell contact. Additionally, we performed a preliminary multicolor FACS- analysis to asses T-cell differnetiation. Herein, we observed no relevant intergroup difference in Th_1/Th_2-polarization or frequency of T_{reg}, apart from a relative trend towards Th_2 in T-cells deriving from LX-2/PDAC-line 3D-cocultures vs. matched control (Th_1/Th_2 ratio 1:8 vs. 1:4).

Conclusion: Our preliminary findings indicate that the effect HSCs may exert on T-cell-TEM depend on the type of malignant cells they interact with in the context of TEM-based TIE. Given the inter-individual heterogeneity it is equally mandatory for future studies to include personalized approaches (i.e. patient-derived organoides) to overcome subtype-specific TIE in PDAC.

A088: Cancer cells secreted TGF-b increases cdd8 t cells TCR activation threshold allowing immune escape

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The low immunogenicity of established solid tumors can be explained by the weak ability of CD8 T cells to efficiently monte a response to cancer cell antigens. This impairment of CD8 T cell functions is thought to be conditioned for a large part by the tumor micro-environment (TME). One particularity of the TME of solid tumors is to be highly enriched in Transforming Growth Factor Beta (TGF-β1) secreted mainly by the cancer cells. However, whether TGF-β present in the TME affects the ability of CD8 T cells to react to cancer cell antigens remains unknown.
Here, using gain and loss of TGF-β signaling selectively in CD8 T cells as well as cancer cells bearing antigens with different affinity for a given CD8 TCR, we reveal that TGF- β signaling in CD8 T cells controls their TCR activation threshold. TGF-β signaling promotes the LCK conformation in its inactive form by stabilizing CSK. Subsequently, the strength of the signal given to the TCR to activate CD8 T cells cytotoxic function in presence of TGF-β needs to be stronger. CD8 T cells escaping TGF-β signaling control efficiently eliminate cancer cells even thus bearing weak affinity antigen for their TCR, whereas CD8 T cells receiving TGF-β signaling fail to respond to weak antigens expressed cancer cells, leading to a selective advantage of these cells. Importantly, we identified cancer cells as the main source of TGF-β1 controlling TCR activation threshold in the TME.
Thus, this study reveals that secreted TGF- β1 by cancer cells increases TCR activation threshold of CD8 T cells present in the TME, and allows the immune escape of the cancer cells bearing antigens with weak affinity.

A089: High dimensional analyses of intratumoral myeloid cells highlights presence of distinct myeloid cell phenotypes in immune checkpoint-sensitive and resistant tumors

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Background: Immune checkpoint therapy (ICT) has revolutionized cancer treatment, however, has produced only 20% durable clinical response rates so far, urging a need to understand the resistance mechanisms against effective therapy. Multiple preclinical and clinical studies demonstrate high myeloid cell abundance in the tumor microenvironment (TME) correlates with poor outcome in ICT resistant cancers such as pancreatic ductal adenocarcinoma (PDAC). Myeloid subsets can also inhibit T-cell function thereby reducing therapy efficacy. Despite the wealth of knowledge regarding the biology of these cells, our ability to distinguish immune-suppressive versus immune-stimulatory myeloid cells remains a major challenge and, thus, targeting myeloid cells has had limited clinical success. Our objective is to better understand the phenotype of these immunosuppressive myeloid cells to identify potential combination strategies to improve response to ICT.

Methods: We performed comparative analyses of intratumoral myeloid cell subsets in orthotopic murine models of ICT sensitive (B16F10 melanoma) and resistant (MT4 PDAC) tumors at baseline and after treatment with anti-PD-1 and anti-CTLA-4 checkpoint therapy. Due to high plasticity of myeloid cells, we used single cell RNA sequencing (scRNAseq) to delineate cell phenotypes with higher sensitivity and specificity. Additionally, we performed longitudinal scRNAseq on ICT resistant MT4 tumors to determine the evolution of suppressive myeloid cells with tumor progression.

Results: The abundance of macrophages and neutrophils was two-fold higher in baseline MT4 tumors as compared to B16F10 tumors. Overall, we identified 4 distinct macrophage subsets and 1 neutrophil subset. Mac_1 and mac_2 were the dominant macrophage clusters in MT4 tumors and while mac_1 expressed *mmp14, axl and mafb*; mac_2 had high expression of *vegfa, arg1, ccl24* and *fn1*.Macrophages with such phenotypes have been shown to inhibit intratumoral T cell function. In contrast, mac_3, the most abundant macrophage cluster in B16F10 tumors, expresses antigen presenting genes (*cd72*) and interferon-induced genes (*cxcl10, isg15*) suggestive of an immunostimulatory phenotype. GSEA analysis indicated upregulation of TGF beta signaling, angiogenesis, hypoxia, glycolysis in mac_1 and mac_2. Contrastingly, mac_3 which upregulated oxidative phosphorylation and interferon gamma and alpha response pathways. These data indicate that baseline MT4 macrophages possess characteristics associated with T cell inhibition whereas baseline B16F10 macrophages show T cell activating phenotypes. The neutrophil subset was present specifically in MT4 tumors and expressed *cd24a, cxcl2, il1b* and *cd274*; consistent with the suppressive tumor-associated neutrophil (TAN) phenotype. Upon treatment with ICT antibodies, all the macrophage subsets decrease moderately, however, the abundance of TANs increases, possibly indicating of compensatory adaptive resistance mechanisms in MT4 TME. Longitudinal analyses performed on day 5, 7, 10 and 15 of MT4 tumor growth indicated that the abundance and suppressive phenotype of neutrophil and macrophage subsets were high at early stages of tumor initiation and persisted during tumor progression.

Conclusion: Overall, our data highlights that distinct myeloid subsets are present in ICT sensitive and ICT resistant tumors which may contribute to overall resistance to ICT. Targeting these suppressive myeloid subsets prior to ICT treatment might be necessary to generate an effective immune response against checkpoint antibodies in such resistant tumor types.

A090: The intratumoral microbiota impacts the spatial, transcriptional, and functional heterogeneity of human oral and colorectal cancers

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The tumor-associated microbiota has gained relevance in recent years with a wealth of studies demonstrating its contribution to the initiation and progression of a range of cancer types including colorectal cancer (CRC). The presence of the dominant bacterial species belonging to the *Fusobacterium*, *Bacteroides* and *Treponema* genera in the tumor tissue has been associated with. cancer development, metastasis, immunosurveillanc and chemoresistance. However, the precise identity of these cell-associated organisms along with the specific host cell types they interact in the naïve tumor tissue context remain to be elucidated. Herein, by adapting existing technologies such as 10x visium spatial transcriptomics, we have mapped the spatial distribution of the bacterial communities that populate the tumor tissue. Using GeoMx Digital Spatial Profiling to combine targeted imaging with *in-situ* spatial protein profiling we show that the tumor-associated microbiota resides in necrotic, less vascularized, and largely immunosuppressed regions of the tumor tissue with an enrichment of neutrophils, reduction of T cells and increased expression of the immune checkpoint molecules CTLA4 and PD1. Further, by using a single cell RNAseq approach, we have determined the signaling pathways in host cells that are associated with intratumoral bacteria including inflammation, metastasis, cell dormancy and DNA repair activity among others. Finally, through functional studies we show that bacteria-infected cancer epithelial cells invade their surrounding environment as single cells and recruit myeloid cells to the site of bacterial localization. Together, this work demonstrates that the tumor-associated microbiota colonizes tumor regions that coincide with dormant cancer cells and T cell exclusion. The presence of intratumoral bacteria could generate transcriptional heterogeneity in cancer cells by activating genes involved in metastasis including SERPIN molecules along with the metalloproteinase MMP3 and MMP9. Intracellular bacteria also induced functional cell heterogenicity by promoting cell invasion in transformed cells with a broad range of velocities and cell displacements as they migrate through the collagen matrix.

A091: HR-deficient ovarian cancer exhibits immune suppression by tumor cell inflammasome activation

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In cancer cells, DNA damage is sensed by innate immune pattern recognition receptors (PRRs) in a manner reminiscent of cellular responses to viral infection. This has important consequences in the context of immune activity against tumor cells. For example, IR induces DNA damage-mediated clearance of melanoma cells. This contrasts with the situation in HR-deficient high grade serous ovarian cancer (HGSOC), where DNA damage is persistent, but T-cell mediated

anti-tumor immunity does not occur and cannot be induced by treatment with immune checkpoint inhibitors (ICI). Indeed, the immune microenvironment in this cancer is exhibits abundant suppressive cells such as M2-like macrophages and other myeloid suppressor cells. This strongly suggests that PRRs are engaged in a different manner in this cancer. We hypothesize further that modulation of this signaling can ameliorate immune suppression and induce susceptibility to ICI therapy. To address this, we proposed to use a syngeneic, physiological relevant model of HGSOC to perform CRISPR/Cas9 screening of all known PRR molecules. Further, we suggested to investigate the contribution of such candidate molecules to genomic instability, the immune suppressive microenvironment of tumors, and ultimately tumor progression *in vivo*. Finally, we draw biopsy material from >45 HGSOC tumors to examine whether this mechanism is relevant and translatable to HGSOC. CRISPR screening data indicates inflammasome PRRs NLRP1 and NLRP9 as novel sensors of DNA damage in this context. In line with this, we show that DNA damage-driven inflammasome activation occurs in HGSOC cells. Intriguingly, analysis of candidate NLRP9 KO tumor cells using *ex vivo* co-culture with immune cells and *in vivo* study suggests that indeed, this protein serves an immune suppressive, pro-tumorigenic role in HSGOC. Analysis of HGSOC patient material shows that such tumors exhibit NLRP9 inflammasomes and these structures correlate with M2-like immune suppressive markers in tumors. Taken together, our work has identified NLRP1/9 inflammasomes as novel PRR-driven immune suppressors in HGSOC.

A092: Delineation of the cellular connectivity network in human tumors – from cells to biomarkers

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Most research focused on the discovery of predictive or prognostic biomarkers utilizes immunohistochemical evaluation of tissue slides stained for a single antigen, or uses bulk tumor transcriptomic approaches. Neither of these methodologies identifies which cell types are found within the tumor, nor connect the expression of a predictive antigen with the cell type expressing it.

We developed a tool to directly study these critical parameters at the highest resolution currently available. We harness the power of multiparametric (up to 16-color) flow cytometry to directly quantify and sort immune, stromal and tumor cell subsets from viably dissociated human tumors. The attained frequency data is highly reproducible with technical replicates demonstrating correlation of Rp>0.98. The flow-cytometric-identified cells are then sorted and utilized for deep transcriptomics (>10,000 genes) even from 50-100 sorted cells.

We assembled a database that included demographic, mutation and full immune frequency data attained by multicolor flow cytometry. The database included 103 brain tumor patients (gliomas metastases, and non-tumorous epileptic brain lesions), 53 of the patients had Isocitrate dehydrogenase (IDH) wildtype glioblastomas (GBM) and had full survival data.

We found that a popular transcriptiomic de-convolution algorithm which aims to perform "digital cytometry" by deducing tumoral cellular frequencies from RNA analysis, performs

poorly when compared to FCM-attained data. The ranges of cell frequencies were considerably different between the two methodologies and correlations which were very strong using FCS, became very weak, or even reversed in the deconvoluted analyzed data.

Using the FCM-attained frequencies we found the IDH mutation strongly affects the entire lymphocytic immune infiltrate independently of tumor type or grade. We also identified paradoxical inverse correlation between the frequencies of cytotoxic- and helper-T cells (CTL, Th) and the patients' overall survival.

Lastly, we found that a parameter composed of the frequencies of 4 types of tumoral lymphocytes had managed to separate a seemingly monolithic IDH^{wt} GBM patient population surviving a median 1.5y, to two equal-sized groups, separated by 10 months of survival. This cell frequency-derived biomarker was then validated using deconvoluted TCGA data. As expected, the estimated (deconvoluted) cell frequency data had reduced the differences in survival between groups from 10 months to 4.3 months. The FCM-based composite parameter identified is currently strongest survival prognosticator for newly-diagnosed GBM.

A093: Understanding the role of Interleukin 1 alpha in HER2 positive breast cancer

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The role of cytokine- Interleukin 1 alpha (IL1α) produced by the host in HER2 positive tumor progression is poorly understood. In the present study, we report that *IL1a*$^{-/-}$ mice regress HER2 positive breast cancer completely within 3-weeks from tumor implantation, while the tumor in Wildtype (WT) mice grows exponentially with time. At the 2- week time point, the immune profile of *IL1a*$^{-/-}$ tumor showed significant immune cell infiltration and dominantly they were myeloid cells and CD8 T cells. We identified using flow cytometry that myeloid cells were the only source of IL1a in the tumor microenvironment. Moreover, we observed monocytes failed to differentiate into Tumor Associated Macrophages (TAMs) and remained as inflammatory monocytes in *IL1a*$^{-/-}$ tumor, while in WT the differentiation was successful into TAMs. The CD8 positive T cells flow cytometry of the *IL1a*$^{-/-}$ tumor showed higher expression of proliferative marker (Ki67), memory T cell markers (CD44+Ly6C) and significant reduction in immune inhibitory marker (PD1). To study if CD8 cells played an important role in *IL1a*$^{-/-}$ tumor regression, we injected anti-CD8 antibody until 30-day time point. We observed significant tumor growth and the monocytes were able to convert into TAMs in CD8 depleted *IL1a*$^{-/-}$ tumor. These findings show that myeloid cells are the source of IL1a and their absence can affect TAM formation resulting in better T cell activity in the tumor microenvironment.

A094: Tumor Microenvironment: Suppressing Antitumor Response: PIEZO1 Deletion in CD11b+ Cells Suppresses Rhabdomyosarcoma Tumor Rejection and Generates a Tumor-Permissive Immune Landscape

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The solid tumor microenvironment (TME) is a dense and specialized niche where malignant cells interact with local populations, including immune cells, to create a ‘pro-tumor’ and immunosuppressive environment. In several cancers, including the aggressive pediatric sarcoma,

rhabdomyosarcoma (RMS), tumor-associated myeloid cells (TAMCs) are the most numerous immune populations in the TME and has been correlated with poorer survival outcomes and chemotherapeutic resistance. TAMCs constitute a heterogeneous and phenotypically plastic population that can sense changes in their environment, including enhanced ECM stiffness found in the TME, and change their functioning accordingly. Despite the clear clinical influences these plastic cells have and the detrimental effects that elevated intra-tumoral stiffness has on immune cells' phenotype, little is known about the proteins that sense and mediate these changes. Here, *we propose that inhibition of the mechanosensitive cation channel, PIEZO1, promotes a pro-tumor phenotype in TAMCs while its activation skews this population to anti-tumor functioning thereby enhancing tumor rejection.* PIEZO1's influence on tumor rejection and myeloid plasticity was assessed by inoculating a novel murine CD11b-conditional homozygous PIEZO1 knockout strain (CD11b-Cre^{+}; PIEZO1$^{-/-}$) with syngeneic RMS cells and performing several phenotypic analyses. Tumor volume analysis and longitudinal survival analyses has shown found that our RMS-bearing CD11b-Cre^{+}; PIEZO1$^{-/-}$ mice developed significantly larger tumors and had diminished overall as compared to their wild-type counterparts. Flow cytometric analysis of tumors after 21 days has revealed increased pro-tumor, 'M2-like' macrophage frequency in the tumor and the spleen. Complementarily, Piezo1 KO mice had increased monocyte frequency in the peripheral blood and decreased anti-tumor, 'M1-like' macrophage frequency in the spleen compared to tumor-bearing wild type mice. RT-qPCR analysis of the tumor supports these findings as there is marked elevation of the macrophage marker, F4/80, the anti-inflammatory pseudo-marker, Arginase-1, and CCL2, a chemokine critical for monocyte recruitment. Together, these findings suggest PIEZO1 inhibition on CD11b+ cells promote recruitment of monocytes that polarize to a pro-tumoral phenotype in the TME. Consequently, the anti-tumor response is stunted leading to diminished survival and increased tumor burden.

A095: Molecular mechanisms of natural killer cell desensitization

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Cancer is a significant global health burden and is the second leading cause of death in the United States. Natural killer (NK) cells can exhibit powerful antitumor effector functions with great therapeutic potential. NK cells can eliminate tumor cells that evade control by CD8^{+} T cells, such as cells that lose expression of some or all self-MHC class I (MHC I) molecules. However, NK cells in MHC I-deficient *B2m*$^{-/-}$ mice are desensitized, presumably as a result of persistent stimulation, in a process that is called "education" or "licensing." NK cell education operates as a tunable rheostat by which NK cells can constantly adjust their responsiveness to stimulation based on continuous re-evaluation of MHC I expression on surrounding cells in the environment throughout their lifespan. While NK cells initially recognize MHC I loss and kill tumor cells, persistent exposure to MHC I-deficient environment gradually makes NK cells desensitized and incapable of controlling tumors. The molecular bases of NK cell education, desensitization or licensing are not understood mechanistically, and represent one of the most important unresolved issues in NK cell biology. We aimed to develop an understanding of molecular modulators of NK cell desensitization. By comparing the gene expression profiles in functional and hyporesponsive NK cells in multiple settings, we found that the phosphatases *Ptpn6* (encodes SHP-1) and *Ptpn22* were expressed at higher levels in NK cells desensitized due to persistent stimulation in the absence of inhibitory signaling from MHC I. In contrast, *Ptpn6*

and *Ptpn22* transcripts were significantly reduced when NK cells were treated with NK-activating cytokines, suggesting that SHP-1 and PTPN22 downmodulates NK cell responsiveness. In agreement, treatment with PTPN22 inhibitor enhanced degranulation and IFN-γ production by NK cells in response to diverse stimuli as well as NK cell cytotoxicity. Interestingly, treatment of NK cells with ionomycin to mimic induction of free Ca^{2+} flux caused by excessive stimulation increased the expression of *Ptpn22*. Consistently, we found that drug-mediated inhibition of PTPN22 in ionomycin-treated NK cells prior to stimulation partially restored the compromised degranulation capacity of these cells, supporting a model in which enhanced intracellular calcium flux, possibly upon NK cell activation, leads to the transcriptional upregulation of *Ptpn22*. Furthermore, the unbiased genome-wide profiling of gene expression in functional and desensitized NK cells revealed multiple genes in addition to Ptpn6 and Ptpn22 whose roles in regulating NK cell functional activity could be validated. Once the roles of these genes are extended to NK cells in tumor models, it will shed light on how to engineer tumor-restricted NK cells that can kill tumors better for anti-tumor adoptive cell therapy.

A096: Intratumoral immunotherapy of murine pheochromocytoma shows no age-dependent differences in its efficacy

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Cancer immunotherapy has shown remarkable clinical progress in recent years. Although age is one of the biggest leading risk factors for cancer development and older adults represent the majority of cancer patients, only a few new cancer immunotherapeutic interventions have been preclinically tested in aged animals. Thus, the lack of preclinical studies focused on age-dependent effect during cancer immunotherapy could lead to different therapeutic outcomes. Our previously developed intratumoral MBTA therapy, based on the combination of polysaccharide mannan-BAM, TLR ligands, and anti-CD40 antibody (MBTA therapy) has been tested in various subcutaneous murine tumor models, such as melanoma (B16-F10), pancreatic adenocarcinoma (Panc02), colon carcinoma (CT26), and pheochromocytoma (MTT) with the complete elimination of tumors in 62-83% of mice depending on tumor type. However, all the studies have been performed in 6-8 weeks old mice which is equivalent to human 11-15 years of age. Pheochromocytomas (PHEO) are rare catecholamine-producing neuroendocrine tumors derived from neural crest cells. These tumors can occur at any age, but they are most common between the age 30-50 depending on catecholamine phenotype and genetic background.

Therefore, we assessed the efficacy of intratumoral MBTA therapy in 6-weeks and 71-weeks old mice, which correlates to 11 and 56 years of human age, to evaluate possible age-dependent difference and better simulate a situation in patients with PHEO. First, young and aged mice were subcutaneously injected with pheochromocytoma MTT cells and the incidence of tumors was monitored. Second, young and aged mice bearing PHEO tumor were intratumorally treated with PBS or MBTA therapy and the tumor growth and subsequent survival of mice were evaluated.

The results point out that despite faster growth of pheochromocytoma tumors in aged mice, intratumoral MBTA therapy is effective approach without age dependence and could be one of the possible therapeutic interventions to enhance immune response to pheochromocytoma and perhaps other neuroendocrine tumors also in aged host.

A097: Exploiting the breakdown of tolerance in Aire-deficient mice for isolation of autoantibodies against melanoma

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Breakdown of immunological tolerance mechanisms, represents a very promising and effective approach for cancer immunotherapy. However, the use of different checkpoint inhibitors is often associated with severe side effects. Given that the Autoimmune regulator (Aire) gene is one the most critical players in the establishment of immunological tolerance to self, we exploited the natural occurring humoral response in Aire-deficient mice that develop vitiligo, a disease characterized by an autoimmune-destruction of melanin-producing cells (melanocytes) and subsequent loss of skin pigment, to target melanoma-specific membrane associated proteins. We hypothesized that such melanoma-specific autoantibodies could be harnessed for melanoma immunotherapy for their high specificity and affinity. Indeed, our data demonstrate that Aire-deficient mice develop autoantibodies to several melanoma-specific targets, without the need of immunization. Using a baiting system for the targets of interest, we were able to isolate target-specific B-cells and clone their corresponding Ig loci into human FC plasmids in order to generate chimeric antibodies. These “nature-made and shaped” antibodies recognize human melanoma cancer cells in-vitro, and offer potential candidates for melanoma-specific immunotherapy based on diverse approaches ranging from antibody drug conjugates (ADCs), bi-specific antibodies to CAR-T cells.

A098: Identification of 85 new HLA class I-restricted minor histocompatibility antigens reveals that the repertoire is often shared, confined and unconventional

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Background: Allogeneic stem cell transplantation is a curative treatment for hematological malignancies, but patients may still experience relapse of their disease as well as Graft-versus-Host Disease (GvHD). After transplantation, donor T cells recognize polymorphic peptides presented by HLA surface molecules on patients’ cells. These polymorphic peptides, called minor histocompatibility antigens (MiHAs), are encoded by genetic differences in single nucleotide polymorphisms (SNPs) between patient and donor. Dependent on whether the antigen is presented on tumor cells or healthy non-hematopoietic tissues of the patient, donor T cells may induce the favorable Graft-versus-Leukemia effect or GvHD, respectively. MiHAs, which resemble neoantigens in that they are created by single nucleotide variants, are fundamental to understand and modulate the immune response in patients after transplantation.

Aims: To identify and characterize the dominant repertoire of MiHAs in seven common HLA class I molecules (HLA-A*01:01, A*02:01, A*03:01, B*07:02, B*08:01, C*07:01, C*07:02) for immune monitoring and modulation.

Methods: Activated donor derived CD8+ T cells were single cell sorted from 40 patients with GvHD or disappearance of patient hematopoietic cells after T cell-depleted, HLA-matched allogeneic stem cell transplantation and donor lymphocyte infusion. Growing T cell clones were tested for reactivity against patient and donor cells, and patient-specific T cell clones were tested against an optimized panel of 191 selected EBV transformed B cell lines, which have been sequenced as part of the 1000 Genomes Project. This panel enables identification of MiHAs presented by seven common HLA class I molecules (HLA-A*01:01, A*02:01, A*03:01, B*07:02, B*08:01, C*07:01, C*07:02) by genome wide association study (GWAS) and increases SNP coverage to 11 million (MAF > 0.01). SNPs that strongly associated with T cell recognition patterns were subsequently validated to encode MiHAs.

Results: We identified 85 new MiHAs presented by seven common HLA class I molecules with our GWAS method using an unbiased forward strategy (T cell-to-antigen), thereby more than doubling the previously known repertoire of 65 MiHAs. Remarkably, of the total 150 MiHAs, 39 (26.0%) antigens are not derived from annotated ORFs of protein-coding genes, but cryptic antigens, i.e. out-of-frame antigens or antigens translated from SNPs in 5' UTR or non-coding regions. Ribosomal profiling was performed for six B cell lines to explore its sensitivity to detect ORFs for cryptic antigens.

Our data show that antigens targeted in immune responses after transplantation are often shared, i.e. recurrently found in multiple patients. In total, 177 distinct T cells clones for 109 different MiHAs were isolated in 40 patients. Strikingly, 44 (40.4%) MiHAs were shared and recognized by 112 (63.3%) of the 177 T cell clones. Whole exome sequencing showed that larger fractions of SNP mismatches for known MiHAs were targeted in patients with increased severity of GvHD.

Finally, we investigated tissue distribution and SNP disparities of all MiHAs to identify potential targets for immunotherapy based on whether they are selectively expressed by hematopoietic cells and frequently mismatched. In total 12 hematopoietic-restricted MiHAs were found of which 6 antigens are novel.

Summary/Conclusion: In conclusion, despite many SNP mismatches between patients and donors, our data demonstrate that the repertoire of MiHAs is often shared, confined and unconventional. As the antigens were identified by an unbiased forward strategy (T cell-to-antigen), our collection provides relevant insight to predict antigen immunogenicity by reverse strategies (antigen-to-T cell). A large collection of MiHAs is fundamental to predict, follow or manipulate immune responses after allogeneic stem cell transplantation to improve clinical outcome of transplanted patients.

A100: Engineering Synthetic Gene Circuits for Targeted Ovarian Cancer Immunotherapy

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Synthetic biology has enabled advanced targeting of cancer cells through synthetic gene circuits that can sense and respond to oncogenic signals. We developed a programmable gene circuit platform to target cancer cells with a tumor-localized therapeutic that recruits and activates immune cells: Tumor Immunotherapy by Gene-circuit Engineered Response (TIGER). Using Boolean-logic computation, this circuit can detect multiple intracellular cancer-specific transcription factors to determine whether the cell is cancerous, which would release an immunotherapeutic payload within the tumor.

This platform has been characterized to specifically sense the ovarian cancer-associated transcription factors, cMyc and E2F1. When the activities of both transcription factors are detected by a Boolean AND gate, the circuit is programmed to express the SCIP combinatorial immunotherapy, which consists of the Surface T-cell Engager (a membrane-bound anti-CD3 scFv domain that can activate T cells, leading to T-cell mediated killing of the expressing cell), the chemokine CCL21, the cytokine IL-12, and the anti-PD1 antibody. This circuit has been shown to exhibit high specificity to tumors *in vitro* and high therapeutic efficiency *in vivo*, significantly reducing the tumor burden and increasing survival rates in mouse ovarian cancer models.

To extend our targetable spectra for ovarian cancer, we performed a computational analysis of cancer genomic repositories to identify other relevant transcription factors. We found that out of hundreds of oncogenes, Myc was the most frequently overexpressed transcription factor in ovarian cancer. Additionally, we found that most ovarian cancer tumors lacking E2F1 overexpression had mutations in TP53, which are largely loss-of-function mutations. Thus, sensing the loss of TP53 could provide an orthogonal asset in detecting ovarian cancer tumors. Therefore, we design a novel circuit using Boolean NIMPLY (AND-NOT) logic to detect if cMyc is present and p53 is not present, extending the targetable range of our therapy. This platform can be adapted to target multiple tumor types and to express any genetically encodable immunomodulator. Furthermore, this platform has the potential to be applied beyond cancer to target genetic diseases by designing the circuit to detect disease-specific transcription factors.

A101: Unbiased proteomics approach uncovers novel regulators of the endogenous retrovirus HERVK in melanoma and teratocarcinoma

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The human genome contains 8.9% of endogenous retroviruses (ERVs), also known as LTR retrotransposons[1]. ERVs originated from exogenous retroviruses that infected the germline over millions of years. In humans, the most recently inserted ERV is HERVK. HERVK insertions are

characterized by the presence of LTR5Hs, a distinct type of long terminal repeat that functions as promoter of the viral genome and contains multiple transcription factor binding sites.

Remarkably, an implication in oncogenesis has long been suspected of ERVs in general, and HERVK in particular. HERVK elements are epigenetically silenced in healthy somatic cells, but transcripts, viral proteins, and viral particles originated in these proviruses have been detected in teratocarcinoma, melanoma, lymphoma, breast, ovarian and prostate cancers[2]. Whether activation of HERVK is a byproduct of the widespread epigenetic reprogramming of cancer cells, or if it is a driver of oncogenesis is still unknown. Compellingly, HERVK may be playing dual opposite roles in cancerous cells by either accelerating tumor progression or mimicking viral infections thus boosting the innate immune response[2].

Recently, our lab demonstrated that LTR5Hs elements act as transcriptional enhancers of 275 genes, providing a surprisingly large contribution to gene regulation in a teratocarcinoma model. In fact, some of the genes regulated encode proteins involved in tumor immunogenicity, tumor suppression, and tumor vasculogenesis, invasion and progression[3]. Despite their role in transcriptional control, the molecular mechanisms underlying LTR5Hs reactivation and regulation in cancer are poorly understood. Which transcription factors, cofactors and regulators govern the unspecific activation of LTR5Hs in tumors? To fill this gap, I utilized our LTR5Hs-targeting system (CARGO-CRISPR)[3,4] coupled with a robust proximity biotinylation approach (APEX2)[5] to probe in an unbiased manner the chromatin proteome of the LTR5Hs elements in both teratocarcinoma and melanoma cell lines. Upon induction of the system, a dCas9-APEX2 fusion protein is recruited to the LTR5Hs elements and biotinylates the proteins that are in their proximity. Next, quantitative mass spectrometry allows the identification of potential regulators of the LTR5Hs elements in the assayed cell line.

Using this system, I have shed light on the complex regulatory landscape of LTR5Hs elements in melanoma and teratocarcinoma cell lines. Strikingly, the unbiased nature of this proteomics approach has identified novel regulators of HERVK/LTR5Hs beyond the realm of transcriptional regulation. These hits uncover novel potential targets for endogenous retroviral control in cancer and illuminate the complex interplay between the cellular functions and the still-viral nature of ERVs.

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A102: Contrasting autoimmune and treatment effects reveals baseline set points of immune toxicity following checkpoint inhibitor treatment

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Immune checkpoint inhibitors (ICIs) have changed the cancer treatment landscape, but severe immune-related adverse events (irAEs) can be life-threatening or fatal and may prohibit patients from receiving further ICI treatment. While the clinical features of irAEs are well documented, molecular signatures, predictive biomarkers, and mechanisms of impending irAEs are largely unknown. In addition, the markers and mechanisms of ICI-induced antitumor immunity often overlap with those for irAEs. It is thus critical to uncover signatures associated specifically with irAEs but not with antitumor immunity. To identify circulating immune cell states associated with irAEs, we applied multimodal single cell analysis (CITE-seq) to simultaneously measure the transcriptome and surface proteins from peripheral blood mononuclear cells (PBMCs) collected before and after treatment with an anti-PD-L1 antibody (avelumab) in patients with thymic cancers (thymic epithelial tumors). All patients had an antitumor response, yet a subset developed muscle autoimmunity (myositis), a potentially life-threatening irAE. Mixed-effect modeling disentangled cell type-specific transcriptional states associated with ICI treatment responses from those of irAEs to identify temporally stable pre-treatment immune set points associated with irAEs only. These pre-treatment baseline signatures of irAE developed post-avelumab irAEs reflect correlated transcriptional states of multiple innate and adaptive immune cell populations, including elevation of metabolic genes downstream of mTOR signaling in T-cell subsets. Together these findings suggest putative pre-treatment biomarkers for irAEs following ICI therapy in thymic cancer patients and raise the prospect of therapeutically dampening autoimmunity while sparing antitumor activity in cancer patients treated with ICIs. Together, pre-treatment biomarkers and interventional therapeutics could help mitigate treatment discontinuation and improve clinical outcomes.

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A103: Concentration dependent Runx transcription factor binding site choice in early T-development and its functional significance in regulating selective gene network modules

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Runx transcription factors are widely expressed in all hematopoietic lineage cells, and mutations in genes encoding Runx factors are causally associated with various lymphoma and leukemia. Despite a broad expression pattern, Runx factors perform cell-type specific functions both in normal hematopoiesis and carcinogenesis. It remains unclear how ubiquitously expressed Runx factors control such a range of context-specific gene networks. We utilize early thymic T cell development system, which spans diverse regulatory contexts; therefore, Runx factors can provide unique or common functional inputs at different stages. Runx factors are stably expressed throughout T-development, and they are implicated at most enhancers active at any stage in thymic T-development. Despite constant activity, Runx factors preferentially regulate genes that are dynamically changing during T-lineage commitment, and they control different, stage-specific genes. We have found that this is closely associated with global redistributions of Runx DNA binding site choices before and after T-lineage commitment. This prompts the question what mediates redeployment of Runx factors in these sequentially-related stages.

Our previous studies showed that PU.1 dominates Runx1 site choice in pro-T cells by diverting Runx1 to PU.1 sites and depleting Runx1 from alternative high quality Runx motif sites. Therefore, we hypothesized that transcription factor partners compete to recruit a limited amount of Runx to different sites. If so, mutually exclusive binding of Runx1 to different classes of sites might be overruled if Runx availability is increased. To test this, exogenous Runx1 was introduced to pro-T cells in pre-commitment stage and the Runx1 binding profile was measured by CUT&RUN. Overexpressed Runx1 still interacted with pre-commitment sites, but it also occupied three different types of additional sites. First, it prematurely established occupancy at more than 50% of normal post-commitment sites. In addition, a high level of Runx1 induced new binding sites within open chromatin and new binding sites within normally closed chromatin.

Notably, the new binding site choices with increased Runx1 resulted in substantial changes in the transcriptional program by significantly accelerating expression of genes associated with T-lineage commitment. Single-cell transcriptome analysis showed that Runx1 overexpression caused deviation from the normal trajectory, suggesting that a high level of Runx1 directs T-development by regulating transcriptional programs of individual cells rather than by changing subpopulation distributions. Also, the T-development pseudotime trajectory analysis followed by gene network inference implied that Runx1 overexpression advanced early T-development by prematurely activating gene regulatory modules associated with the T-lineage/lymphoid program, even while the stem/progenitor gene networks still remained active, and genes involved in cytokine/environmental signaling pathways were not fully induced yet. Of importance, differentially expressed genes resulted from increase of Runx1 showed complete reversal of transcriptional profile when Runx1 and Runx3 were deleted together, and the core Runx target genes sensitive to both loss- and gain-of Runx functions were strongly enriched for T-lineage commitment pathways. Consistent with these analysis, co-culture of control vs. Runx1 overexpressing progenitor cells in mixed chimeric artificial thymic organoid cultures showed that increased Runx1 availability resulted in overall faster progression though commitment, and

accelerated development was sustained beyond b-selection. Together, our data show that availability of Runx factors tunes Runx-binding site choice between different developmental stages, and this Runx-dosage dependent DNA binding controls the activity of selective gene regulatory modules directing T-lineage commitment and early T cell development speed.

A104: 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 and myeloid malignancies.

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 extra-medullary compartments, with exacerbated loss of lymphoid potential during aging. 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 *Mfn2*$^{-/-}$ knock-out (KO) mice compared to wild-type (WT). Detectable Interferonα (IFNα) was furthermore found in the serum of *Mfn2*$^{-/-}$ 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 cycling. Interesting, aged *Mfn2*-deleted BM has 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 IFNa production in a STING dependent fashion.

Taken together, these findings suggest that manipulating mitochondrial dynamics might constitute an approach to alleviate some aspects of aging, myeloproliferative diseases as well as genetic interferonopathies.

A105: Birnaviruses as potential candidates for oncolytic virotherapy of breast cancer

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Introduction

Oncolytic virotherapy, involving the usage of oncolytic viruses (OVs) can that selectively replicate in and lyse cancer cells, is emerging as a promising immunotherapeutic modality. Our group discovered that Infectious Pancreatic Necrosis Virus (IPNV) and Infectious Bursal Disease Virus (IBDV), members of the *Birnaviridae* family, have potent oncolytic activity. Since Birnaviruses are animal viruses, they possess the advantages of exerting prolonged therapeutic action due to the lack of preexisting antiviral immunity and non-pathogenicity. Further, the dsRNA genome of Birnaviruses can induce strong immunostimulatory activity *in vivo*. Preliminary studies of the viruses against a range of cancer cell types showed significant cancer-selective replication of the virus (Oncoselectivity) and selective lysis of cancer cells (Oncotoxicity). Since the oncolytic action of Birnaviruses was particularly effective against epithelial cancers, this study was undertaken to evaluate the efficacy of the oncolytic action of Birnaviruses against breast cancer, the most common incident cancer in humans and dogs, using *in vitro* studies and *in vivo* testing in the murine triple-negative breast cancer (TNBC) model, 4T1.2.

Methods

Human breast cancer cells (HCC1500 (Estrogen receptor (ER)+, Progesterone Receptor (PR)+, HER2-), HCC1187 (TNBC), canine mammary tumor cells (CMT-U27), and non-cancerous primary cell controls were infected with IBDV and IPNV at MOI ranging from 0.001 to 5. At 72 hours post-infection, the cell viability was assessed using MTS assay, apoptosis was measured using Caspase-Glo 3/7 assay, viral replication was quantified using qRT-PCR, and the presence of viral protein in the cells was assessed by immunofluorescent (IF) staining. Female BALB/c mice were injected with 4T1.2 cells into the left fourth mammary fat pad. At 19 days post-tumor implantation, 10^5 $TCID_{50}$ units of IPNV and IBDV in 50 µl were injected half intratumorally and half intraperitoneally. Mice in the mock treatment group received 50 µl of vehicle control. Tumor sizes were measured regularly, and on day 35, mice were sacrificed, and the number of live cells in the tumor was quantified by flow cytometry.

Results

A significant decline in viability and increase in apoptosis of human breast cancer cells with IPNV and IBDV (MOI $\geq$ 0.1) and canine breast cancer cells with IBDV (MOI $\geq$ 1) were observed. There was a significant increase in viral replication inside the above-mentioned cells, starting at 24h post-infection. There was no notable change in viability, apoptosis, or viral replication in the non-cancer controls. In the 4T1.2 tumor-bearing mice treated with IBDV and

IPNV, there was a significant decrease in live cells in the tumor. A substantial decline in tumor sizes was also observed in the IPNV-treated group.

Conclusion and Future Directions

The results strongly support the susceptibility of human mammary cancers to Birnavirus oncolytic action and the susceptibility of canine mammary cancers to IBDV. Hormone receptor-positive and triple-negative breast cancer are susceptible, and this implies a broad range of efficacy of Birnaviruses against breast cancer. Further experiments are underway to study the *in vivo* immune responses in the 4T1.2 model and the efficacy of recombinant Birnaviruses containing GM-CSF to enhance immunostimulatory properties.

A106: In vivo Crispr-screen of mouse cell surface proteins reveals important biomarkers of cancer immunotherapy

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Cell surface proteins play critical roles in numerous biological processes and is an essential gateway for a cell to interact with its microenvironment. They also act as important biomarkers for cell identity and potential targets for pharmacological intervention. Xenograft-based CRISPR screens accurately reconstruct tumors biological development and their environment *in vivo*, such as cell-matrix interaction, cell-cell contact, and the immune microenvironment. Hereby, we designed a *de novo* small library targeting mouse cell surface proteins and conducted in vivo CRISPR screens with or without PD-1 blockade to explore essential and novel cell surface proteins on tumor growth and immunotherapy. MC38 mouse colon cancer cells were infected with library virus (multiplicity of infection [MOI] < 0.3). After selection, cells were divided into three groups, *in vitro* cultured, subcutaneously transplanted into C57BL6 mice with or without PD-1 blockade treatment. At certain time points, the genomic DNA was extracted from the cells/tissues, indexed with barcode primers, and subjected to NGS. Our screen results detected several known proteins involved in tumor immunotherapy such as CD274 and CD47. Moreover, we found a bunch of novel genes, such as GLUT1, Fut10, CLTN1, and elt highly involved in PD1 blockade treatment. Gene ontology (GO) and KEGG analysis revealed these genes were highly involved with cellular immunology. Collectively, our integrated screens revealed several cell surface genes involved in immunotherapy and with immense potential for clinical translational study.

A107: Immunity to the microbiota promotes sensory neuron regeneration

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The microbiota plays a fundamental role in the induction, education, and function of the mammalian immune system. In turn, the immune system operates to sustain and restore tissue

function in the context of microbial or environmental exposures. Host-microbiota dialogue is of particular importance at barrier sites that are both home to the microbiota and primary targets of environmental stressors. All barrier tissues, including the skin, are home to a dense network of sensory nerve fibers that are involved in the perception of touch, temperature, pain and itch. Recent work reveals that sensory neurons can also influence other biological processes including host metabolism, inflammation, and protective immunity. These emerging observations underscore our growing understanding of the profound interconnection among biological systems and more particularly between the immune and nervous systems. In the context of infection or injury, host survival requires protection and restoration of all tissue components, each requiring specific repair programs. Based on the profound alliance between the microbiota and its host, we hypothesized that the microbiota could play an important role in bridging biological systems to reinforce tissue protection and restore barrier integrity. In this context, whether immunity to the microbiota can promote neuronal regeneration remains unclear. Here, we show that, upon injury, adaptive responses to the microbiota directly promote sensory neuron regeneration. At homeostasis, commensal-specific T helper 17 (Th17) cells colocalize with sensory nerve fibers within the dermis and express a transcriptional program associated with neuronal repair. Following injury, commensal-specific Th17 cells promote axon growth and local nerve regeneration. Mechanistically, our data reveal that the cytokine interleukin 17 A (IL-17A) produced by commensal-specific T cells directly signal to sensory neurons via the IL-17 receptor A, the transcription of which is specifically upregulated in injured neurons. Collectively, our work reveals that microbiota-specific T cells can bridge biological systems by directly promoting neuronal repair, and identifies IL-17A as a major determinant of this fundamental process. Our findings that upregulation of the IL-17A/IL-17RA axis represents a conserved response in injured neurons open the door to novel therapeutic approaches to potentiate sensory recovery after injury, or limit neuropathies in the context of diabetes and chemotherapy.

A108: Ligand-biased trafficking and signaling of C-C chemokine receptor 5 (CCR5) revealed by APEX2 proximity labeling coupled with tandem mass tagging (TMT) mass spectrometry

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In most cancers, immune cells are guided to tumor sites through interactions between chemokines and chemokine receptors. C-C chemokine receptor 5 (CCR5) is a G protein-coupled receptor (GPCR) and one of the major chemokine receptors involved in inflammation associated with malignant diseases. It is expressed on both immune and cancer cells, and has been shown to have pro-tumor effect in cancers, promoting tumor growth and metastasis by affecting both immune and cancer cells. This renders CCR5 a highly attractive therapeutic target. However, it is challenging to design effective drugs against CCR5 in the context of cancers because the precise signaling mechanism of CCR5 is still poorly understood. CCR5 has the capacity to sense multiple stimuli in the tumor microenvironment and elicit distinct ligand-specific signaling responses and receptor trafficking behavior of the receptor that significantly impact tumor fate. To gain a comprehensive understanding of the CCR5 interactomes involved in ligand-specific signaling and trafficking responses of the receptor, we have employed a powerful proximity

labeling method using APEX2 peroxidase that reveals time and spatially dependent protein-protein interactions. For this purpose, a stable HEK293T cell line expressing APEX2 conjugated CCR5, which is capable of labeling proteins in proximity to CCR5, was generated and characterized to ensure it closely mimics the trafficking and signaling behaviors of wild-type CCR5. Using this cell line and tandem mass tagging (TMT) coupled with mass spectrometry, we generated a comprehensive dataset of CCR5 interactors under with three different ligands treatment conditions (including the endogenous ligand CCL5 and two engineered CCL5 variants - a super agonist 6P4 and a partial/biased agonist 5P14), and four different timepoints (3, 10, 30, 60 minutes after stimulation). The proteomic data revealed distinct trafficking behaviors of CCR5 under different ligand treatment with exquisite spatio-temporal resolution that has not been described previously. Moreover, gene ontology analysis showed that CCR5 is interacting with or /within proximity of different functional groups of proteins that are well-matched with the ligand-biased trafficking patterns. Among these, we have prioritized for follow up studies around ~10 proteins that have previously been shown to interact with other GPCRs and affect their signaling and trafficking, but have not been studied in association with CCR5 for follow up studies. Currently, we are generating knockdown cell lines of these targets in both model cell line HEK293T cells and more relevant cell lines such as monocyte and T cell lines and testing them for functional roles in CCR5 trafficking and signaling. The successful execution of this project will provide valuable information for developing drugs with therapeutically desirable consequences, such as internalization and persistent intracellular sequestration of CCR5 in the context of tumor suppression. This work is supported by Cancer Research Institute Irvington Postdoctoral Fellowship.

A109: Association of TP53 mutation status and sex with clinical outcome in NSCLC treated with immune checkpoint inhibitor: a retrospective cohort study

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TP53 mutation has been suggested as a prognostic biomarker associated with longer survival in Non-Small Cell Lung Cancer (NSCLC) patients receiving immune checkpoint inhibitor (ICI) therapy. Recent studies have suggested that TP53 mutation may impact sex disparity in several cancer types including NSCLC. Hence, we hypothesized that TP53 mutation might serve as a sex-dependent genomic biomarker for response to ICI treatment in NSCLC patients. We conducted a retrospective study on 100 consecutive metastatic NSCLC patients treated with ICI monotherapy at Seoul National University Bundang Hospital. Clinical data including tumor programmed death ligand-1 (PD-L1) expression, TP3 mutation status assessed by panel-based next-generation sequencing, and clinical outcomes were collected from the electronic medical record. We also analyzed the genomic and clinical data of a MSKCC ICI-treated cohort from cBioPortal database. The median age at ICI initiation was 62 (range: 36-84) years; 63 (63%) were male; 57 (57%) were harboring TP53 mutations; 74 (74%) were adenocarcinoma. There was no statistically significant difference in disease control rate (DCR: CR+PR+SD ≥6 months) according to TP53 mutation status (45.8% in TP53 MT vs 39.0% in TP53 WT, P=0.503). In univariate analysis for PFS, there was no significant difference in the median PFS between NSCLC patients with or without TP53 mutations in all patients (3.9 months in TP53 WT vs 3.4

months in TP53 MT, P=0.930). However, when stratifying by sex, female patients with TP53 mutation had a significantly prolonged median PFS compared to wild-type female patients (female with TP53 MT, 6.1 months, 95%CI 2.2-10.1, TP53 WT, 2.6 months, 95%CI 1.5-3.7; P=0.021). In multivariate analysis, TP53 mutation was independently associated with longer PFS in female patients (HR=0.41, 95%CI 0.18-0.96, P=0.039). PD-L1 high (≥50%) expression was significantly enriched in female patients with TP53 mutation (58.8% vs. 5.0%, P=0.001). In the ICI-treated cohort from MSKCC, NSCLC patients with TP53 mutation were associated with prolonged PFS. When stratifying by sex, female patients with TP53 mutation showed significantly longer PFS than that of TP53 wild-type female patients, while there was no significant difference in PFS between male patients with or without TP53 mutations (female with TP53 MT, 5.4 months, 95%CI 3.3-7.5, TP53 WT, 2.4 months, 95%CI 2.0-2.8, P<0.001; male with TP53 MT, 3.6 months, 95%CI 2.7-4.6, TP53 WT, 2.8 months, 0.0-5.7, P=0.760). This study found that female NSCLC patients with TP53 mutation were associated with favorable clinical outcomes after ICI therapy and high expression of PD-L1. The role of TP53 mutation in sex disparities in NSCLC treated with ICI should be further validated.

A110: Interleukin-2 signaling at the immunological synapse

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The immunological synapse is the molecular platform for signal integration that controls T cell activation. Interleukin-2 (IL-2) is an essential signal for optimal T cell activation and proliferation in response to antigen recognition. IL-2R signaling is polarized to the synaptic cleft and trans-presentation of IL-2 at the synapse promotes high-affinity IL-2 signaling for the priming of naive T cells. However, the role of IL-2 on synapse architecture remains largely unexplored. In addition, while a crosstalk between IL-2R and TCR signaling has been studied, insight into how these events are coordinated in space and time at the synapse is missing. Here, we analyse the distribution and function of IL-2R at the synapse of different subsets of CD8 T cells. A supported-lipid bilayer system is used to reconstitute the surface of a target cell and generate antigen-specific synapses with CD8 T cells. We image the effects of IL-2 on the recruitment of different receptor molecules at the synapse, as well as the spatiotemporal organization and function of the IL-2R complex. To further study how IL-2R and TCR signaling are integrated in the synapse, we use a new generation of biologic therapeutics termed Immuno-STATs™, that are composed of a Fc-formatted peptide-HLA complex and a modified IL-2 with reduced affinity. Our data sheds light into the cellular and molecular mechanisms of early IL-2 action and improves our knowledge to engineer optimal therapeutic approaches against cancer and autoimmune diseases.

A111: APOBEC3A impacts Interferon Stimulated Gene expression

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Retrotransposons are selfish mobile genetic elements that closely resemble or directly descend from retroviruses and make up ~35% of the human genome. Evolutionarily young retrotransposon expression and mobilization has been implicated in cancer emergence and progression. Conversely, some more ancient retrotransposons are involved in coordinating innate immunity-associated gene expression programs in several species. Given the significant influence retrotransposons can have on cell homeostasis, it is critical to understand the mechanisms by which these selfish genetic elements are regulated.

It has been reported that the APOBEC3 family of cytidine deaminases restrict retroviral replication and contribute to the repression of retrotransposon activity. Of this family, APOBEC3A (A3A) has been reported to canonically restrict retroviral replication by introducing lethal mutations into the viral genome via cytidine deamination. A3A can also repress retrotransposon replication by a deaminase-independent mechanism that is poorly understood.

Our lab previously discovered that A3A can bind the regulatory sequences of proviral Human Immunodeficiency Virus, and recruit chromatin remodeling and transcriptional repression machinery without causing cytidine deamination of genomic DNA. Since retrotransposons are broadly distributed throughout the genome and have been implicated in regulating transcription of host innate immunity-associated genes, we hypothesized that A3A may be able to regulate retrotransposon and gene transcription. We used a combination of computational and experimental approaches in cell culture to characterize the sequence features required for A3A genome binding and transcriptional repression, and identify genes impacted by A3A. Using *in vitro* DNA-binding assays, we discovered that A3A preferentially binds paired TTTC motifs separated by spacers of 1-10 nucleotides. Such motifs are present in Interferon-Stimulated Response Elements (ISRE) which are core binding sequences for innate immunity-associated transcription factors like pSTAT1. These data suggest that A3A genome binding is most likely to occur in regions where single-stranded DNA is exposed, such as in R-loops that are formed during transcription. Our *in silico* genome-wide scan for TTTC motif pairs revealed that these motifs are significantly enriched in the promoters of Interferon Stimulated Genes (ISG). ChIP-qPCR and luciferase reporter experiments showed that A3A binds ISRE motifs present in the proximal promoters of ISG15 and OAS1. RNAseq analyses in IFNa-treated WT and A3A-KO JLat cells indicated that a subset of ISGs are differentially upregulated in the absence of A3A. Computational motif enrichment analysis of these promoters indicated that differentially upregulated ISG promoters contain significantly higher numbers of TTTC motifs compared to ISGs not impacted by the presence or absence of A3A.

Combined, these data suggest that A3A may play a role in modulating TypeI-IFN dependent ISG expression. This activity may contribute to the necessary downregulation of ISG expression following immune stimulation or viral infection. Future work will aim to identify where A3A binds within the genome and characterize what effect this binding may have on retrotransposon and genome regulation.

A112: Determining the liver immune microenvironment in fibrolamellar carcinoma by non-invasive live imaging

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Fibrolamellar carcinoma (FLC) is a rare type of liver cancer without approved therapeutically options that affects mostly healthy young people. FLC is associated with the overexpression of the fusion protein DNAJB1-PRKACA. Immunotherapeutic approaches are taking more relevance as an option to treat FLC; however, the immune regulatory mechanisms involved in the disease are unknown. Our aim is to characterize the liver immune landscape in FLC and investigate the contribution of immune cells and inflammasome to FLC progression. Using the zebrafish animal model, we previously have shown that overexpression of the fusion protein DNAJB1-PRKACA specifically in hepatocytes triggers carcinogenesis and induces liver inflammation and inflammasome activation as early as 7 days post-fertilization. Combining this FLC model with different available zebrafish transgenic lines and using genomic approaches and confocal fluorescent microscopy, we are now determining the liver immune cell composition and the role of myeloid-specific inflammasome in early progression of FLC. Consistently with our previous findings we observed that, FLC animals show sustained liver inflammation at later development stages characterized by increased infiltration of neutrophils and pro-inflammatory macrophages in the liver suggesting that innate immune cells might play a role at early FLC progression. Interestingly, at 2-week-old animals we started to observe a decrease in the infiltration of total T cells and CD4 T cell populations to the FLC liver comparing to control siblings. In addition, using gene expression data publicly available from FLC mice tumors we found an upregulation in many inflammasome related genes. Using non-invasive live imaging of the liver area, we observe an increased number of neutrophils expressing *pycard*, a protein adaptor key component on canonical inflammasome in FLC livers. Finally, we performed a proteomic analysis comparing livers from control and FLC sibling zebrafish finding 44 significantly altered proteins in FLC. Among these proteins are Usp7, previously described target for FLC treatment, Cdc42 and Catenin β1 (Ctnnb1), which is one of the main members of the Wnt signaling pathway involved in hepatocyte proliferation and liver carcinogenesis. Overall, our findings suggest that innate immune cells and myeloid-specific inflammasome activation might be important players in early FLC progression. Our studies could have a significant impact in the FLC field by helping to develop new-targeted immunotherapies but also on how to make better use of currently available ones.

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A113: Metabolic dysregulation explains the diverse impacts of obesity on the immune system in male and female patients with gastrointestinal cancers

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Background: Epidemiologic studies have shown that obesity is associated with an increased risk of thirteen types of cancer, including esophageal, stomach, and colorectal cancers. Our prior collaborative research has revealed that obesity leads to T-cell dysfunction in cancer by upregulation of the PD-1/PD-L1 pathway that is at least partially driven by leptin. Recent evidence suggests that obesity disproportionally impacts males and females with cancer, resulting in different levels of transcriptional and metabolic dysregulation. Based on the data presented above, we hypothesized that obesity leads to metabolic changes both systemically as well as in the tumor microenvironment of cancer patients, and these metabolic changes are associated with differential immune dysregulation in males vs. females. Given the strong association between obesity and different gastrointestinal (GI) cancers, this study aimed to elucidate the differences in not only the obese metabolic milieu but also those that are related to sex in the context of obesity in GI cancers.

Methods: We utilized serum metabolomics data from GI cancer patients (N=8, normal weight=5, overweight=3) to understand the metabolic differences between obese and non-obese patients. To further elucidate this, we compared these changes with TCGA transcriptional metabolic alterations, using our metabolic pipeline to demonstrate pathway parallels between metabolomics and transcriptomics. To address the role of gender and obesity in GI cancers, we utilized the transcriptional metabolic pipeline to investigate TCGA data for multiple GI cancers [Stomach (STAD), Esophageal (ESCA), and Colorectal (CRC)], comparing differences in obese and non-obese males and females.

Results: Our serum metabolomics results demonstrate dysregulation of several key metabolic pathways, including lipid metabolism, amino acids, and kynurenine, in obese GI cancer patients, compared to non-obese patients. This corresponds to obese vs. non-obese transcriptional metabolic enrichment, where we see dysregulation of lipid and kynurenine metabolism. Metabolic pipeline analysis of all three GI disease sites, considering age, BMI, and gender (STAD, ESCA, and CRC), revealed dysregulation of several immune-related metabolic pathways like tryptophan and kynurenine metabolism and steroid hormone metabolism in all studied cancers and both genders, albeit to a differing extent. The analysis also revealed unique metabolic dysregulation of pathways by disease sites (Glycan Biosynthesis in ESCA) and gender (Pyrimidine Metabolism in obese females with CRC).

Conclusion: Our analysis revealed that obese patients show differences in major metabolic pathways, which are also associated with immune dysfunction, providing a rationale for the varied impact of obesity in a sex-related manner and evidence for continued study of this phenomenon.

A114: Multimodal analysis of signaling-coupled transcription factor networks regulating human B cell responses

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Activated B cells bifurcate into antibody-secreting plasmablasts (PB) or germinal center B cells (GCBC), the balance of which determines the quality and magnitude of humoral immune responses. While short-lived PB can provide a rapid burst of low-affinity antibodies, GCBCs

delay differentiation in secondary lymphoid organs where their immunoglobulin genes undergo somatic hypermutations and affinity-based selection to generate long-lived plasma cells capable of secreting high-affinity antibodies. The purpose of our study is to comprehensively elucidate the dynamic signaling-coupled transcription factor (TF) network that regulates the bifurcation of human activated B cells into PBs (short-lived, low-affinity) or GCBCs (potentially long-lived, high-affinity). We and others have previously found that reciprocal negative feedback between signaling-induced TFs (IRF4, IRF8, and Bcl-6) regulate this primary bifurcation event in mice. While elevated IRF4 expression promotes PB differentiation, elevated IRF8 and Bcl-6 expression restrain PB differentiation and enable GCBC formation instead. This bifurcation of activated B cells also occurs in the context multiple signaling inputs including through the B cell receptor, toll-like receptors, and T cell-derived ligands and cytokines. Therefore, to investigate how signaling and TF networks are dynamically integrated to program human B cell fate determination, we are coupling two highly tractable *in vitro* systems with temporal, single-cell multimodal profiling. In striking similarity to our previous studies in mice, we have found that activated human B cells also bifurcate into $IRF4^{hi}/IRF8^{lo}/Bcl6^{lo}$ PB and $IRF4^{lo}/IRF8^{hi}/Bcl6^{hi}$ GCBC precursor-like cells after several days of activation and clonal expansion. We are currently using single-cell transcriptomics and proteomics approaches to temporally profile human B cells as they progress through activation, clonal expansion, and differentiation. We will then use high-throughput CRISPR activation and interference approaches to functionally validate the predicted signaling-coupled TF network and its regulation of the activated human B cell bifurcation process. We anticipate that this work will uncover the regulatory apparatus that controls the quality humoral immune responses in humans and will provide a foundational systems immunology approach to design more rational and precise patient-specific vaccination and immunotherapy strategies.

A115: Strategic Ig kappa locus modification to study long range Ig kappa V(D)J recombination

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Exons that encode IgH and IgL chain variable regions are assembled in developing B lymphocytes from V, D, and J segments. V(D)J recombination is initiated by RAG endonuclease, which generates DNA breaks between gene segments and flanking recombination signal sequences (RSSs). In progenitor B cells, RAG binds a J_H-based recombination center (RC) and then joins upstream Ds to form a DJ_H-RC. Joining of J_Hs to Ds occurs by a linear RAG chromatin scanning mechanism in which D-containing chromatin is presented to the J_H-RC-bound RAG by cohesin-mediated loop extrusion. DJ_H-RC-bound RAG can similarly employ loop extrusion to scan the upstream 2.4 Mbs V_H domain to locate V_Hs. Long-range scanning is facilitated by neutralization of abundant V_H locus CTCF-binding elements (CBEs), which impede scanning, through down-regulation of the WAPL cohesin-complex unloading factor. Inversion of the 2.4 Mbs V_H locus showed that, convergent ("deletional") orientation of V_HRSS relative to DJ_HRC-RSS is required for their recognition and rearrangement during RAG scanning. Ig Kappa (*Igk*) variable region exons are assembled in the subsequent precursor B cell stage. The 3.2 Mbs *Igk* locus undergoes direct Vk-to-Jk joining between 92 functional Vks and 4 functional Jks. While proximal and distal Vks are oriented for deletional joining, middle Vks are

mostly oriented for inversional joining. Based on our *Igh* locus studies, long-range inversional joining of distant Vks to Jks is not consistent with direct linear RAG scanning. In theory, short-range diffusion-based mechanisms our lab described previously for low-level inverted D-to-J_H joining, may be structurally-optimized in the *Igk* locus to bring Vk-RSSs close enough to the Jk RC for a short-range diffusion-mediated joining.

My current *Igk* locus V(D)J recombination experiments were mostly done with Abelson MuLV-transformed pro-B cells (*v-Abl* cells) that robustly rearrange Vks to Jks with patterns like those of normal pre-B cells. Analyses of Igk V(D)J recombination have been hindered by complex chromosomal and extrachromosomal secondary recombination events. Thus, we generated a single Jk5 *v-Abl* cell that joins to deletional and inversional oriented Vks across the locus and excludes confounding secondary joining events. Using this simplified locus, we used a cas9/gRNA approach to delete the CBE-based Cer/Sis elements in the Vk-Jk interval. Based on our high throughput repertoire-sequencing approach, deletion of Cer/Sis nearly abrogated distal deletional and inverted Vk rearrangements, but increased use of proximal deletional-oriented but not inversional-oriented Vks, consistent with activating a mechanism in which Jk-RC-based RAG linearly scans upstream proximal Vk- containing chromatin. Deletion of Cer or Sis individually revealed that activation of RAG scanning into the proximal Vk locus was mainly due to inactivation of Cer. We also inverted the entire 3.0 Mbs Vk locus and found, in dramatic contrast to inversion of the V_H locus, modest effects on rearrangement of Vk utilization across the locus. Upon inversion, all Vks formerly oriented for deletion were oriented for inversion and all formerly oriented for inversion were oriented for deletion. Yet, all still rearranged, with relatively modest differences in relative utilization. Finally, in a non-simplified locus and in great contrast to experiments done in our lab for the *Igh* locus, inversion of the Jks also had relatively little effect on the level of Vk rearrangement across the locus. Our studies clearly demonstrate that Igk and *Igh* use, at least some, distinct mechanisms to incorporate their Vs into the long-range V(D)J recombination process. Ongoing studies to further address the mechanistic basis for these differences will be presented. Overall, our studies should provide new insights into mechanisms that establish highly diverse primary antibody repertoires and how the process goes awry to generate oncogenic translocations.

A116: A novel TIL therapy product enriched for CD39-CD69- CD8+ T cells

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Adoptive cell transfer (ACT) with autologous tumor-infiltrating lymphocytes (TILs) has proven to be one of the most successful immune therapy modalities with overall response rates of around 50% and complete responses in up to 20% in clinical trials with patients with malignant metastatic melanoma.

Current protocols combine a first expansion of TILs from tumor fragments or tumor digest with high-dose IL-2, followed by further expansion with a rapid-expansion-protocol (REP) using allogeneic feeder cells, αCD3 and IL-2. Following this protocol, TIL production takes 4-7 weeks,

and many patients deteriorate before they can receive therapy. With success rates of TIL expansion ranging from 70-90%, a TIL product cannot be generated for every patient. Furthermore, clinical response to TIL therapy is lower in other solid tumor types such as Cervical Cancer, Ovarian Cancer or Renal Cell Carcinoma, likely due to a lower number of expanded TILs, higher numbers of Tregs or lower frequencies of tumor reactive CD8+ T cells.

Therefore, there is a clinical need for improvement of current TIL expansion protocol and product qualities to make this therapy available to other tumor entities and more cancer patients.

In this project, the *ex vivo* addition of various TIL stimulators was tested in T cell expansions from tumor fragments of patients with Lung Cancer, Cervical Cancer, Ovarian Cancer and Renal Cell Carcinoma obtained commercially or in collaboration with Odense University Hospital. TIL phenotype, cytotoxic functionality and T cell repertoire were compared to TILs expanded following the standard protocol.

Using this novel culture protocol, success rates of expansion across tumors increased from 48 to > 96%. Additionally, significantly higher frequency and total numbers of viable CD8+ T cells per fragment were obtained compared to standard expansion with IL-2 alone. Expanded CD8+ cells exhibit a higher fraction of cells expressing the costimulatory marker CD28 and exhaustion markers BTLA and TIM3, while simultaneously containing a higher frequency and number of CD39-CD69- cells that have been shown to be present in higher numbers in the TIL product of ACT-responders and that exhibit a stem-like phenotype. Additionally, more T cell populations specific for tumor-associated antigens are present when TILs are expanded with TIL stimulators, thus showing a broader T cell repertoire associated with better outcome of ACT.

With this study, we show that by adding a cocktail of different TIL stimulators, we can increase success rate and shorten expansion time while simultaneously improving the characteristics of the TIL product.

A117: Exploiting viral pre-existing immunity to enhance oncolytic immunotherapy

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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. When the virus starts to replicate within the tumor, it might

render the tumor a good target for a pre-existing T cell response and could potentiate treatment. Although logical from the immunological point of view, this mode of action of the oncolytic viruses needs to be elucidated. Here, we addressed the role of viral pre-existing immunity upon treatment with an oncolytic vaccine platform previously developed in our lab named PeptiCRAd. This latter consists of an oncolytic adenovirus decorated with tumor MHC-I restricted peptides. 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 functional characterization assay (ELISPOT IFN-g). 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-g 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, we are exploiting the mechanism of action in the 4T1 tumor model. In the future, we aim to better understand the crosstalk between pre-existing immunity and APCs in the sustainment of anti-tumor response. Furthermore, we will translate our investigation into human setting through the study of human macrophages and human DCs pulsed with our technology. We hope to capitalize on the pre-existing anti-viral immune response to enhance cancer oncolytic therapy.

Poster Session B

B001: Cooperation of gene therapy and delivery of neoantigens for the inhibition of tumor progression and enhancement of anti-tumor immunity

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INTRODUCTION: We have shown that combined gene transfer of p19Arf (functional partner of p53; p19Arf in mice; p14ARF in humans) and interferon-β (IFNβ, pleitropic cytokine) using a nonreplicating adenoviral vector induces immunogenic cell death (ICD) and promotes a Th1 response. While our approach acts as an immunotherapy, we wish to further strengthen the immune response by directing cytolytic T cell activity towards tumor specific antigens. In this context, we evaluated whether tumor neoantigens would enhance the immune response induced by our gene therapy approach.

PROCEDURES: B16F10 cells were modified with a lentiviral vector expressing mCherry or five tandem minigenes encoding known neoantigens (Tubb3, Atp11a, Mthdfl1, Pbk and Asf1b). The B16-mCherry and B16-NeoTAMPA cells were treated with the adenoviral vectors *ex vivo* and dying cells applied s.c. as a single dose vaccine in C57BL/6 mice. Alternatively, three doses of the vaccine were applied s.c. in association with a pool of synthetic neoantigen peptides (40 µg each). The mice were then rechallenged (s.c., 7-9d after initial vaccine/peptide application) with B16 cells expressing or not the neoantigens and observed for tumor progression and survival. *In situ* gene therapy of s.c. B16F10 tumors was performed using 3 doses, $1x10^9$ TU each, at 48h intervals of the native viral vectors or vectors coated by extrusion in the presence of cell membrane derived from the B16 cells expressing or not the neoantigens. *, $p<0.05$; Mantel-Cox Log rank test.

UNPUBLISHED DATA: *In vitro* studies verified that the B16F10, B16-mCherry and B16-NeoTAMPA cell lines respond equally to treatment with the adenoviral vectors encoding p19Arf and IFNβ in terms of induction of cell death and emission of ICD markers. Vaccination using B16-NeoTAMPA treated *ex vivo* with p19Arf and IFNβ prolonged survival in mice rechallenged with B16-NeoTAMPA as compared to B16-mCherry (on d20, 3/4* vs. 0/4 survivors, respectively). Vaccination using parental B16F10 cells treated *ex vivo* with p19Arf and IFNβ in association with neoantigen peptides also prolonged survival upon rechallenge with B16-mCherry or B16-NeoTAMPA as compared to vaccination without peptides (on d20, 5/5* or 2/3* survivors vs. 0/5, respectively). In a separate assay, mice were treated similarly and recovered splenocytes showed elevated CD8+ viability, perforin expression and IFNγ ELISPOTs only upon stimulation with the PBK peptide. Evaluation of cytokines (Luminex) showed elevated IL1β, IL2, IL4, IL6, IL13 upon stimulation with the Atp11a peptide. Before the *in situ* gene therapy model, we verified that adenoviral vectors coated with cell membrane maintained their capacity to transduce cells *in vitro*, yet provided improved transduction of s.c. B16F10 tumors upon intratumoral treatment while reducing off target gene transfer in the liver. For the *in situ* gene therapy assay, s.c. B16F10 tumors were treated with intratumoral injection of naked virus or virus coated with cell membrane derived from B16F10 or B16-NeoTAMPA (presumably

presented by MHC-I). Strikingly, response (tumor volume <300 mm^3 on day 12 post treatment) was seen in 100% of the tumors treated with virus coating containing the neoantigens, yet only 50% responded when the coating did not present the neoantigens. At the conclusion of the assay, elevated numbers of CD19+CD22+ cells were found in the spleen or while increased CD8+ cells were recovered from the tumor when the vectors had been coated with membrane derived from B16-NeoTAMPA or B16F10, respectively.

CONCLUSIONS: Here we have shown that the association of p19Arf/IFNβ gene therapy with neoantigens supplied either in the context of cell membrane or as synthetic peptides resulted in improved control over tumor progression associated with enhanced immune activity.

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B002: Assessment of methods to direct T cells toward a newly established HPV16 E6/E7-dependent orthotopic tumor model in A2.DR1 mice

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Therapeutic vaccines against HPV16 (human papillomavirus 16)-associated malignancies that previously showed promise in *in vivo* experiments often fail to translate into the clinic. One reason might be the suitability of the used murine tumor models, which are either located at a heterotopic site or are immunologically murine. In our group, we recently demonstrated the therapeutic efficacy of amphiphilic peptide vaccinations in a subcutaneous HPV16 tumor model in MHC-humanized A2.DR1 mice. To better mimic the situation in patients, we now established another model at an orthotopic location, namely the vaginal mucosa. This new tumor model is based on HPV16 E6/E7-dependent lung cells from A2.DR1 mice. The cells were transduced and immortalized with the vaccination target proteins HPV16 E6 and E7. Additionally, the cells were transfected with the oncoprotein $HRAS^{G12V}$ for tumorigenicity and firefly luciferase for intracorporeal tracking of tumor growth. Subsequent to successfully validating the expression of said proteins, tumorigenicity of the novel cell line, termed E6/7-lucA2, was tested first subcutaneously and then intravaginally. Monitoring of the genital region revealed the growth of tumors in the vaginal cavity. We also determined the minimal cell number needed for reliable tumor formation. Additionally, HPV16 epitope presentation was verified by immunopeptidomics, and specific killing of E6/7-lucA2 cells by HPV16-specific T cells was assessed. Concurrently to tumor model establishment, novel ways of eliciting an HPV16-specific T cell response in the genital mucosa were investigated. This was achieved either by directly inducing tissue-resident T cells on-site or by influencing the trafficking of vaccination-induced T cells towards the vaginal mucosa. Vaccine candidates were peptide-loaded nanoparticles, peptides encapsulated in liposomes or amphiphilic peptide constructs. The trafficking of T cells towards the vagina was achieved by utilizing immunomodulating substances or by induction of inflammation. These approaches will now be combined to tackle the previously established vaginal tumors.

Taken together, the new HPV16 orthotopic tumor model in MHC-humanized A2.DR1 mice will help to develop effective therapeutic HPV16 vaccinations by allowing to study the trafficking of T cells to the mucosal tumor site.

B003: Development of an arenavirus vector-based immunotherapy for prostate cancer
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Prostate cancer is one of the most common malignant neoplasms in men. Despite initially responding to androgen deprivation therapy, advanced disease inevitably progresses to castration-resistant prostate cancer, a patient population with a high unmet medical need. Immunotherapy with checkpoint inhibitors, such as anti-PD-1/PD-L1 has limited efficacy in prostate cancer, most likely due to its highly immunosuppressive nature and the lack of an effective T cell response.

Preclinical and clinical studies demonstrated that non-oncolytic, recombinant arenavirus vectors have the potential to dramatically enhance tumor antigen-specific T cell responses. To address whether these vectors can overcome peripheral tolerance to self-antigens in preclinical tumor models, we compared three replication-competent vectors based on lymphocytic choriomeningitis virus (LCMV) encoding either a foreign antigen (HPV16 E7E6), an antigen derived from an endogenous mouse retrovirus (gp70) and a self-antigen (Trp2). Tumor antigen-specific CD8 T cell responses were of similar magnitude in all three tumor models (TC1, MC38 and B16F10, respectively) after a single intravenous dose. In line with the strong CD8 T cells response, tumors were temporarily controlled, and the mean survival time was extended compared to control mice. These data provide a preclinical proof of concept that engineered arenavirus vectors can overcome peripheral self-tolerance and induce anti-tumor responses. To employ this platform for therapy of prostate cancer, vectors based on the two distantly related arenaviruses, LCMV and Pichinde virus (PICV), have been engineered to encode the human self-antigens prostate-specific antigen (PSA) and prostatic acid phosphatase (PAP). Antigen expression and immunogenicity were confirmed by Western blot and single-dose immunization of mice, respectively. Alternating prime/boost immunization with a PICV prime followed by an LCMV boost could further enhance PAP- and PSA-specific CD8 T cell responses.

Following preclinical development, this alternating 2-vector regimen will be tested for safety and tolerability in a Phase 1 clinical trial with the aim to define the recommended Phase 2 dose in metastatic castration-resistant prostate cancer patients who have progressed following standard of care.

B004: Therapeutic vaccine Pseudocowpoxvirus: Effects of cytokine arming
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Introduction: We have recently described Pseudocowpoxvirus (PCPV) as a novel poxviral vector to enhance efficacy of antitumor vaccination (Clinical and Translational Immunology 2022 doi: 10.1002/cti2.1392):  PCPV was selected for its capacity to efficiently induce IFN alpha secretion from human primary cells from healthy donors, as well as from cancer patients. PCPV produced efficient activation of human monocytes and dendritic cell, degranulation of NK cells and reversed MDSC-induced T- cell suppression, without being offensive to activated T cells. A PCPV-based vaccine, encoding the HPV16 E7 protein (PCPV-E7), stimulated strong antigen-specific T-cell responses in TC1 tumor-bearing animals. Complete regression of tumors was obtained in a $CD8^+$ T-cell-dependent manner after intratumoral injection of PCPV-E7, followed by intravenous injection of the corresponding Modified virus Ankara vaccine MVA-E7. Here we sought to further harness the PCPV vector inserting the gene for the cytokine (CYT), active in mouse and man, playing a role in T cell regulation. The vector PCPV-CYT encoding or not epitopes of the model antigen chicken Ovalbumin (Ova) were assessed *in vitro* in human and murine cells and in murine tumor models.

Experimental procedures: Recombinant PCPV vectors were generated by homologous recombination: the expression cassette for CYT was placed under the control of the vaccinia promotor pSE/L (PCPV-CYT). The virus PCPV-Ova encodes the epitopes of ovalbumin (MHC I: SIINFECKL, MHC II: ISQAVHAAHAEINEAGR, separated by GSG linkers) under control of the vaccinia promoter p7.5K. PCPV-CYT-Ova contains both expression cassettes. Murine splenocytes or PBMCs from healthy blood donors were isolated and infected with these viruses. Live NK and $CD8^+$ and $CD4^+$ T cells were identified by appropriate antibody staining. The percentage of live activated ($CD69^+PDL1^+$) cells within selected cell populations was measured by flow cytometry. IFN-gamma concentration in cell culture supernatant was determined by Luminex. Naïve C57BL/6 mice or mice with subcutaneously (sc) grafted MC38 or E.G7-Ova tumors were used in this study. Viruses were injected sc or intratumorally, tumor growth and survival were followed. For the detection of antigen-specific response, whole blood cell suspensions were labeled with fluorescently labeled peptide-MHC multimers. The presence of antigen-specific T cells *ex vivo* was studied by intracellular staining. Activation of NK and T cells in tumors and draining lymph nodes were followed.

Summary of unpublished data: Infection of murine splenocytes or human PBMCs with PCPV-CYT conferred an incremental gain of activation of NK and T cells compared to the empty PCPV vector. Arming of PCPV by CYT further increased anti-tumor efficacy of PCPV vectors in antigen-independent tumor model (MC38). Vaccination of naïve mice with PCPV-CYT-Ova allowed to detect more antigen-specific T cells 45 days after vaccination than with PCPV-Ova. Both vectors are very efficient in tumor control and increase of survival in the antigen-dependent tumor model (E.G7-Ova). Functionality of antigen-specific T cells will be presented.

Conclusions: Our results demonstrate further assets of a therapeutic PCPV vaccine vector: Arming with a suitable cytokine can further enhance efficacy. Homologous prime boost with PCPV vectors is efficient and well tolerated.

B005: Dendritic cell reprogramming of cancer cells restores tumor immunogenicity

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Immunotherapy changed the cancer treatment paradigm, but only a minority of patients respond. Growing evidence indicates that tumor immunosurveillance as well as the success of immunotherapy relies on effective presentation of tumor-associated antigens by conventional type 1 dendritic cells (cDC1). However, tumors employ multiple mechanisms to evade immunity including downregulation of antigen presentation and exclusion of cDC1s, leading to immunotherapy failure. Previously, we identified a combination of transcription factors, PU.1, IRF8, and BATF3 (PIB), which reprograms fibroblasts into cDC1-like cells endowed with the ability to present and cross-present antigens. Here, we hypothesize that cDC1 cell fate can be imposed directly on tumor cells, generating functional tumor-antigen presenting cells (tumor-APCs) able to reinitiate anti-tumor immune responses.

We demonstrated that expression of PIB induces expression of hematopoietic and cDC1-specific markers in a broad range of mouse and human tumor cell lines derived from leukemia and multiple solid malignancies. We further show that reprogramming restored the expression of antigen presentation complexes (MHC-I and MHC-II) along with the co-stimulatory molecules CD40, CD80 and CD86 at cell surface required for productive T cell activation.

Transcriptomic analysis using mRNA sequencing of 17 cell lines before and after reprogramming showed that PIB expression gradually overwrote cancer transcriptional program and imposed common antigen presentation and cDC1 signatures. Furthermore, step-wise mRNA sequencing and Assay for Transposase-Accessible Chromatin (ATAC) sequencing analysis revealed that PIB-mediated cDC1 reprogramming elicited rapid epigenetic remodeling followed by gradual rewiring of transcriptional program and stabilization of cDC1 identity. Mechanistically, we demonstrated that reprogramming factor engagement is dependent on histone acetylation. HDAC inhibition with valproic acid during the initial reprogramming phases increased cDC1 reprogramming efficiency and speed by facilitating PU.1 binding.

Functionally, tumour-APCs presented endogenous antigens on MHC-I and became prone to $CD8^+$ T cell mediated killing. Tumor-APCs secreted the pro-inflammatory cytokines IL12p70, TNFα, IL-29, and CXCL10 and adopted hallmark cDC1 functional features including phagocytosis of antigens and dead cells and the ability cross-present endogenous antigens to naïve CD8+ T cells. Importantly, intratumoral injection of reprogrammed tumor-APCs elicited

tumour growth control *in vivo.* Adoptive transfer of tumor-APCs is translated in higher frequencies of activated CD8+ T-cells and NK cells infiltrating B16-OVA tumors. Remarkably, alongside with antigen presentation and stabilization of cDC1 identity, tumor-APCs harboring *TP53, KRAS* and *PTEN* mutations downregulated proliferation and impaired tumorigenicity *in vitro* and *in vivo*. To support the translation of our approach, we showed that tumor-APCs were efficiently generated at the single cell level from primary cancer cells derived from melanoma, lung, breast, pancreatic, urothelial, and head and neck carcinoma as well as cancer-associated fibroblasts.

In summary, cDC1 reprogramming imposes cDC1 cell fate directly in a wide array of human and mouse cancer cells, generating functional tumor-APCs endowed with cDC1-specific functions and the ability to reinstate anti-tumor immunity. Our approach elicits the immune system against cancer and counteracts major tumor evasion mechanisms including tumor heterogeneity and impaired antigen presentation, laying the foundation for developing immunotherapeutic strategies based on cellular reprogramming of human cancer cells.

B006: Blockade of tumor derived CSF1 promotes an immune-permissive tumor microenvironment

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The macrophage colony stimulating factor 1 (CSF1) is a chemokine essential for the survival, proliferation, and differentiation of especially mononuclear phagocytes, such as macrophages and monocytes. However, within the tumor microenvironment, CSF1 has been shown to promote tumor progression partly by recruiting M2 macrophages. Overexpression of CSF1 also correlates with poor prognosis in breast, prostate, and ovarian cancer patients and has been shown to regulate tumorgenicity, invasiveness, and accelerate metastasis. Inhibition of the CSF1 receptor (CSF1R) is currently being evaluated clinically in multiple cancer types either as a monotherapy or in combination with checkpoint inhibition. In this study, we demonstrate for the first time, that treating established murine MC38 colon and 4T1 breast carcinoma tumors with a CSF1R blocking antibody promotes the expansion of neoepitope-specific T cells. CSF1R blockade also associates with an increase in CD8+ T cells within the tumor microenvironment. Combining CSF1R blockade with a therapeutic cancer vaccine associates with enhanced CD8+ T cell numbers within the tumor and correlates with a reduced tumor burden. CSF1 can be produced by multiple cell types within the tumor microenvironment; to assess the role of tumor derived CSF1 in these model systems, we generated and characterized CSF1 CRISPR-Cas9 knockouts in the 4T1 and MC38 carcinoma cell lines. In both models, elimination of tumor derived CSF1 results in a decreased rate of tumor growth in syngeneic animals, along with a coordinate increase of CD8+ T cells within the tumor microenvironment. We also observed an expansion of neoepitope-specific T cells in mice bearing $CSF1^{-/-}$ tumors as compared to animals bearing parental tumors. This data demonstrates that targeting the CSF1/CSF1R axis promotes the generation of an immune permissive tumor microenvironment and demonstrates the potential of combining this treatment regimen with therapeutic cancer vaccines to facilitate the generation of effective anti-tumor immunity.

B008: Extended length KRAS mutant peptides bind stably to MHC Class I molecule

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Robust methods to elucidate cancer antigens are the starting key for developing cancer vaccine and T cell therapy approaches. Traditionally, this involves the identification of MHC-I peptides as likely antigens guided by either direct characterization using HLA-based mass spectrometry (HLA-MS) or indirect computational predictions. More importantly, the low sensitivity of HLA-MS has ultimately led to several computational improvements in affinity- or stability-based NetMHC predictors and an outperforming MHCFlurry 2.0 predictor, which includes both affinity and antigen processing algorithms for 8- to 11-mers.

As part of our effort to advance MHC-I peptide discovery and antigen prediction, we have developed a peptide MHC-I (pMHC-I) complex stability assay, the EZ MHC-I assay performed in seconds (Lim, 2020). Here, the pMHC-I complex stability is directly measured. First, we have benchmarked its performance to identify peptide binders that are also immunogenic but remained unresolved by the modern NetMHCpan algorithm. Moreover, its R^2 correlation greatly improves from NetMHCstabpan to MHCFlurry 2.0, the current best-in-class predictor. Motivated by the experimental accuracies by the EZ MHC-I assay, we next seek to identify extended peptides > 10-mer that could be missed by even the MHCFlurry 2.0 approach.

Extended peptide MHC-I complexes are becoming an important class of antigens that may also augment CD4+ T cell responses. However, extended peptides are often missed *in silico* due to their unconventional length and unpredictable bulge-like binding conformations. With a specific focus on two KRAS mutations, G12C and G12D, we sought to measure pMHC stability with EZ MHC-I discovery engine with all possible 8-mer, 9-mer, 10-mer and 11-mer and 12-mer using a pool of 100 KRAS-derived peptides. The EZ MHC-I discovery engine was conducted in a blinded approach. We have already identified several potential antigens, which include canonical peptides that are in strong agreement with computational approaches. Of note, one of our hits (VVVGADGVGK), a 10-mer is a recurrent (KRAS G12D) neoantigen in cancer but is predicted to be a weak binder. More importantly, two extended 12-mer peptides that are as stabilizing as VVVGADGVGK were actually missed by both NetMHCpan and MHCFlurry predictors. As stable ternary pMHC-I complexes are needed to encounter an antigen-specific CD8 + T cells or to mount immunogenicity as peptide vaccines, even one new antigen could make a big difference.

Unbiased exome sequencing combined with more accurate MHC-I peptide measurements could potentially favor more neoantigens to be discovered and effective multi-epitope cancer vaccines to be developed. A complementary high-throughput, cost-effective approach to better depict actual pMHC-I complex biophysical stability can validate cancer neoantigen predictions as well as identify new antigens. The EZ MHC-I discovery engine creates new starting points such as extended peptides missed by current approaches and facilitates a more reliable peptide library that can serve as a bedrock to discover extended peptide reactive $CD8^+$ T cells. Overall, this assay, can support the discovery of extended $CD8^+$ T cell antigens as well as the future selection of cancer peptide vaccine candidates for any HLA-I allele.

B009: Vaccination targeting post-translational modification homocitrullination can induce CD8 T cell responses and confer anti-tumor immunity

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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, thereby resulting in altered immune responses. One such modification is homocitrullination that results in the formation of homocitrulline (Hcit) residues in a non-enzymatic reaction of cyanate with lysine residues in the polypeptide chain. We have shown that CD4-mediated immune responses to Hcit epitopes can target stressed tumor cells and provide a potent anti-tumor response in mouse models. In this study, we have identified Hcit peptides from aldolase and cytokeratin, and used human leukocyte antigen (HLA) transgenic mice to assess immunogenicity of the peptides by enzyme-linked immunosorbent spot (ELISpot) assay. Anti-tumor efficacy was assessed in tumor therapy studies by challenging HLA-A2 transgenic mice with B16F10 HHDII cells. To determine that the induced anti-tumor response was CD8-T cell driven, an anti-tumor study was performed along with CD8 T cell depletion. Modified peptides showed enhanced binding to HLA-A2 compared to the native sequences and immunization of HLA-A2 transgenic mice with Hcit peptide generated high avidity modification specific CD8 responses that possessed the ability to kill peptide expressing target cells. Importantly, *in vivo* the Hcit aldolase specific response was associated with efficient tumor therapy in the aggressive murine B16 tumor model indicating that this epitope is naturally presented in the tumor. The *in vivo* anti-tumor response was demonstrated to be CD8 T cell dependent. This is the first evidence that Hcit peptides can be processed and presented via MHC-I and targeted for tumor therapy. Thus, Hcit specific immune responses have the potential to be harnessed in the development of future anti-cancer therapy.

B011: cDC1 vaccines drive tumor rejection by direct presentation independently of host cDC1

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As a cell-based cancer vaccine, dendritic cells derived from peripheral blood monocytes or bone marrow (BM) treated with GM-CSF (GMDCs), were initially thought to induce antitumor immunity by presenting tumor antigens directly to host T cells. Subsequent work revealed that GMDCs do not directly prime tumor specific T cells, but must transfer their antigens to host DCs. This reduces their advantage over strictly antigen-based strategies proposed as cancer vaccines. Type 1 conventional DCs (cDC1s) have been reported to be superior to GMDCs as a cancer vaccine, but whether they act by transferring antigens to host DCs is unknown. To test this, we compared anti-tumor responses induced by GMDCs and cDC1 in *Irf8* $+32^{-/-}$ mice, which lack endogenous cDC1 and cannot reject immunogenic fibrosarcomas. Both GMDCs and cDC1 can cross-present cell-associated antigens to CD8 T cells *in vitro*. However, injection of GMDCs into tumors in *Irf8* $+32^{-/-}$ mice did not induce anti-tumor immunity, consistent with their reported dependence on host cDC1. In contrast, injection of cDC1s into tumors in *Irf8* $+32^{-/-}$

mice resulted in their migration to tumor-draining lymph nodes, activation of tumor-specific CD8 T cells, and rejection of the tumors. Tumor rejection did not require the *in vitro* loading of cDC1 with antigens, indicating that acquisition of antigens *in vivo* is sufficient to induce anti-tumor responses. Finally, cDC1 vaccination showed the abscopal effect of rejection of untreated tumors growing concurrently on the opposite flank. These results suggest that cDC1 may be a useful future avenue to explore for anti-tumor therapy.

B013: Metabolically armored CAR-T cells counter dysfunction and promote stemness for solid tumor clearance

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Chimeric antigen receptor (CAR)-T cell therapy has achieved a remarkable clinical success in treating B cell malignancies. However, CAR-T cells have so far shown limited efficacy against solid tumors and long-term disease control remains rare. One of the major factors that hinders the efficacy of CAR-T cell therapy is T cell dysfunction in tumors due to T cell exhaustion. Emerging evidence suggests that metabolic alteration and deficiency of intratumoral T cells drive T cell exhaustion. We recently reported that in vivo administration of an IL-10–Fc fusion protein reprograms intratumoral T cell metabolism towards OXPHOS, leading to potently enhanced expansion and effector function of terminally exhausted $CD8^+$ tumor-infiltrating lymphocytes (TILs). The metabolic reprogramming strategy could therefore be exploited to engineer metabolically armored CAR-T cell therapy to better treat solid tumors. Here, we designed metabolically armored CAR-T cells to counter exhaustion-associated dysfunction in the tumor microenvironment (TME) for enhanced anticancer immunity. CAR-T cells engineered to secret IL-10 preserved intact mitochondrial structure and function in the TME, and exhibited increased oxidative phosphorylation activities in a mitochondrial pyruvate carrier-dependent manner. IL-10 secretion promoted proliferation and effector function of CAR-T cells, leading to complete regression of established solid tumors and metastatic cancers in syngeneic and xenograft mouse models. Notably, infusion of IL-10-secreting CAR-T cells induced stem cell-like memory responses that imparted durable protection against tumor rechallenge. Our results establish a generalizable approach to counter CAR-T cell dysfunction through metabolic armoring, which may overcome a major barrier in the current adoptive T cell therapy in the clinic.

B014: TCR-engineered CD4 T cell transfer for optimized cancer immunotherapy

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While cancer immunotherapy has mainly focused on exploiting CD8 T cells given their role in the direct elimination of tumor cells, increasing evidence highlights the crucial roles played by CD4 T cells in anti-tumor immunity. However, their very low frequency, the lack of robust algorithms to predict peptide binding to MHC class II molecules and the particularly high

polymorphism of MHC class II molecules render the study and use of circulating tumor antigen-specific CD4 T cells challenging. In this regard, the HLA-DRB3*02:02 gene is encoding an HLA allele that is expressed by half of the Caucasian population, thus offering a way to identify CD4 T cell-defined tumor antigens with broad cancer patient coverage.

Here, we have screened HLA-DRB3*02:02 melanoma patients for tumor-specific CD4 T cell responses. We have observed strong NY-ESO-$1_{123-137}$/HLA-DRB3*02:02 CD4 T cell responses, both in the peripheral blood and in the tumor bed. By characterizing a large collection of NY-ESO-$1_{123-137}$/HLA-DRB3*02:02-restricted CD4 T cell clones we detected an unexpectedly high cytotoxic potency of these cells and a recurrent TCRαβ usage across patients and healthy donors, as well as at different anatomic localizations (blood and tumor). We cloned this TCR, transduced it in primary human CD4 T cells and we demonstrated its direct anti-tumor efficiency both in *in vitro* killing assays and in *in vivo* using NSG mice implanted with HLA-DRB3*02:02 melanoma cells.

Overall, we believe that the usage of this specific TCRs in ACT in the clinic represents a new, attractive, and safe immunotherapy for the large number of NY-ESO-1 expressing adult and pediatric cancers.

B015: Addition of PARP Inhibitor to Temozolomide Increases ULBP-1/MULT-1 and Increases Gamma Delta T-Cell-mediated Cytotoxicity

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INTRODUCTION: Drug Resistant Immunotherapy (DRI) is a novel cancer treatment strategy: cytolytic chemotherapy for bulk mass reduction and forced upregulation of stress-associated tumor Natural Killer Group 2D Ligands (NKG2DL) is combined with concurrent administration of ex vivo expanded and activated γδ T cells genetically modified for resistance to the cytolytic therapy employed. We have recently shown substantial benefit of DRI in mice bearing primary glioblastoma (GBM) xenografts, a strategy which is currently being evaluated for primary GBM in a Phase I clinical trial (NCT04165941). In the current *in vitro* study, we sought to determine whether the addition of the PARP inhibitor niraparib to the DNA alkylating agent temozolomide (TMZ) could further upregulate NKG2DL, thereby increasing the vulnerability of the targeted malignancy to expanded and activated γδ T cells modified for TMZ-resistance with a lentivirus vector expressing O^6 methylguanine methyltransferase (MGMT)

METHODS: GBM cells cultured under cancer stem cell maintenance conditions as neurospheres were isolated from two patient derived xenografts (PDXs) or a murine GBM cell line was used. Changes in viability and NKG2DL expression were determined after treatment with vehicle, TMZ (100mM), niraparib (5-15mM), or the combination. Transcriptional levels of NKG2DLs were evaluated by RT-qPCR, and changes were further validated at the protein level via flow cytometry. Using cells from a representative PDX that were pretreated for 24hrs with single and combined agents, a 4hr cytotoxicity assay with γδ T cells was performed. *Ex vivo* expanded/activated/MGMT-modifiedγδ T cells T cells were added at a 10:1 effector to target (E:T) ratio and viability determined using the nucleic acid stain SYTOX orange.

RESULTS: In the GBM neurospheres and murine cell line tested, treatment with niraparib, but not TMZ, significantly decreased cell growth at 72hrs ($p<0.001$). The addition of TMZ to niraparib significantly decreased cell growth in comparison to the vehicle control in all cells ($p<0.0001$) and significantly decreased cell growth in comparison to either drug alone in two of the three cell types tested ($p<0.05$). Importantly, decreases in viability did not always correlate with increases in NKG2DL mRNA. There was a significant increase in ULBP-1/MULT-1 mRNA upon treatment with TMZ and niraparib in comparison to the single agents in two of the three cell types tested. Treatment with the combination also led to increases that were not statistically significant in the mRNA of several NKG2DL (MICA, MICB, ULBP2, ULBP5) in one of the GBM neurospheres. In these cells, cytotoxicity in the presence of γδ T cells was significantly increased upon pretreatment with TMZ and niraparib in comparison to the control or either drug alone: the percent change was an approximately 30% increase in cytoxicity with the combination. Microscopy revealed γδ T cells localized to neurospheres and cytolysis was observed.

CONCLUSION: Combined PARP inhibition, DNA alkylation, and γδ T cell therapy represents a promising therapeutic approach for GBM and extracranial tumors that warrants additional investigation.

B016: Evaluation of CAR-T cells targeting CD276 in medulloblastoma

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Brain tumors are the most frequent category of solid tumors in children and have the highest mortality rate among all pediatric cancers. Although diagnosis and treatment have improved prognosis over the past decades for some childhood brain tumors, others remain lethal and current treatments are highly toxic to the developing brain, resulting in severe sequelae and considerably affecting the patient's quality of life. Thus, new therapeutic options with reduced secondary effects are urgently needed.

From this perspective, immunotherapies have gained a lot of attention due to their effectiveness in targeting tumor cells specifically. Chimeric-antigen receptor (CAR) T-cells recognize the target antigen on the surface of. CD276 is an immune checkpoint molecule that is expressed in a variety of solid tumor entities, including pediatric brain tumors. We analyzed the CD276 expression in our Patient-Derived-Xenograft (PDX) biobank of brain tumors and found that CD276 is ubiquitously expressed (Atypical Teratoid Rhabdoid Tumor-ATRT, Medulloblastoma-MB, Ependymoma-EPN, Gliomas, Embryonal tumor with Multilayered Rosettes-ETMR, etc).

Flow cytometry of MB PDX (n=4) confirmed CD276 expression of 97-99% of tumor cells, indicating that CD276 might be a good antigen target for CAR-T cell therapy of MB_{G3} and MB_{SHH} subgroups. We found that second generation CAR-T cells (CD28) targeting CD276 antigen significantly decreased tumor burden of the most aggressive MB subgroups (G3 and SHH-TP53mut PDX models) in NSG mice. We further treated NSG mice carrying a high tumor burden of the aggressive SHH-TP53 mut PDX BT084 with second (CD28) and third generation (CD28-41BB) CD276-CAR-T cells. While both 2nd and 3rd generation improved the survival rates compared with CD19-CAR-T control cells, we found no difference in survival between the CD276 CAR-T generations, with no severe secondary effect during treatment. In conclusion, CD276 is a good antigen target for medulloblastoma, and warrants further evaluation for the treatment of medulloblastoma patients at relapse or as a maintenance therapy after standard treatment.

B017: Complementary determining region clustering causes CAR-T cell dysfunction

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Chimeric antigen receptor (CAR)-T cells are successful against hematologic malignancies. All parts of the CAR-molecule are important for CAR-T cell functionality. This include the extracellular single chain variable fragment (scFv), the linker, transmembrane and intracellular signaling domains. The complementarity-determining region (CDR) loops of the scFv determines the antigen specificity while the framework impacts CAR stability. It has previously been found that the scFv of the CAR can mediate clustering of CAR-molecules and thereby induce antigen-independent tonic signaling. CAR clustering has been attributed to the framework region of the scFv. We report for the first time that the CDR loops of the scFv can also mediate CAR clustering and induce antigen-independent tonic signaling, which led to CAR-T cell dysfunction.

Five different IL13Rα2-specific scFvs, which only differed in the CDR loop sequences, were used to generate CAR-T cells. One construct exhibited antigen-independent T cell activation, as evident by increased cell size, interferon-γ (IFN-γ) release, phenotypical exhaustion and apoptosis. Mutagenesis in the CDR loops of this construct prevented CAR clustering and antigen-independent tonic signaling. In summary, we highlight the importance of the CDR loops of the scFv in driving CAR clustering and thereby antigen-independent tonic signaling. We also propose that evaluation of cell size and IFN-γ release of unstimulated CAR-T cells could be used for large-scale screening for tonically signaling CAR-T cells.

B018: Improving precision and safety of CAR-T cell therapy for Acute Myeloid Leukemia using synthetic Notch receptors

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The presence of tumor-specific antigens is critical for the success of Chimeric Antigen Receptor (CAR) T cell therapy. Many pediatric cancers lack unique antigens and are therefore considered high risk for on-target off-tumor toxicities from CAR-T cells. In acute myeloid leukemia (AML), most targetable antigens are expressed on hematopoietic stem and progenitor cells (HSPCs). Potential myeloablation resulting from the use of conventional CAR-T cells remains a significant obstacle to the development of CAR-T cells for AML. Recently, combinatorial targeting approaches have been developed to improve the precision and safety of CAR-T cells. As such, a synthetic Notch (synNotch) receptor was engineered to recognize the first antigen, thereby activating the expression of a CAR protein that target a second antigen. This "gated" approach improves specificity by requiring both antigens to be present, therefore limiting toxicity to normal cells. We developed a logical synNotch circuit that recognizes the combination of two antigens overexpressed on AML cells: CD33 and CD123. Primary human T cells were genetically engineered to express anti-CD33 synNotch receptor that transcriptionally regulates CAR against CD123. Phenotype analysis by flow cytometry revealed that the synNotch circuit prevents T cell differentiation and maintains T cell stemness. In comparison to conventional CAR-T cells, co-expression of exhaustion markers was significantly lower in gated CAR-T cells, suggesting that these gated CAR-T cells possess superior fitness. Gated CAR-T cells demonstrated antigen-specific cytotoxicity *in vitro* against CD33+CD123+ AML cell lines and AML patient samples, secreting cytotoxic cytokines such as TNF-α or IFN-γ. *In vivo* studies also showed that gated CAR-T cells were effective against AML cell lines and significantly prolonged survival. Safety was assessed by colony-forming unit (CFU) assays with human CD34+ HSPCs cocultured for 24 hours with CD33-CD123 gated or CD123 conventional CAR-T cells. While CD123 conventional CAR-T cells eradicated colony formation, CD34+ HSPCs obtained from three different donors and cocultured with gated CAR-T cells formed as many colonies as CD34+ cells cocultured with untransduced T cells. Finally, using a model of humanized mice engrafted with human CD34+ cells (huCD34-SGM3), we showed that conventional CAR-T cells impacted normal cells and caused cytokine release syndrome (CRS); however, gated CAR-T cells had little effect and protected mice from CRS. In summary, we generated effective CD33-CD123 gated CAR-T cells that can improve the precision and safety of CAR-T cell therapy by preventing hematologic toxicities and overcome the challenges of this therapy in AML.

B020: An innovative long-term re-challenge model for in vitro evaluation of survival and exhaustion of modular reverse chimeric antigen receptors

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T cells genetically modified to express chimeric antigen receptors (CARs) have shown encouraging outcomes in the treatment of B cell malignancies. Despite initial clinical efficacy, the adoptive transfer of conventional CAR T cells (conCAR-T) is often limited by serious side effects and lack of long-term disease control. Reasons for the latter include tumor heterogeneity and limited survival of the conCAR-T. While conCAR-T directly and continuously target the tumor antigen, our modular reverse CAR (RevCAR) platform separates tumor recognition and T cell activation. RevCAR-T are equipped with an inert CAR that is exclusively activated when crosslinked to a tumor cell through a bi-specific soluble adapter molecule (targeting module,

TM). By separating tumor recognition and T cell activation, the RevCAR platform enables flexible targeting of various antigens with a single T cell product. Additionally, by ceasing administration of the TM, RevCAR activity can be rapidly switched off and back on in case of disease relapse. Despite overcoming multiple limitations of conCAR-T, the long-term persistence of RevCAR-T requires further investigation.

To our knowledge, evaluation of conCAR-T persistence thus far lacks suitable profound *in vitro* assessment and is primarily performed *in vivo.* However, *in vivo* testing of human conCAR-T grafts in immunocompromised mice has several pitfalls that potentially confound results. Hence, we sought to establish an innovative model for in-depth analysis of RevCAR-T persistence *in vitro* to provide additional predictive value. For that purpose, we repeatedly stimulated RevCAR-T with fresh cancer cells for four days and alternatingly let the RevCAR-T rest for three days. This re-challenge was performed for a total of 18 days. After each cycle, RevCAR-T were intensively characterized regarding functional persistence using co-culture experiments and T cell phenotype. Since CAR-T persistence is primarily influenced by T cell survival and exhaustion, we examined the impact of multiple distinct intracellular signaling domains (ISD) on long-term persistence of RevCAR-T. We designed eight unique RevCAR variants comprising CD28, 4-1BB and/or ICOS costimulatory domains followed by CD3ζ. We investigated 2nd generation (2G: 28ζ, ICOSζ), 3rd generation (3G: 28BBζ, ICOSBBζ) and lastly 5th generation (5G: 28IL2ζ, 28IL7ζ, IL728ζ) RevCAR-T. Engineered T cells were manufactured by lentiviral transduction of primary T cells of five independent healthy donors yielding comparable transduction efficiencies of 60-70 %. Re-challenging RevCAR-T showed that expansion of RevCAR-T was improved for 3G and 5G RevCAR-T. Intriguingly, activation of RevCAR-T bearing an ICOSBBζ CAR signaling domain increased over time and is delayed compared to other RevCAR-T variants. Functional assessment at the end of each cycle revealed 3G RevCAR-T (28BBζ and ICOSBBζ) as most functionally persistent. In depth phenotypic characterization identified both decreased CD25 and consequently decreased PD1 expression and lower terminal memory differentiation as potential indications of retained persistence for 3G RevCAR-T.

Taken together, we established a novel and profound *in vitro* model enabling us to investigate a comprehensive range of RevCAR-T variants avoiding excessive number of animals used for *in vivo* trials. In depth *in vitro* evaluation indicates prolonged cytotoxic potential of 3G RevCAR-T accompanied by fewer features of exhaustion. We hypothesize that 3G RevCAR-T will display higher persistence in an *in vivo* evaluation compared to 2G RevCAR-T.

B021: Developing Flt3 ligand secreting chimeric antigen receptor T cells for the clinic

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Adoptive cell therapy using chimeric antigen receptor (CAR) T cells is a form of immunotherapy where T cells are genetically modified to recognize tumor antigens and specifically target cancer. While CAR T cells have achieved remarkable therapeutic efficacy for some blood cancers, its effect against solid cancers has remained limited. One confounding issue is the variable expression of target antigens on solid tumors (i.e. tumor antigen heterogeneity). CAR T cells may eliminate antigen-expressing but not antigen-negative tumor cells, which can consequently lead to disease relapse involving the latter. We have previously shown that CAR T cells engineered to secrete the dendritic cell (DC) growth factor Fms-like tyrosine kinase 3 ligand (FL) induces DC proliferation in tumors, leading to enhanced host T cell responses against antigen-negative tumors (i.e. epitope spreading) when combined with immune-stimulatory adjuvants. Here, to translate our findings into the clinic, we have successfully generated human FL-secreting CAR T cells targeting the tumor antigens CD19, as well as Her2 and Lewis-Y. Concurrently, we are exploring a number of strategies that synergizes with FL-secreting CAR T cells to further enhance DC recruitment and activation. Our work suggests that engagement of host anti-tumor immunity represents a promising strategy to improve the overall efficacy of CAR T cell therapy against solid tumor antigen heterogeneity, which may help to combat the clinical problem of antigen-negative tumor relapse following therapy.

B022: SUV39H1 disruption reprograms CAR T cells 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 expansion and persistence. Both, resistance to treatment and tumor relapses reduce the efficacy of CAR T cells, especially against solid tumors. SUV39H1, a histone 3, lysine 9 methyltransferase represses stemness and memory genes in $CD8^+$ T cells in a *Listeria monocytogenes* infection mouse model. Here, we investigate how depletion of SUV39H1 affects human CAR T cell phenotype, anti-tumor efficacy and persistence in immunodeficient mice. Our results show that SUV39H1 gene disruption induces expression of stemness genes and enhances memory phenotype on human CAR T cells. In an orthotopic lung human tumor model, SUV39H1-deficient CAR T cells reject established tumors better than SUV39H1-sufficient CAR T cells, displaying stronger persistence and protection against relapses. Single-cell RNA-seq and single-cell ATAC-seq analyses of tumor infiltrating CAR T cells revealed increased chromatin accessibility and expression of stem/memory genes in SUV39H1 deficient T cells at early time points, and increased persistence of a stem/memory, self-renewing, population. This stem-like SUV39H1 KO CAR T cells persist in lung and spleen, protecting mice against tumor re-challenges, even several months after infusion. SUV39H1 deficient mRNA CAR T signature correlates with effective clinical response to CAR T cells and immunotherapy in general. Altogether, our findings demonstrate the potential of epigenetic reprogramming by SUV39H1 depletion for adoptive T cell therapies.

B024: Prerequisites of an efficient adoptive T cell transfer in the B16 melanoma model

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A complex network of pro-and anti-tumorigenic factors within the tumor microenvironment determines tumor rejection and persistence. Tipping the balance towards tumor eradication requires precise therapeutic strategies and multi-pronged approaches. We investigated the prerequisites of virotherapy employing engineered arenavirus vectors for breaking immune tolerance to the melanoma self-antigen TRP2 in a syngeneic mouse tumor model. Replication competency was identified as a key discriminator for the induction of TRP2-specific CD8 T cell responses and anti-tumor efficacy. Despite initial control of tumor growth, most tumors eventually relapsed in this monotherapy setting. Combining adoptive transfer of TCR transgenic TRP2 specific CD8 T cells and a single dose of replicating arenavirus vectors, however, synergized and resulted in complete tumor eradication in 100% of mice. Of note, the combination therapy was successful without lymphodepletion or administration of IL-2, a standard regimen for adoptive T cell therapy. A significant increase of TRP2-specific CD8 T cells with lower PD-1 expression was observed in the tumor upon combination therapy with replicating but not with non-replicating vectors. These results demonstrate for the first time the requirements for and potential of combining two clinically explored therapies, namely active immunization with arenaviral vectors and adoptive T cell transfer.

B025: Synthetic receptors enabling soluble signal sensing in engineered cell-based immunotherapies

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The challenge of differentiating tumor cells from healthy tissue remains one of the foremost obstacles in expanding the efficacy of cell-based immunotherapies beyond the scope of hematological malignancies. Despite significant efforts in antigen discovery, few cell surface targets have been found to be uniquely expressed by cancer cells. The ability to integrate multiple environmental signals would enable engineered T cells to restrict their activity to highly specific locales, enabling more efficacious dosing regimens and limiting potential toxicity. Toward this aim, the Synthetic Notch Receptor (SynNotch) enables sequential AND gating when paired with a Chimeric Antigen Receptor, restricting CAR-T cytotoxicity toward cells expressing both the CAR and SynNotch antigens. However, several of the most broadly conserved

molecular targets across solid tumor types are secreted soluble factors which fail to trigger SynNotch due to its mechanosensitive mode of activation.

Recently, a next-generation receptor platform dubbed the SyNthetic Intracellular Proteolysis Receptor (SNIPR) has been developed. Like SynNotch, this receptor family is able to couple ligand-mediated activation to custom transcriptional output; however, its structural modifications impart additional useful properties including compactness and a tunable activation profile. Notably, SNIPRs are capable of responding to secreted proteins in addition to conventional cell surface ligands. Here we demonstrate that SNIPRs can respond to key tumor-relevant secreted targets including TGF-β, and that SNIPR/CAR genetic circuits can integrate extracellular and membrane-bound target signals to precisely localize CAR-T activity to solid tumors in models which display significant toxicity upon treatment with constitutive CAR-T cells. These receptors may provide a route to design broadly tumor-restricted safety circuits, thereby enhancing the ability of engineered immune cells to combat cancers that are currently refractory to immunotherapeutic treatment.

B026: Novel human EGFRvIII-specific CAR T cells completely eliminate glioblastoma tumors

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Glioblastoma is an aggressive and malignant brain tumor. Cases of glioblastoma account for approximately 60% of all adult brain cancer diagnosis and five-year survival rates are estimated at only 5%. Consequently, new, and targeted treatments are required to enhance patient survival, beyond the 14-months provided by the current standard of care treatments. We have developed a novel target discovery platform using cell surface proteomics that will be discussed, and we have discovered several novel protein targets for glioblastoma. In addition, we present our work validating a new Chimeric Antigen Receptor (CAR) against the Epidermal Growth Factor Receptor (EGFR) truncation mutation, Variant III (EGFRvIII) which is one of very few identified tumor specific mutations, and is expressed in a subset of glioblastoma tumors, providing a safer target than antigens with co-expression on healthy tissue.

CAR T cells have revolutionized the treatment of many types of hematological malignancies. This therapy provides a patient's own immune cells with a synthetic receptor; specific for a target antigen, enabling recognition and lysis of antigen expressing malignant cells. CARs specific for EGFRvIII have been developed and have been generated from re-deriving pre-existing monoclonal antibodies. We have previously published on the development and characterization of a novel high affinity EGFRvIII specific CAR (GCT02) in primary murine T cells. This construct was found to have an approximate 300-fold higher affinity than the previously published constructs and demonstrated in vitro and in vivo efficacy. We now extend this work and demonstrate the function of primary human T cells engineered with this construct.

These human GCT02-CAR T cells demonstrated comparable target specific cytotoxicity to the clinical benchmark CAR Clone 2173 EGFRvIII scFv (an EGFRvIII-specific single chain being currently used in clinical trials) against three glioblastoma cell lines engineered to express EGFRvIII. An analysis of the secreted cytokine profile revealed the $CD8^+$ GCT02 CAR T cells to secrete comparable quantities of effector cytokines such as IFN-γ and IL-2. However, the $CD4^+$ GCT02 CAR T cells secreted a reduction of less desirable inflammatory cytokines such as TNF-α and a loss of IL-4 release compared to the CAR containing the 2173 scFv clone.

In two orthotopic in vivo models of glioblastoma, a single intravenous infusion of GCT02 CAR T cells was found to mediate elimination of implanted glioblastoma cells two weeks post treatment in both models. An analysis of infiltrating T cells in one of these models demonstrated the presence of infiltrating T cells within the brain, in the critical window before complete tumor regression.

Finally, we have performed a safety screen against primary human keratinocytes, known to express high levels of the EGFR protein, and found our GCT02 CAR T cells to display a favorable safety profile.

In summary, we have characterized the high affinity EGFRvIII-specific CAR; GCT02 in primary human cells. The GCT02 CAR was found to have effective anti-tumor functions in vitro and in two in vivo orthotopic models of glioblastoma. Furthermore, safety testing demonstrates this construct to likely to safe against human tissue with high EGFR expression.

B027: CD276 CAR T cells and CD276/FGFR4 Dual CAR T cells eradicate rhabdomyosarcoma in an orthotopic mouse model

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Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma of childhood. The most frequent RMS subtypes are fusion-negative RMS (FN-RMS; 60-70%) and fusion-positive RMS (FP-RMS; 20-30%). Current cancer treatments have contributed to improve survival in patients, but are insufficient for metastatic RMS and the more aggressive FP-RMS. Therefore, there is urgent need of new therapies for patients with high-risk and recurrent solid tumors.

Chimeric Antigen Receptor (CAR) T cells are a promising immunotherapy based on *ex vivo* engineering of T cells from patients to confer T cells MHC-independent anti-tumor activity via cytokines release.

So far, six CAR Ts therapies have been approved to treat hematological malignancies. Despite this remarkable success, CAR Ts application in solid tumors remain challenging, mainly due to a lack of ideal tumor specific targets, and to the immunosuppressive tumor microenvironment. One strategy to improve CAR Ts efficacy in solid tumors aims at using expressing CARs against multiple targets (dual-CAR, bi-specific CAR, tandem CAR).

To identify novel RMS targets, we performed a surfaceome profiling of eleven RMS cell lines and three RMS PDXs. Among the identified RMS surface proteins, we detected the RMS targets CD276 and FGFR4, validating our approach. CD276 CAR Ts have already shown good pre-clinical activity against several pediatric sarcomas, but have not been tested against RMS. FGFR4 is a well-known RMS specific antigen, but FGFR4 CAR Ts have so far shown limited activity against orthotopic RMS mouse models.

Since each CAR domain plays a functional role in enhancing CAR Ts activation, persistence, and tumor eradication, we generated a pool of eight different CD276-CAR constructs, with Hinge/Transmembrane (HD/TM) domains deriving from either CD8α or CD28 proteins; and CD28 and/or 4-1BB as CSDs of second- and third-generation CARs. Moreover, to address RMS heterogeneity, we generated Single- and Dual-CAR Ts targeting CD276 and FGFR4. CD276 was targeted by the humanized CD276.MG-scFv, whereas FGFR4 by the F8-nanobody selected from four FGFR4-nanobodies previously identified in our lab. Their activity was compared *in vitro* and *in vivo* in an orthotopic RMS mouse model.

Methods

T cells isolated from three different donors were transduced with lentiviruses to generate CD276- and FGFR4-CAR Ts. CARs expression was validated by Western Blot and FACS. Cytotoxicity of CAR Ts was determined at day 14 of expansion by 48h co-incubation with firefly luciferase positive (fLuc) RMS cells. Cytokines release assays were performed to detect IL-2 and IFN-γ. For the orthotopic RMS mouse model, $5x10^5$ FP-RMS Rh4 cells were injected into the gastrocnemius muscle of NSG mice. 5 days after tumor inoculation, the mice were treated with $5x10^6$ CAR Ts, and tumor growth was monitored by caliper measurement and bioluminescence.

Results

Killing and Cytokine Release Assays revealed CD276-CD28HD/TM.CD28.3ζ (276.28.28.z) as the CAR construct showing the highest killing capacity against FP-RMS and FN-RMS cell lines. Keeping the 28.28.z CAR backbone, we generated four FGFR4-CAR Ts and selected the most specific and effective F8-nanobody based CAR. CD276- and F8-CAR Ts were tested *in vivo* in an orthotopic Rh4 RMS mouse model. Treatment with 276.28.28.z resulted in complete tumor eradication in 5/5 mice. F8.28.28.z CAR Ts achieved a significant delay in tumor growth but showed lower efficacy than CD276-CAR Ts. Dual-CAR Ts targeting both CD276 and FGFR4 showed complete tumor remission in 5/5 mice and no side effects.

Conclusions

These results show the promising therapeutic potential of CD276-CAR Ts for RMS and reveal the capacity of F8-CAR Ts targeting FGFR4 to delay RMS growth. The efficacy of F8-CAR Ts needs to be improved. The Dual-CAR strategy showed that co-expression of CD276 and FGFR4 CARs does not impair CD276 tumor controlling potential and that it represents a valuable strategy to address the heterogeneity of RMS.

B028: Humanized CD30 chimeric antigen receptor T Cells with novel 4-1BB derived spacer have improved activity and safety against CD30 positive lymphoma models

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Autologous T cells expressing chimeric antigen receptors (CARs) against CD30 have produced promising clinical efficacy in the treatment of relapsed or refractory CD30 positive classical Hodgkin lymphoma (cHL) patients. Similarly, we have treated nine CD30-positive lymphoma patients in an ongoing Phase 1 study (NCT04288726) with allogeneic Epstein-Barr virus specific T cells (EBVST) expressing a murine HRS3 antibody derived CD30 specific CAR. The allogeneic CD30.CAR EBVSTs showed promising signs of efficacy with a favorable safety profile and no evidence of graft versus host diseases (GVHD). However, the allogeneic CD30.CAR EBVSTs showed low persistence in the peripheral blood of patients, with cells not detected in most patients seven days post infusion. In this study we seek to improve the persistence of the CD30.CAR EBVSTs with the humanization of the murine HRS3 scFv to minimize immunogenicity, and the replacement of the current IgG1 spacer with a novel 4-1BB derived spacer to eliminate any off-target interactions. We show that CD30.CARs with the humanized scFvs exhibited improved stability, preserved specificity and efficacy as compared to the parent HRS3 CAR. Here we also describe the use of a novel spacer domain derived from 4-1BB (CD137). The 4-1BB spacer did not exhibit any nonspecific interactions with $CD16^{+}$ immune cells, displayed improved efficacy in vitro, improved persistence *in vivo* in various humanized mouse models and more importantly an improved safety profile in a leukemia model with high tumor burden. Thus, we believe the re-engineered CD30.CAR can potentially improve allogeneic CD30.CAR EBVST performance.

B030: Compound-triggered degradation switches for the regulation of engineered cell therapies

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A principal challenge in increasing the efficacy of chimeric antigen receptor (CAR) T cell therapy is preventing T cell exhaustion. Although previously postulated to be an irreversible state, recent studies have shown that exhausted T cells can be rescued by transient rest where their CAR levels are down-regulated. Efforts to control CAR expression have included fusion with compound-triggered degron motifs derived from known neo-substrates of the E3 ubiquitin ligase $CRL4^{CRBN}$. Moreover, controlled production of various cytokines is also known to increase the tumor-killing ability of T cells. For the simultaneous regulation of several proteins using a single compound, we have designed multiple orthogonal sets of compound-inducible switches of varying switch characteristics. To create OFF and ON switches, we have designed artificial degron tags that can be fused to the protein of interest and either get degraded or stabilized upon compound treatment with drugs that are approved or in advanced trials.

We generated novel degrons using computational design to bind the E3 ligases $CRL4^{CRBN}$ and $CRL4^{DCAF15}$ at higher affinities than known neo-substrates. Libraries of designed switches were

screened using flow cytometry and hits were validated using cellular degradation assays and *in vitro* time-resolved Forster resonance energy transfer (TR-FRET). In Jurkat cells, designed OFF switch degrons required as little as one-fiftieth the concentration of the compound E7820 to be degraded compared to known neo-substrate, RBM39. Proteins fused to ON switch degrons were stabilized by up to two-fold upon compound addition. Moreover, we demonstrate the simultaneous up- and down-regulation of two proteins using a single compound. In addition, degrons of five new protein folds were designed that are degraded via $CRL4^{CRBN}$ upon addition of different immunomodulatory imide compounds. Thus, we have built a library of switches of various strengths and polarities. Being post-translational, the regulation is rapid, reversible, and dose-dependent. Moreover, having switches triggered by different compounds gives us the flexibility to choose the best switch to control the levels of CARs, cytokines, and inhibitory elements such as PD-1, TIM-3, and LAG-3.

B031: Development of a novel neoantigen-reactive CD8+ T cell adoptive cell transfer (ACT) model in murine B16F10 melanoma

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Adoptive cell transfer (ACT) of tumor-reactive $CD8^+$ T cells has had some success in the clinic but is hampered by the immunosuppressive tumor microenvironment (TME) and limited understanding of what constitutes an ideal, targetable antigen. Neoantigens (NeoAgs) are derived from genomic alterations and can be highly immunogenic peptides uniquely presented by tumors, while tumor-associated antigens (TAAs) tend to be less immunogenic, and are expressed highly, but not exclusively, by tumors. Murine models of anti-tumor $CD8^+$ T cell immunity in the context of artificially introduced antigens and TAAs are widely used, but this fails to recapitulate neoantigen-reactive $CD8^+$ T cell immunity. To address this gap, we developed a model of neoantigen-reactive $CD8^+$ T cell immunity in the immunotherapy-resistant murine melanoma tumor, B16F10. Charge-modified TLR7/8 conjugate neoantigen vaccines were then constructed and administered to tumor-bearing C57BL/6 mice. Neoantigen vaccination induced neoantigen-reactive $CD8^+$ T cell immunity and modest tumor growth control. T cell receptors (TCRs) were isolated from T cell clones; from this, a high affinity TCR reactive against a neoepitope from the transcription factor heat shock factor 2 (Hsf2) was identified. Importantly, we also utilized the same vaccination and TCR cloning pipeline to yield TAA-reactive TCRs to study in tandem. Retroviral transduction of neoantigen- or TAA-reactive TCRs of murine $CD8^+$ T cells was performed to create neoantigen- or TAA-reactive $CD8^+$ T cells, respectively. Collectively, our neoantigen- and TAA-reactive $CD8^+$ T cells span the spectrum of affinity/avidity towards target peptide-MHC complexes. All T cells were determined to recognize B16F10 tumor *in vitro.* Co-culture of B16F10 and T cells revealed tumor antigen expression and TCR avidity as determinants of T cell killing (IncuCyte assay), in which optimal killing of tumor was achieved in scenarios of dually high tumor antigen expression and TCR avidity. Further, significant reduction in tumor growth was observed upon transfer of neoantigen- or TAA-reactive $CD8^+$ T cells to mice bearing tumors that abundantly expressed antigen. In

summary, we have developed a novel model of neoantigen-reactive $CD8^+$ T cell immunity in B16F10, an immunotherapy-resistant tumor. Our model enables comparison of attributes of neoantigen- and TAA-reactive $CD8^+$ T cells; future research will center on understanding the hierarchy of antigen abundance, TCR avidity, and other parameters, in influencing ACT outcomes.

B032: Combination of EGFRvIII-targeting CAR T cells and concurrent Pembrolizumab in de novo GBM disrupts PD1 correlation with engraftment

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Glioblastoma is an aggressive, malignant brain tumor with no definitive cure. While combination therapies, such as temozolomide and radiation, have shown an increase in survival, patients still do not survive long, with a median overall survival of 14.6 months. With that in mind, this study elucidated the effect of chimeric antigen receptor T (CAR-T) cells to combat GBM. CAR-T cells are genetically engineered T cells designed to target desired antigens via production of an artificial T cell activating receptor complex. Initially, the effect of programmed cell death protein 1, or PD1 - an inhibitory protein expressed by T cells to keep the immune response in check during periods of inflammation or injury - alongside CAR-T cells was studied. Given that PD1 offers a protective factor for cells against T cell attacks by decreasing the interaction time between cytotoxic T lymphocytes and target cells, PD1 is implicated in playing a vital role in immunotolerance and T cell exhaustion, making it a desired target in cancer treatment. Using the mutated EGFRvIII protein as our target and PD1 as a readout of CAR-T cell phenotypes, patients with de novo GBM were given three infusions of CAR-T cells with concurrent administration of pembrolizumab and their apheresis and infusion products analyzed using flow cytometry. Initially, we found a strong, positive correlation between PD1 expression and both area under the curve (AUC) ($r=0.7849$) and progression-free survival (PFS) ($r=0.8004$) in a previous trial involving recurrent GBM patients who received only one infusion of CAR T cells. In this study, however, no such correlations were seen within these parameters whatsoever. In addition to AUC ($r=-0.4069$) and PFS ($r=-0.2025$), the correlation between PD1 expression and overall survival (OS) ($r=0.1957$) was explored, and all three were found to be weak. These findings may imply a causative rather than correlative factor for the relationship between PD1 expression and CAR-T cell engraftment. Given that patients were simultaneously infused with pembrolizumab and CAR-T cells, infusing patients with both may have been a setback since pembrolizumab may be acting on CAR-T cells as well as cancer cells and inhibiting their function when targeting the immunosuppressive PD1 protein. While there was no correlation, we believe that there is significance in the lack of association that warrants further exploration and that the concept of injecting CAR-T cells concurrently with pembrolizumab may need to be revised.

B033: A modular strategy for targeting pancreatic cancer with nanobody-based CAR macrophages and T cells

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While chimeric antigen receptor (CAR) T cells have had clinical success in treating hematological cancers, their use against solid tumors has proved challenging. The extracellular portions of conventional CARs are modeled after antibody-derived single-chain variable fragments (scFvs), and these receptors can be prone to improper folding and aggregation and may require extensive optimization for proper cell surface expression in addition to their high immunogenicity. The Ploegh laboratory has recently developed CARs that use alpaca antibody-derived nanobodies, which are smaller, are displayed more efficiently at the cell surface, and are less immunogenic than scFvs, as antigen recognition components to address these limitations. Notably, previous work showed that nanobody-based CAR (nano-CAR) T cells can effectively kill B16 melanoma allografts in immunocompetent mice. Building on this work, the current study focuses on the development of nano-CAR macrophages capable of antigen-dependent phagocytosis to further diversify and enhance adoptive transfer strategies to combat solid tumors. In particular, pancreatic ductal adenocarcinoma (PDAC) is one of the deadliest cancers and is resistant to chemo- and immuno-therapies, owing to protection by dense, encasing stroma. Through use of nanobodies that target extracellular matrix components that are highly and selectively expressed by PDAC cells and their surrounding milieu, we aim to turn this protective stroma into a targetable vulnerability. Also, given that pancreatic tumors have an abundance of myeloid cells and express high levels of myeloid cell chemoattractants, as is common for a range of solid cancers, CAR macrophages may have an advantage over CAR T cells in infiltrating and persisting within the PDAC tumor microenvironment. Nano-CAR macrophages generated from the J774 mouse cell line or primary bone marrow-derived macrophages through lentiviral transduction demonstrate markedly enhanced phagocytosis of antigen-coated microbeads and killing of human PDAC cells. Furthermore, we are testing whether the inclusion of endodomains from pattern recognition receptors in macrophage nano-CARs can couple the activation of pro-inflammatory signaling pathways with target cell phagocytosis. Pertinently, such endodomains may trigger the production of type I interferons, which enhance T cell proliferation and cytotoxic activity as well as stimulate production of lymphocyte chemoattractants by macrophages through autocrine signaling. Thus, while our preliminary results show that nano-CAR T cells demonstrate tumoricidal activity against PDAC xenografts in mice, nano-CAR macrophages may work synergistically with these modified T cells by providing them with proper chemotactic cues to invade deep within tumors as well as enhance their persistence within the tumor microenvironment. This dual therapeutic approach is compatible with current clinical pipelines for generating CAR T cells, as multiple immune populations can be extracted by apheresis from a single patient and genetically modified in parallel.

B034: 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 designs. While many ongoing efforts are focused on the identification of cell surface target antigens, we have taken advantage of our recent super-resolution microscopy finding to modulate CAR signaling. 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, 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 against 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*. First generation CAPs containing ZAP70 domains, and second-generation CAPs, containing ZAP70 and CD28 costimulatory domains, were evaluated for their ability to eliminate CD19+ leukemia in an NSG xenograft model. Second generation CAPs exhibited high anti-tumor efficacy, and significantly enhanced *in vivo* persistence of tumor clearance in leukemia-bearing NSG mice as compared with conventional CD19-28zeta CAR-T. In this regard, it is interesting to note that increased tumor clearance and persistence may be due to the direct downstream activation of CAP-T; they differ from CAR-T which activate upstream TCR signals that induce T cell exhaustion molecules such as PD1.

B035: 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. STAT3 can form homodimers, or heterodimerize with STAT1 or STAT5. To understand the role of STAT3 homodimers and heterodimers, we made use of knockout models in which NK cells lack STAT3 expression or specific cytokine receptors. 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-a 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. RNA-seq analysis further corroborated that in high-dose infection STAT3-deficient NK cells are nearly indistinguishable from WT NK cells during regular-dose infection by transcriptome. Thus, we believe STAT3 operates as a rheostat, modulating the sensitivity of NK cells to their inflammatory environment.

B036: Role of antiviral program in the control of host microbiota interaction within the skin

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All multicellular organisms are meta-organisms which have evolved to facilitate a symbiotic dialogue between the host and the microbiota. This is especially important at barrier sites such as the skin, where communication between the microbiota and commensal-specific T cells is essential to promote protective immune responses, including anti-tumor immunosurveillance. Further, because of the skin's constant exposure to UV radiation, which has been shown to lead to the reactivation of potentially oncogenic viruses such as Merkel cell polyoma virus and HPV, understanding homeostatic mechanisms of anti-viral immunity in the skin will be essential in improving current therapeutic strategies and the prevention of skin cancer. Integrated retroviral elements, known as endogenous retroviruses (ERVs), comprise up to 8% of the human genome. We found that encounter with commensal microbe reactivated ERVs, and that inhibition of ERV cDNA synthesis abrogated immune responses to the skin microbiota. In models of microbiota-independent sterile inflammation, such as high fat diet, CD8 T cell recruitment to the skin was also ERV dependent. Here we hypothesize that ERV-specific CD8 T cells constitute a novel

class of immune cells present in the skin at steady state, and that they may contribute to barrier immunity. We first established a model of sterile injury where ERVs can be reactivated specifically in the skin, independently of the microbiota. Topical application of brain-heart infusion broth dissociated in tween detergent robustly reactivated ERVs, and recruited CD8 T cells to the skin in an ERV dependent manner. We next sought to identify potential ERV antigen presenting cells. RNAseq analysis of ERV expression revealed Langerhans cells (LCs) have the most abundant ERV expression amongst skin dendritic cells. LCs are an abundant skin-resident population, whose function remains unclear. Using mice depleted of LC, we found that CD8 T cell responses observed in the context of sterile injury were highly dependent on this call of antigen presenting cells. This observation supported the idea that LCs could be involved in driving CD8 T cell responses to sterile injury by presenting ERV antigens. To identify ERV-reactive T cells, we selected peptides from our RNAseq that were uniquely expressed in LCs, but not expressed in thymic epithelial cells capable of mediating negative selection. Several peptides promoted cytokine production from injury-elicited CD8 T cells, confirming the presence of ERV-reactive CD8 T cells within the skin. Ongoing work involves generating tetramers and TCR transgenic mice to track ERV-specific T cells and define their role in barrier protection. We expect this work will uncover how the immune system recognizes vestigial viruses through a novel class of T cells, called to the skin by LCs, to contribute to anti-viral immunity and immunosurveillance.

B038: Learning dynamic ensemble host-virus interactions in the nasal mucosa at single-cell resolution

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To advance novel mucosal vaccines that protect both healthy and immunocompromised individuals in the tissues that first encounter a virus, we must develop a comprehensive, temporal understanding of the effective cell-virus, cell-cell, and tissue ensemble interactions following natural infection. Our recent work studying the nasopharyngeal response to SARS-CoV-2 has identified several unique subsets of epithelial and innate immune cells associated with disease outcomes, but with currently unknown functions in viral immunity. Beyond cataloging these cells at a static snapshot of infection, we hypothesize that we can leverage the timing of host-viral interactions within the nasal mucosa to propose causality in cellular interactions and test contributions to disease trajectory. Moreover, by better understanding the contributions of non-adaptive immune cells to fighting infection, we stand to inform vaccines for immunocompromised individuals (e.g., leukemia).

To pinpoint critical cell-virus and cell-cell interactions in time, and establish a comprehensive understanding of the nasal mucosa, we applied single-cell RNA-sequencing to longitudinal samples of respiratory viral infection. Using the well-studied strain of influenza PR8 (H1N1), we developed a mouse model of upper respiratory tract infection and captured >156,000 single cells from samples at baseline and throughout acute infection (n=15 animals from Day 0 to Day 14). As the nasal mucosa has distinct anatomical regions with specific functions, we micro-dissected

the respiratory, olfactory, and glandular epithelial regions as distinct samples and optimized antibody-based hashing approaches to retain animal identity and increase biological power.

To realize dynamic responses to infection, we developed a cell cluster composition analysis approach to understand how tissue regions change in cellular identity, cell-cell interactions, and gene programming over time. Infection was largely restricted to the respiratory epithelium and induced coordinated longitudinal changes in cell cluster frequencies. We identified a polyfunctional subset of neutrophils as the earliest response to infection (D2) preceding most other antiviral responses (D5-8). By aligning to a custom host and viral genome, we measured influenza reads restricted to epithelial and myeloid cells that were concordant with high numbers of interferon-responsive cells (both viral read+ and bystander) and enriched at D5 and D8. Our total cell numbers also enabled us to identify a rare and previously undescribed epithelial subset expressing multiple co-inhibitory ligands that increase in frequency concurrently with tissue resident memory T cells at D14. Together, our work establishes a resource for contextualizing future experiments studying infection of the nasal mucosa and nominates several unique epithelial and myeloid cell subsets potentially impacting infection trajectory for further study. Future research will seek to test the causal relationships discovered here and in complementary human data.

B039: Targeting regulatory CD4 T cells in glioblastoma (GBM) using ADCC optimized anti-CD25 promotes tumour control and synergizes with anti-EGFRvIII tumour-targeting antibodies

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Despite the success of anti-CTLA-4 and anti-PD-1 for melanoma and lung cancer, to the date immunotherapy for GBM has been ineffective. Although at a small frequency, effector CD8 and CD4 T cells are present in GBM tumours, 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. EGFRvIII is the most common rearrangement of amplified EGFR genes, located in the cell surface of tumour cells which makes it a good candidate for the development of therapeutic antibodies. As EGFRvIII is expressed in up to a 60% of all GBMs, these tumours could be therapeutically targeted with antibodies against Tregs and/or EGFRvIII. Our laboratory has developed the new anti-$CD25^{NIB}$ that depletes Tregs via antibody dependent cell cytotoxicity (ADCC) and/or phagocytosis (ADCP) by engaging activating Fc gamma receptors on macrophages and NK cells, whilst preserving IL2 signalling on effector CD4 and CD8 T cells. In this study we have derived a Treg specific GBM signature that when applied to the GBM TCGA predicts worst survival, suggesting that these cells are actively suppressing the immune response in these tumours and highlighting their potential as

targets for immunotherapy. Next, we used both human GBM single cell RNA sequencing data together with a recently developed mouse model to analyze look at the expression of CD25 on Tregs and to quantify the expression of the Fc gamma receptors on the different innate immune populations. We found that both in human and mice, Tregs express the highest levels of CD25 compared to effector CD4 and CD8 T cells and that the myeloid compartment expresses the highest levels of Fc gamma receptors, suggesting that the anti-$CD25^{NIB}$ could be tested in GBM. In fact, the use of the anti-$CD25^{NIB}$ in our mouse model of GBM showed that one dose led to effector CD8 and CD4 T cell activation and proliferation that resulted in a 60% survival. This effect is accompanied by the recruitment of monocytes and myeloid derived suppressor cells expressing high levels of activating Fcg receptors which can be concomitantly engaged with another ADCC optimized antibody. Coadministration of anti-$CD25^{NIB}$ together with an Fc-optimized anti-EGFRvIII antibody led to a synergistic effect with complete elimination of all GBM tumours in the mice. Finally, we have used human GBM explants to test the human version of the anti-CD25NIB demonstrating that Tregs can be depleted in this system and suggesting that the use of this antibody holds promise for its future use in the clinic.

B040: Dissecting innate immune memory using single cell transcriptomics

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Antigen specificity and immune memory have long been considered defining characteristics that separate the functions of the adaptive and innate arms of the immune system. However, mounting studies have shown that cells of the innate immune system, including hematopoietic stem and progenitor cells (HSPC), undergo epigenetic and metabolic modifications that result in significantly altered responses to subsequent heterologous challenges. Indeed, alterations in myelopoiesis downstream of innate immune memory responses to microbially-derived compounds have been associated with improved anti-tumor and anti-pathogen immunity. Conversely, innate immune memory in chronic inflammation has also been linked to dysregulated immune responses. Despite its importance, the cellular and molecular mechanisms underlying innate immune memory are poorly defined. Thus, in this work we utilized scRNA-seq, RNA-seq, and ATAC-seq to identify cell populations activated in response to relevant memory-inducing inflammatory stimuli. These experiments defined intercellular signaling pathways upregulated in response to innate immune memory and pointed towards innate lymphocytes as one source of cytokine signals which promote innate immune memory *in vivo*. Altogether, our data reveal molecular pathways modulated in innate memory and point towards a potential key role for innate lymphocytes in the instruction of memory in HSPCs.

B041: Identifying molecular targets for rational immunosuppressive strategies in severe immune checkpoint therapy-induced myocarditis, myositis, and myasthenia gravis

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Background: Immune checkpoint therapies (ICTs) can induce anti-tumor T cell responses that improve survival but also life-threatening immune-related adverse events (irAEs) requiring ICT discontinuation. Myocarditis, myositis, and myasthenia gravis (MG) are rare irAEs with reported mortality up to 40% and no predictive biomarkers. The only standard treatment is high-dose steroids, which can cause complications with prolonged use, e.g. infection, hyperglycemia, delirium, and osteoporosis. We and others have found serum autoantibodies in these irAEs, suggesting plasma B cells contribute to toxicity. Further, we showed biologically selective immunosuppression in other severe irAEs enabled ICT resumption and led to durable responses. We aimed to 1) determine if targeting B cells and T cells would improve outcomes in these irAEs and 2) characterize immune cell phenotypes in inflamed patient tissue to identify molecular targets for more effective immunosuppressive strategies.

Hypothesis: Plasma B cells and T cells contribute to pathogenesis of severe ICT-induced myocarditis, myositis, and MG.

Methods: In May 2019, we implemented an algorithm for patients in Genitourinary (GU) Medical Oncology (prostate, kidney, and bladder cancers) with severe myocarditis, myositis, and MG. Patients received high-dose steroids, plasma exchange (to eliminate autoantibodies and ICTs), rituximab (anti-CD20 for B cell depletion), and infliximab (anti-TNF-α for T cell suppression). Diagnosis required: 1) symptoms requiring hospitalization; 2) elevated serum markers (creatine kinase ≥5x normal, troponin >99th percentile or autoantibodies for MG); and 3) positive biopsy, electromyography, or cardiac MRI. From Nov. 2020, GU and non-GU patients admitted with these irAEs enrolled in a laboratory protocol with fresh cardiac and skeletal muscle tissues and peripheral blood mononuclear cells stored following standard diagnostic testing.

Results: 31 patients with GU cancers and severe irAEs were analyzed (n=15 pre-algorithm [myositis: 13, myocarditis: 4, MG: 8, overlap: 8], and n=16 post-algorithm [myositis: 13, myocarditis: 10, MG: 9, overlap: 11]). The most common ICT in both groups was anti-CTLA-4 + anti-PD-(L)1. Serum autoantibodies to striated muscle or acetylcholine receptor were detected in 58% (18/31). Median serum cytokine levels at admission were TNF-a: 27.5 pg/mL (95% CI: 18.0-51.0, ref: <22), IL-6: 14.0 pg/mL (95% CI: 6.0-20.0, ref: <5), and IFN-γ: 0.0 pg/mL (95% CI: 0.0-3.0, ref: <5) (n=26). Treatments pre-algorithm were variable. All patients received steroids except 1 post-algorithm with severe diabetes. All post-algorithm patients received plasma exchange, rituximab, and infliximab. Successful taper to physiologic steroid doses (≤10 mg prednisone) was achieved in 67% (10/15) of patients pre- and 93% (14/15) post-algorithm (p=0.17). In these patients, the median time to physiologic steroid dose was 8.5 weeks pre- and 3.9 weeks post-algorithm (p=0.005). 37 GU and non-GU patient tissue samples were collected, with 29 samples from 22 patients evaluable for single cell RNA sequencing (scRNASeq) (n=6 patient-matched inflamed cardiac and skeletal, n=7 non-matched inflamed cardiac or skeletal, and n=10 ICT-treated, non-inflamed control cardiac or skeletal muscle). Immunohistochemistry of inflamed tissue showed infiltrating T cells and myeloid cells.

Conclusion: An algorithm of steroids, plasma exchange, anti-CD20 and anti-TNF-a antibodies was associated with shortened time to physiologic steroid doses in patients with severe ICT-induced myocarditis, myositis, and MG. Mechanism-based immunosuppressive strategies will be

needed to abrogate toxicity and enable ICT resumption. Elevated IL-6 levels in serum and infiltrating myeloid cells in affected tissue suggest that myeloid populations contribute to these irAEs. scRNASeq analysis of inflamed cardiac and skeletal muscle is ongoing to identify molecular targets for rational immunosuppressive strategies.

B042: Metabolic adaptation and glutamine metabolism drives regenerative and tumor-associated myelopoiesis

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Tumor-associated myelopoiesis is a critical regulator of the tumor-immune microenvironment, particularly for tumor-associated neutrophils, given their short half-life and tissue residency time. Under conditions of stress, HSCs activate emergency myelopoiesis (EM) pathways to rapidly regenerate myeloid cells and maintain homeostasis. EM involves the overproduction of myeloid-biased multipotent progenitors (MPP), the myeloid reprogramming of lymphoid-biased MPP, and the formation of granulocyte-macrophage progenitor (GMP) clusters in the bone marrow microenvironment, which serve as hubs of myeloid cell differentiation and overproduction. In the context of solid tumor development, systemic inflammation hijacks these regenerative EM pathways to drive the overproduction of myeloid cells entrained with immunosuppressive and prometastatic effector functions. Understanding how EM regulates hematopoietic stem and progenitor cell (HSPC) and myeloid cell behavior will be critical for developing new treatments to improve anti-tumor immunity and enhance immunotherapy response rates.

To investigate the molecular mechanism regulating EM pathway activation, we generated an atlas of EM using single-cell RNA sequencing to profile bone marrow HSPCs following myeloablation with the chemotherapeutic compound 5-fluorouracil (5FU) and upon breast cancer development using the MMTV-PyMT mouse model. In the context of both regenerative and tumor-associated myelopoiesis, pseudotime analysis revealed precocious activation of OXPHOS genes in HSCs and MPPs as part of a larger c-Myc driven transcriptional signature. We confirmed increased c-Myc and mitochondrial biogenesis in HSPC populations during regeneration using *c-Myc-GFP* and *MitoDendra2* reporter lines. Real-time cell metabolic analysis revealed that regenerative HSPCs had greater baseline respiration and spare respiratory capacity. Furthermore, we observed that the metabolic adaptations driving the expansion of myeloid progenitors upon EM pathway activation are heritable. While neutrophils generally have low levels of mitochondria and rely on glycolytic metabolism, we observed that regenerative and tumor-associated neutrophils had elevated levels of mitochondria. Notably, EM neutrophils had decreased reliance on glycolysis during oxidative burst assays due to compensatory mitochondrial metabolism. We next sought to determine the necessity of c-Myc activity and metabolic adaptation in EM pathway activation and found that hematopoietic specific c-Myc haploinsufficiency ($Myc^{+/flox}$; Scl-CreER) was sufficient to impair regeneration. Using liquid culture assays, we observed that EM GMPs demonstrated increased metabolic flexibility but were critically reliant on glutamine to fuel their expansion. Acute *in vivo* inhibition of glutamine metabolism with the small molecule inhibitor 6-Diazo-5-oxo-L-norleucine (DON) as well as hematopoietic-specific loss of *Gls1* ($Gls1^{flox/flox}$; Scl-CreER) impaired EM activation following 5FU, delaying the emergence of GMP clusters and suppressing myeloid output. Collectively these results demonstrate that the activation of EM pathways triggers c-Myc-driven metabolic

adaption through the HSPC compartment, including increased mitochondrial biogenesis, OXPHOS, and metabolic fuel flexibility. Glutamine metabolism, however, remains a therapeutic vulnerability, and chemical or genetic inhibition of glutaminolysis suppressed EM. Finally, EM-associated metabolic adaptation is heritable, and regenerative and tumor-associated neutrophils display altered immunometabolism and effector functions. Work is ongoing to evaluate the therapeutic efficacy of targeting hematopoietic c-Myc activity and glutaminolysis to suppress tumor-associated myelopoiesis.

B043: Phenotypic screening platform identifies statins as enhancers of immune cell-induced cancer cell death

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High-throughput screening of small molecule drug libraries has greatly facilitated the discovery of new cancer drugs as it has produced several chemotherapeutics that are currently used in the clinic. However, most phenotypic screening platforms used in the field of oncology are based solely on cancer cell populations and do not allow for the identification of immunomodulatory agents. To address this, we developed a phenotypic screening platform based on a miniaturized co-culture system with human cancer- and immune cells. The human colorectal cancer (CRC) cell line HCT116 constitutively expressing green fluorescent protein (GFP) was cultured as monoculture or co-cultured with anti-CD3/IL-2 activated peripheral blood mononuclear cells (PBMCs) in a 384-well plate format, providing a model that recapitulates part of the tumor immune microenvironment (TIME) complexity while simultaneously being compatible with a simple image-based readout. Using this platform, we screened 1,280 small molecule drugs and identified statins, a group of widely prescribed cholesterol lowering drugs, as enhancers of anti-tumor immunity. The lipophilic statin pitavastatin had the most potent anti-cancer effect, as well as the greatest impact on the secretion of inflammatory cytokines, and was therefore selected for further analysis. Transcriptome-wide gene expression analysis demonstrated an increased expression of tumor suppressor genes and an overall pro-inflammatory expression profile post-treatment with pitavastatin in our in vitro tumor-immune model. A considerable amount of data from both preclinical and clinical studies demonstrate that statins have tumor-suppressing properties. Our findings added, we speculate that the clinical benefits described for cancer patients receiving statins are dependent on the combined effect exerted on both cancer- and immune cells; a notion supported by recent studies suggesting that statin treatment is associated with improved clinical outcomes for patients receiving therapy with immune checkpoint inhibitors. In conclusion, we developed a phenotypic screening platform for in vitro identification of immunomodulatory agents, thus addressing a critical gap in the field of immuno-oncology. Furthermore, our pilot screen identified statins, a drug family gaining increasing interest as repurposing candidates for cancer treatment, as enhancers of immune cell-induced cancer cell death.

B044: A novel double-humanized PD-1/PD-L1 mouse model reveals the quality of tumor-specific T cells that is crucial in mediating the antitumor effects of PD-1 or PD-L1 inhibitors.

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Though the current FDA-approved anti-PD-1 or PD-L1 blocking antibodies have demonstrated encouraging clinical responses, their underlying mechanisms of action still need to be better defined, particularly for optimizing therapeutic timing and identifying new opportunities for rational combination therapy. To that end, we produced novel double-humanized PD-1 and PD-L1 mice in which the human sequences of the extracellular domains of PD-1 and PD-L1 were used to replace the mouse sequences. In this model, we found that it is the *quality* rather than the quantity of tumor-specific T cells that is crucial in mediating the anti-tumor effects of FDA-approved PD-1 or PD-L1 inhibitors in vivo. Using an in vivo priming assay, we found that both control mice and mice treated with anti-PD-1 or PD-L1 antibodies produced equivalent numbers of antigen-specific T cells. However, we identified the increased generation of CX3CR1+ cytotoxic CD8+ T cells in the anti-PD-1/L1-treated mice, the same subset of effector T cells known to increase in clinical responders to PD-1/L1 therapy. Thus, our model permits the direct comparison of FDA-approved anti-PD-1/L1 monoclonal antibodies and further correlates successful tumor rejection with the level of CX3CR1+ CD8+ T cells, making this model a critical tool for future studies to optimize and better utilize anti-PD-1/L1 therapeutics. We also demonstrated the potential of combining tumor vaccine therapy and anti-PD-1/PD-L1 antibodies that could be explored in future clinical trials.

B045: A neoepitope DNA vaccine induces anti-tumor effect and synergizes with Checkpoint Inhibitor blockade in preclinical mouse models

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Tumor mutations giving rise to neoepitopes have recently emerged as promising targets for cancer immunotherapy. Vaccines delivering tumor-specific neoepitopes have demonstrated promising efficacy and safety results in numerous preclinical and early clinical studies. However, the identification and selection of therapeutically relevant neoepitopes present a big challenge for designing effective immunotherapies.

In the presented work, we assessed the ability of a plasmid DNA (pDNA) vaccine encoding neoepitopes to induce anti-tumor effect and neoepitope immunogenicity in preclinical cancer models. To identify relevant vaccine epitopes, the tumor mutanome was mapped via next generation sequencing and the neoepitopes were scored based on their probability of being presented on MHC molecules for recognition by the immune system using a diverse set of computational features. The highest ranked neoepitopes were selected and used for vaccine design and subsequent testing. To assess anti-tumor effect, mice were vaccinated with neoepitope-encoding pDNA or with empty plasmid prior to subcutaneous tumor challenge. The study showed that neoepitope pDNA vaccination completely prevented establishment of the

tumors and, importantly, conferred long-lasting protection against subsequent tumor re-challenge. Complementary immunological analyses confirmed the induction of neoepitope-specific, polyfunctional CD4+ and CD8+ T cells in the blood, spleen, and tumor compartments in immunized mice.

To delineate the importance of CD4+ and CD8+ T-cell engagement in mediating tumor growth control, T-cell subsets were depleted *in vivo* and the mice were vaccinated prior to tumor inoculation. Engagement of both T-cell subsets was documented to be indispensable for optimal anti-tumor effect, as depletion of either subset compromised the ability to contain tumor growth.

Finally, to assess the outcome of combining the neoepitope pDNA vaccine with Checkpoint Inhibitor (CPI) blockade treatment, mice were co-administered with anti-PD-1 antibodies and the neoepitope pDNA vaccine. Impressively, combining a suboptimal dose of the neoepitope pDNA vaccine with CPI led to improved anti-tumor effect compared to either monotherapy alone and strongly potentiated the neoepitope-specific immune responses.

Taken together, these results demonstrate that plasmid DNA is a versatile vaccine platform that allows encoding of multiple neoepitopes in a single formulation and highlights the feasibility of *in silico* identification, prioritization, and selection of therapeutically relevant tumor-specific neoepitopes for personalized immunotherapy. Our observations contribute to understanding the dynamic interplay between vaccine-induced T cells and the evolving tumor. Finally, our findings underline that neoepitope pDNA vaccine and CPI treatment cooperatively impair tumor growth in an additive, non-redundant manner and importantly, highlight the potentiation of the vaccine-induced immune responses.

B046: Increased recognition of primary melanoma tumor cell lines grown as spheroids compared to 2D monolayers by autologous tumor infiltrating lymphocytes

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In this study we evaluated differences in tumor recognition between monolayer and spheroid grown tumor cells by autologous tumor infiltrating lymphocytes (TIL).

Immunotherapy using immune checkpoint inhibitors (ICI), or adoptive cell therapy based on tumor infiltrating lymphocytes (TIL) to treat patients with metastatic melanoma and other immunogenic cancer types has shown promising results with a ~40-60% response rate. However, there is still room for improvement and an urgent need to define biomarkers making it possible to predict who could benefit from these immunotherapies. To improve current immunotherapy treatment regimen and to be able to predict who to treat, it is important to have the accurate research tools that mimic the tumor microenvironment to evaluate the reactivity of immune cells against autologous tumor cells.
In contrast to tumor cells grown as monolayers (2D), multicellular spheroids (3D) mimic the tumor microenvironment in terms of nutrient and oxygen gradients as well as cell-cell interactions. We were interested to understand how these two *in vitro* models might differ

regarding their interactions with immune cells and if 3D models could be a better predictor for TIL- tumor recognition. To study this, we used autologous TIL- tumor pairs generated from two melanoma patients.
Notably, we found that 3D tumor cells induce stronger anti-tumor responses by autologous TIL compared to 2D grown tumor cells, evaluated by cytokine release and cytotoxicity. In addition, these differences were observed using either mechanically disrupted or intact spheroids during the TIL- tumor coculture. Blocking MHC class I confirmed that the TIL- tumor recognition was antigen dependent. Furthermore, pre-treatment with IFNγ, known to upregulate the antigen presentation machinery, MHC class I and antigen processing, led to an increased tumor recognition of 2D grown tumor cells by autologous TIL, similar to 3D grown tumor cells.
To further refine the tumor recognition, dextramer enriched TIL recognizing a specific, clinically relevant neoantigen, ETV6 (identified in Wickström et al. Frontiers in Immunology 2019), were used and recognition of autologous tumor cells grown as 2D vs 3D spheroids was investigated. We observed that ETV6 enriched T cells displayed increased recognition of spheroids compared to 2D grown tumor cells. To conclude, we hypothesize that tumor cells grown as multicellular spheroids have increased presentation of tumor antigens, including neoantigen(s), exemplified by ETV6, and that this could be explained by increased antigen presentation, MHC class I expression and/or antigen processing. These findings show the importance of choosing the appropriate *in vitro* system and the results suggest that multicellular spheroids might serve as a better model to evaluate immune cell – tumor cell interactions to assess different immunotherapies.

B047: Modelling resistance of colorectal peritoneal-metastases to immune checkpoint blockade in humanized mice

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Background: The immunogenic nature of metastatic colorectal cancer (CRC) with high microsatellite instability (MSI-H) underlies their responsiveness to immune-checkpoint blockade (ICB). However, resistance to ICB is commonly observed, and is associated with the presence of peritoneal-metastases and ascites formation. The mechanisms underlying this site-specific benefit of ICB are unknown.

Methods: We created a novel model for spontaneous multi-organ metastasis in MSI-H CRC tumors by transplanting patient-derived organoids (PDO) into the caecum of humanized mice. Anti-PD1 and anti-CTLA4 ICB treatment effects were analyzed in relation to the immune context of primary tumors, liver-metastases, and peritoneal-metastases. Immune profiling was performed by immunohistochemistry, flow cytometry and single cell RNA sequencing. The role of B-cells was assessed by antibody-mediated depletion. Immunosuppressive cytokine levels (IL10, TGFb1, TGFb2, TGFb3) were determined in ascites and serum samples by enzyme-linked immunosorbent assay (ELISA).

Results: PDO-initiated primary tumors spontaneously metastasized to the liver and the peritoneum. Peritoneal-metastasis formation was accompanied by the accumulation of ascites. ICB completely cleared liver-metastases and reduced primary tumor mass but had no effect on peritoneal-metastases. This mimics clinical observations. After therapy discontinuation, primary tumor masses progressively decreased, but peritoneal-metastases displayed unabated growth. Therapy efficacy correlated with the formation of tertiary lymphoid structures (TLS) – containing B-cells and juxtaposed T-cells – and with expression of an interferon-γ signature together with the B-cell chemoattractant CXCL13. B-cell depletion prevented liver-metastasis clearance by anti-CTLA4 treatment. Peritoneal-metastases were devoid of B-cells and TLS, while the T-cells in these lesions displayed a dysfunctional phenotype. Ascites samples from cancer patients with peritoneal-metastases and from the mouse model contained significantly higher levels of IL-10, TGFb1, TGFb2 and TGFb3 than serum samples.

Conclusions: By combining organoid and humanized mouse technologies, we present a novel model for spontaneous multi-organ metastasis by MSI-H CRC, in which the clinically observed organ site-dependent benefit of ICB is recapitulated. Moreover, we provide empirical evidence for a critical role for B-cells in the generation of site-dependent anti-tumor immunity following anti-CTLA4 treatment. High levels of immunosuppressive cytokines in ascites may underlie the observed resistance of peritoneal-metastases to ICB.

B048: PI3Kγδ inhibitor plus radiation enhances the antitumor immune effect of PD-1 blockade in syngenic murine breast cancer and humanized patient-derived xenograft model

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Introduction: We hypothesized that combined use of radiation therapy and a phosphoinositide 3-kinaseγδ inhibitor to reduce immune suppression would enhance efficacy of immune checkpoint inhibitor.

Methods: Murine breast cancer cells (4T1) were grown in both immune-competent and -deficient BALB/c mice, and tumors were irradiated by 3 fractions of 24 Gy. A PD-1 blockade and a PI3Kγδ inhibitor were then administered every other day for 2 weeks. Same experiments were performed in humanized patient-derived breast cancer xenograft (PDX) model, and its tumor was sequenced to identify immune-related pathways and to profile infiltrated immune cells. Transcriptomic and clinical data were acquired from The Cancer Genome Atlas (TCGA) pan-cancer cohort, and the deconvolution algorithm was used to profile immune cell repertoire.

Results: Using a PI3Kγδ inhibitor, RT, and PD-1 blockade significantly delayed primary tumor growth, boosted the abscopal effect, and improved animal survival. RT significantly increased CD8+cytotoxic T-cell fractions, immune-suppressive regulatory T cells (T_{regs}), myeloid-derived suppressor cells (MDSCs), and M2 tumor associated macrophages (TAMs). However, PI3Kγδ inhibitor significantly lowered proportions of T_{regs}, MDSCs, and M2 TAMs, achieving dramatic gains in splenic, nodal, and tumor CD8+ T-cell populations after triple combination therapy. In a humanized PDX model, triple combination therapy significantly delayed tumor growth and

decreased immune suppressive pathways. In TCGA cohort, high T_{reg}/CD8+ T cell and M2/M1 TAM ratios was associated with poor overall patient survival.

Conclusion: These findings indicate PI3Kγ and PI3Kδ are clinically relevant targets in an immunosuppressive TME and combining PI3Kγδ inhibitor, RT, and PD-1 blockade may overcome the therapeutic resistance of immunologically cold tumors.

B049: Immune functions of CXCR3 in chemo-resistant glioblastoma

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The objective of this study is to investigate the effect that temozolomide will have on CXCR3 expression in glioblastoma tumour cells and its related immune cells.

C57BL/6 mice will be implanted with murine glioma cell line GL261-luc2 intracranially and randomized into 4 treatment arms:

i. Control wild type
ii. Control CXCR-3 deficient mouse
iii. Temozolomide in wild type
iv. Temozolomide in CXCR-3 deficient mouse

In the intracranial model, pre-operative skin preparation would first be performed with an incision at 2cm lateral and 1mm dorsal to bregma, with mice anaesthetized) intraperitoneally. A right-sided craniotomy would be completed with the mouse mounted on a stereotactic frame to expose the right forebrain. A 24-gauge needle attached to a Hamilton syringe would be inserted at 3.5mm depth and withdrawn 0.5mm to make space for tumour cell accumulation. 5x10^5 GBM cells will be injected, with 3uL of culture media will be used to resuspend tumour cells for injection. The needle would be left in place for one more minute after finishing the inoculation of the cell and then is slowly removed. The skin incision would be closed with a 6-0 suture.

Post-injection, tumour-bearing mice will be treated with TMZ at 55 mg/ kg oral gavage (equivalent to human dosage) for 3 days (consecutive)/ week, for 2 cycles.

Every 2 days post-treatment, tumour size and immune activation will be assessed. Tumour size is measured by in-vivo imaging with the Xenogen system to measure bioluminescence signalling from luciferase-expressing tumour cells. Expression of CXCR3 will be quantified using flow cytometry analysis of the brain tissue. After obtaining single-cell suspensions and post-red blood cell lysis, the cell is suspended in TruStain FcX™ PLUS (anti-mouse CD16/32, 0.25ug per in 30ul PBS) before incubation with antigen-specific antibodies at 1:50 dilution for 30 minutes. The cell suspension is transferred to a PP cell flow tube and ready for flow cytometry analysis.

In initial models, we have demonstrated that CXCR3 expression is detectable intratumorally with western blot and qPCR in different glioma cell lines as well as tissue culture. With western blot, we also see a corresponding increase in LRP1, supporting the CXCR3/ LRP1 cross-talk invasion. We illustrate that temozolomide, similar to cancers such as melanoma also induces expression of

CXCR3 ligands, thus upregulating CXCR3 expression and also favouring T cell infiltration, on the foundation that the mechanism of temozolomide depositing methyl groups on DNA bases and typical resistance due to over-expression of O6-methylguanine methyltransferase (MGMT) in GBM comparable to that of other cancers. Similarly, other studies have shown that TMZ induced expression of other CXCR ligands in glioblastoma, and we have shown that ligands of CXCR3 (CXCL4,9,10) also increase.

CXCR3 expression has been previously shown to play a role in recruiting tumour-producing immune cells in glioblastoma, but it is uncertain how conventional temozolomide may affect its expression, which may or may not favour tumour control as in other cancers such as melanomas. Thus, CXCR3 holds the potential to be further targeted and this study will thus provide essential pre-clinical evidence to support future translational studies on new treatment paradigms for GBM.

B050: Single-cell profiling uncovers features of a proliferative metaplastic cell type sustained by Helicobacter pylori-specific inflammation

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At least 80% of gastric cancer cases are attributed to stomach infection with the bacterium *Helicobacter pylori* (*Hp*), which causes lifelong chronic inflammation. In some individuals, this inflammation can cause gastric atrophy, metaplasia (conversion of one normal cell type to another), dysplasia (presence of abnormal cells) and finally cancer, but the specific mechanism(s) through which *Hp* drives this cascade are not well understood. A similar cascade of preneoplastic changes can be triggered in a transgenic mouse model through tamoxifen-induced expression of active KRAS in the chief cells of the stomach (KRAS+ mice). About 40% of gastric cancers have RAS activity, and more broadly, KRAS in this model serves as a tool to recapitulate human disease phenotypes. We previously found that *Hp* infection of KRAS+ mice exacerbated disease: *Hp*+KRAS+ mice had greater inflammation and dysplasia compared to *Hp*-KRAS+ mice. To probe whether *Hp* accelerated versus altered the trajectory of disease, we performed single cell RNA-sequencing (scRNA-seq) on eight mouse stomachs (+/- *Hp*, +/- KRAS) using the 10x Chromium platform. Cluster analyses followed by UMAP (Uniform Manifold Approximation and Projection) visualization allowed us to identify 25 clusters, including epithelial and immune cell types. *Hp*+KRAS+ mice had a striking expansion of a population of metaplastic cells (most similar to surface mucus-producing "pit" cells), which comprised ~40% of epithelial cells in these mice. Metaplastic pit cells expressed the intestinal mucin *Muc4* and other metaplasia- and cancer-associated genes, and had increased epidermal growth factor receptor (EGFR) signaling relative to classical pit cells. They exhibited a genetic signature of exposure to inflammation, particularly interferon (IFNγ) and tumor necrosis factor alpha (TNFα). We observed a gastric lamina propria population of dual IFNγ+TNFα+ T effector cells in *Hp*+KRAS+ mice that was significantly suppressed by oral dexamethasone treatment. Strikingly, dexamethasone also reduced the abundance of metaplastic pit cells, without impacting *Hp* loads in the stomach. As well, antibiotic eradication of *Hp* prevented metaplastic pit cell development and other disease phenotypes. Thus, *Hp*+KRAS+ mice exhibit an altered

trajectory of metaplasia compared to *Hp*-KRAS+ mice, with the expansion of an aberrant mucous cell lineage. Finally, analysis of published gastric scRNA-seq datasets and samples from our discovery cohort of 47 gastric cancer cases showed that pit cells can develop *MUC4* expression during gastric preneoplasia and can then acquire the expression of another intestinal mucin, *MUC2*, as cancer develops. As well, gastric MUC4 expression is strongly linked with cell proliferation. Taken together, these studies show that chronic inflammation due to *Hp* infection drives the expansion of a metaplastic pit cell lineage that can be targeted by dexamethasone and antibiotics, and that may serve as a risk factor in patients with preneoplasia.

B051: Cancer immunotherapy in three dimensions: Visualization and quantification of tumor explant-immune interactions

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Introduction: Cancer immunotherapy offers potentially lifesaving treatments to cancers, but the identification of new therapeutic strategies is hampered by a lack of validated preclinical models. There are currently *no* preclinical platforms for long-term co-culture of intact tumor-derived explants with autologous immune cells for assessment of patient-specific responses to immunotherapies. The high drug attrition rate in clinical trials often originates from the ambiguous drug response in 2D cell lines, the lack of autologous immune cells in models, and the inaccurate representation of inter-individual heterogeneity in oversimplified 3D spheroid/organoid models[1]. We developed an approach for immuno-oncology experiments with extended preservation of patient tumor explant micro-tumoroids and matched peripheral blood mononuclear cells (PBMCs) coupled with in situ confocal imaging capability to track and quantify immune cell action (T-cell killing, proliferation, and motility) and tumor responses (invasion, proliferation, extravasation, and collective cell behavior).

Materials and Methods: Tumor microexplants (200-400 µm) were created from resected cancers obtained from properly consented donors. Purified PBMCs were labeled with CellTrace CFSE and co-cultured in 3D Liquid Like Solid (LLS)[2] with Organoid liquid media[3] and supplements. Steady perfusion flow of 40-60 µL/hr/well was maintained with an oxygen partial pressure > 90%. Cytokine production was measured using Meso Scale Discovery Small Spot assay and confirmed by metabolomics analysis (GC-MS[4]). 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 5-minute intervals to create sequential time-lapse images that can track 3D positions, motions, infiltration dynamics, and cell death. These data were analyzed using cell tracking AI algorithms and confirmed by immuno-phenotyping using flow cytometry[5].

Results and Discussion: Video Scanning Confocal Microscopy and Custom AI algorithms identified individual cells and enabled dynamic movies of cell tracking, cell fate, and cell-cell interactions to be quantified. 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 demonstrating 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 μm/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 IFNg production as compared to both the untreated negative control groups and tumors with an immune-excluded phenotype.

Conclusions: 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.

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B052: Radiation-induced T cell infiltration in preclinical glioma models

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Resection and radio-chemotherapy remain as the mainstay in the first-line treatment of gliomas and response rates of gliomas to immunotherapies such as immune checkpoint inhibitors remain low. There is evidence that irradiation induces an immune permissive tumor microenvironment. However, the underlying mechanism of T cell infiltration and determinants of T cell function in brain tumors after irradiation are incompletely understood. Type I interferons (IFNs) such as IFN-β and IFN-α have been shown to be important factors in stimulating a successful anti-tumoral immune response in several solid cancer entities. Their expression is induced via activation of the cGAS-STING pathway following radiation. We show, that irradiated glioma cells upregulate type I IFNs after 48h and peaked their secretion at 72h after irradiation *in vitro*. In an experimental syngeneic glioma mouse model, we provide evidence that T cell numbers are reduced by irradiation and increased again approximately 72 hours later. This also correlated with the upregulation of the IFN alpha receptor (IFNAR1) on tumor-infiltrating $CD3^+$ $CD8^+$ T

cells after 72h, suggesting a direct link between tumoral type I IFN and infiltrating T cells. At the same time, the number of intact $CD31^+$ vessels decreased in irradiated tumors. However, T cells re-infiltrating irradiated tumors, formed clusters around the remaining $CD31^+$ blood vessels inside the tumor. Using an *Ifnb1* knockout mouse model and single-cell transcriptomics of untreated and irradiated tumors, we are currently assessing the role of irradiation and tumor cell-derived type I IFN on the phenotype of tumor-infiltrating T cells in ongoing work.

B053: Targeted non invasive in vivo detection of platelet-related pathologies in mice by 64Cu-αGPIX-PET

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For many years it has been known that platelets activation and aggregation cause thrombosis and consequently serious diseases. Besides their critical functions in hemostasis, thrombosis and wound healing, it has become evident that platelets also play a crucial role in various processes in tumor development, including angiogenesis and the development of cancer metastasis. However, tools for the non-invasive *in vivo* examination and diagnosis of platelets related pathologies remain limited and are urgently needed. We have developed a novel antibody-based PET tracer, ^{64}Cu-NODAGA-anti-GPIX mAb (^{64}Cu-αGPIX) which targets GPIX, a membrane glycoprotein expressed by human and murine platelets and progenitor cells. Aim of our study was to evaluate our novel radiotracer for its binding capacities *in vitro* and for the *in vivo* detection of platelets aggregation and smallest lesions in mice using a relevant mouse model of thrombosis. Radiolabeling efficacy, stability and immunoreactivity of ^{64}Cu-αGPIX were tested *in vitro* before *in vivo* biodistribution analysis in naïve mice. ^{64}Cu-αGPIX has a high radiochemical purity (>94%), remained stable in mouse serum for at least 24 h and exhibited an immunoreactive fraction of 50%. First *in vivo* PET scans of naïve mice (5 µg ^{64}Cu-αGPIX, *i.v.*) revealed suitable whole-body ^{64}Cu-αGPIX distribution from 3 to 25 h (n=2 each timepoint, TP) with comparatively low uptake in the brain (1.7±0.4 / 1.4±0.1 %ID/ml) and muscle (1.2±0.1 %ID/ml, both TP). Next, we evaluated the applicability of ^{64}Cu-αGPIX-PET in a recent mouse model for cerebral (sinus) venous thrombosis (CVST), which is well suited to study the rapid dynamics of platelets accumulation *in vivo*. Thus, we performed dynamic (60 min) simultaneous PET/MRI scans. CVST was induced by anti-CLEC-2 small antibody treatment (INU1-fab, *i.v.*) 19 h after ^{64}Cu-αGPIX injection and 15 min after start of dynamic PET/MRI acquisition. The reconstructed dynamic PET images were colocalized to 2D-time-of-flight MR angiography (2D-TOF MRA) and anatomical MRI scans to determine the uptake in the brain (vessels) and peripheral organs (e.g. spleen and liver). After INU1-fab injection mice rapidly developed CVST, which was accompanied by an immediate increase in the cerebral ^{64}Cu-αGPIX signal (+1.0±0.5 %ID/ml, n=7). In comparison, sham-treatment resulted exclusively in a constant low background signal from circulating platelets in the brain (+0.1±0.1 %ID/ml, n=4). Quantification of the final 20 min of the scan revealed a strong cerebral platelet-aggregation due to CVST

(2.4±0.2%ID/ml; sham-treatment: 1.2±0.1%ID/ml, $P<0.04$). Of note, we could clearly assign the ^{64}Cu-GPIX-signal occurring from the small lesions in the brain to the (sinus) veins after colocalization with cerebral 2D-TOF MRA. In conclusion, this study revealed the enormous potential of ^{64}Cu-αGPIX-PET to track platelet activities and even smallest lesions while maintaining a low background signal (confined to blood, spleen and the liver). Thus, ^{64}Cu-αGPIX-PET is highly applicable to investigate platelets-related pathologies within the whole body and is a promising tool for non-invasive, longitudinal *in vivo* examination of thrombus formation. Moreover, we next aim to study the role of platelets in cancer immunotherapies non-invasively *in vivo* in mice to uncover the interplay between platelets, tumor growth and metastasis in a therapeutically setup.

B054: A microfluidic, patient-derived tumor-on chip platform for therapeutic efficacy and safety evaluation of CAR-T cell products

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CAR-T cell immunotherapies in the individualized treatment of patients with solid tumors are not very effective to date. Despite improvements in toxicity management, there is still a lack of understanding of the mode of action and side effects of this therapeutic approach. To overcome these obstacles of cell-based cancer immunotherapies, we need physiologically relevant test systems that can mimic both the patient's tumor and complex human processes including vascularization outside the human body. For this aim, we established a patient-derived tumor-on-chip system consisting of patient-derived microtumors (PDM) integrated into a customized microfluidic platform to enable assessment of personalized CAR-T cell product efficacy and safety. Here, PDM are isolated from fresh primary tumor samples (e.g. cholangiocarcinoma, breast carcinoma, glioblastoma, uveal melamona) using limited enzymatic tissue digestion and subsequent culture in defined media in the absence of serum. We have previously shown that PDM display a heterogeneous cellular composition and complexity by means of extracellular matrix and tumor microenvironmental components similar to the corresponding patient tumor tissue. Here, PDM were embedded in a customized microfluidic chip within a Dextran-Hydrogel. To mimic human vasculature and create a physical barrier for CAR-T cells migrating towards PDM, the medium perfusion channel was covered with human primary microvascular endothelial cells (mvECs). We established a reproducible protocol for injection of both PDM and mvECs, which enabled the on-chip culture and perfusion with stable viability for > 15 days. Following perfusion of these chips with CD276-targeting CAR-T cells at an effector-target cell ratio of 10:1, therapeutic efficacy was assessed by spinning-disc confocal microscopy and LDH-release cytotoxicity assay. Efficient CAR-T cell products were identified by a significant increase in cytotoxic microtumor killing. Furthermore, CAR-T cell activation in result of target antigen recognition during PDM co-culture was monitored on-chip by quantification of IFN-gamma from chip-perfused media.

Integrating PDM as tumor model into a customized microfluidic chip system equipped with an endothelial cell barrier and vasculature-like perfusion enables the study of immune-cell-cancer interaction and provides a new platform for preclinical efficacy and safety assessment of cell-based cancer immunotherapy products. We are currently expanding the toolbox of analysis methods for this chip platform to include label-free Raman spectroscopy and multiplexed DigiWest®-based protein profiling in order evaluate the quality and stability of cell-based cancer therapeutic products in even greater detail.

B055: Host CD8+ T cells are required for the antitumor efficacy of adoptive cell therapy

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Adoptive cell therapy (ACT) using tumor-specific cytotoxic T lymphocytes (CTLs) has demonstrated great efficacy in hematological cancers. However, ACT does not work in most patients with solid tumors. Hence, understanding the cellular mechanisms underlying effective antitumor immunity in ACT models is key to develop improved cancer immunotherapies. Here, we observed that ACT using *in vitro* activated SIINFEKL(OTI)-specific $CD8^+$ T cells led to total rejection of B16F10-OTI tumors and promoted intratumoral accumulation of both progenitor ($PD\text{-}1^+TCF\text{-}1^+GzmB^-$) and differentiated cytotoxic ($PD\text{-}1^+TCF\text{-}1^+GzmB^+$) host $CD8^+$ T cells, as compared to untreated controls and mice treated with suboptimal ACT, which results in tumor relapse. Moreover, ACT efficacy was greatly reduced in mice lacking host T cells (RAGKO mice) or mice treated with FTY720, which prevents tumor infiltration of host T cells but not transferred $CD8^+$ T cells. The efficacy of ACT was also decreased by depletion of host $CD8^+$, but not $CD4^+$ T cells. Furthermore, blocking the effector cytokine TNF-α reduced the infiltration of both progenitor and cytotoxic differentiated host, but not transferred $CD8^+$ T cells and impaired ACT efficacy. Mechanistically, ACT promoted activation and migration to draining lymph nodes of tumor-infiltrating type 1 conventional dendritic cells (cDC1). These effects were abrogated by TNF-α blockade. Finally, selective depletion of cDC1 in lymph nodes using Langerin-DTR mice led to decreased efficacy of ACT. Our findings reveal an interplay between transferred and host $CD8^+$ T cells, which underlies effective antitumor immunity in the context of ACT.

B056: Advancing NK cell immunotherapies for ovarian cancer through a patient-specific organoid co-culture system

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Ovarian cancer is the fifth leading cause of cancer deaths in females, with a 5-year survival rate below 30% for most patients. Yet, due largely to significant funding disparities, the standard of care remains virtually unchanged after decades. Moreover, while immunotherapy has

revolutionized the treatment of blood cancers and saved countless lives, the benefits have yet to be realized for solid tumors like ovarian. Ovarian cancer brings the unique challenges of frequent relapses, drug resistance, and high variability among tumors. Recent technological advances have enabled the development of organoids from tumor samples, yielding a revolutionary patient-specific cancer model with unlimited expansion capacity and unprecedented potential for personalized research and therapeutic testing. At the NYSCF Research Institute, we have implemented tumor organoid technology using fresh tumor resections from the NCI-supported Cooperative Human Tissue Network (CHTN) and our clinical collaborators at Memorial Sloan Kettering Cancer Center (MSK) and the Englander Institute for Precision Medicine at Weill Cornell Medicine (EIPM). We are building a large biobank of patient-specific organoid models as a collaborative research resource and precision medicine testing platform with the potential for clinical adoption to address the major challenges in ovarian cancer research and treatments. Organoid-based experimental systems capture human, patient-specific tumor biology and are amenable to therapeutic screening including immunotherapies. These features are critical to accelerate the clinical development of novel treatments and combinations and assess their efficacy across patient populations, including ethnic minorities who have been underrepresented throughout the research pipeline. To realize the potential of immunotherapy for ovarian cancer, we have started to leverage our ovarian cancer organoid technology and our human induced pluripotent stem cell (iPSCs) technology platform to test an emerging immunotherapy that uses natural killer (NK) cells to kill tumors, which has begun to show promise for treating solid cancers. In our experimental system, NK cells derived from iPSCs are combined with patient organoids to evaluate how genetically and clinically diverse patients will respond to this treatment, and whether additional targeted therapies enhance NK killing of certain tumors. This study will accelerate the development of NK cell therapies for ovarian cancer and the design of tailored combination therapies that improve patient outcomes.

B057: Oral cancer vaccine using recombinant Bifidobacterium longum displaying HPV16 E6 E7 proteins

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Introduction

Human papilloma virus (HPV) infection is strongly associated with the development of some types of cancers including cervical cancer, pharyngeal cancer, anal cancer. However, the vaccination rate of current parenteral HPV vaccines is lower in Japan than other developed countries. In recent years, the number of cervical cancer patients have been increasing in Japan and some developing countries even though HPV infection is vaccine preventable disease. One of the reasons for low vaccine coverage is vaccine hesitancy among age-eligible individuals, therefore, more accessible and safer vaccine modality may increase the vaccine coverage. Mucosal vaccine route may be more available because of its less invasiveness and utilizing common mucosal immune system to induce systemic immune responses. In this study, we constructed the *Bifidobacterium longum* displaying the HPV16 E6E7 proteins, which are associated with carcinogenesis in HPV-related cancers, for the oral vaccine for HPV and HPV-related cancers.

Methods

Bifidobacterium longum 105-A, a gut commensal probiotic bacteria, was transformed by using a shuttle vector containing the HPV16 E6E7 genes anchored with a gene encoding Galacto-N-biose/lacto-N-biose I-binding protein (GLBP) to display the proteins on bacterial cell surface. The pasteurized recombinant *B. longum* were orally administered multiple times into C57BL/6 mice to induce the HPV16 E6E7-specific cellular immune responses via intestinal immune system. Twenty thousand of TC-1 cells, a murine tumor cell line which were stably expressing HPV16 E6E7 protein, were subcutaneously inoculated into mice, and then the recombinant *B. longum* were orally administered as mentioned above to investigate the anti-tumor effect.

Results

We successfully produced the recombinant *B. longum* displaying HPV16 E6E7 protein and confirmed the protein expression by western blotting and flowcytometry. Oral administration of the recombinant *B. longum* significantly increased the proportion of both HPV16 E6-specific CD8T cells and HPV16 E7-specific CD8T cells which were secreting IFN-γ in mouse spleens compared with parental *B. longum* control treatment in mice ($p<0.05$, respectively). In TC-1 mice model, oral administration of recombinant *B. longum* displaying HPV16 E6E7 protein significantly decreased the tumor growth and prolonged overall survival compared with control treatment ($p<0.05$, respectively).

Discussion

In this study, we constructed the recombinant *B. longum* displaying HPV16 E6E7 proteins and demonstrated the anti-tumor effect against TC-1 by inducing antigen-specific CD8T cells in mice. These findings suggested that effective HPV-specific cellular immune responses were induced by the oral immunization, which were modulating intestinal immune system. Oral administration is considered as easier and safer route for vaccination, therefore, our recombinant *B. longum* may be a new accessible modality for the prevention and treatment for HPV-related cancer.

B058: Alternative splicing of CD20 5' untranslated exons modulates its protein expression and responses to immunotherapy

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CD20 is a B cell lineage marker that is expressed on the surface of most mature B cell neoplasms. CD20-targeting monoclonal antibodies, such as rituximab and more recently developed ofatumumab, have become standards of care for both aggressive lymphomas [Burkitt's (BL) and diffuse large B cell (DLBCL)] and indolent diseases [e.g., chronic lymphocytic leukemia (CLL)]. CD20-directed chimeric antigen receptors-armed T cells (CARTs) are also under clinical development. Despite these advances, up to half of all adult

DLBCL patients and one fifth of CLL patients are not cured by combined immuno/chemotherapy regimens. One known cause of treatment failure is down-modulation of CD20 protein expression, but the underlying molecular mechanisms remain largely unknown. Nor is it clear why surface CD20 levels are lower in CLL compared to BL and DLBCL. Here, we show that down-modulation of CD20 is often driven by alternative splicing of 5' untranslated region (5' UTR) exons of CD20 mRNA, yielding transcript variants with extended 5' UTRs. Such transcripts were found to be poorly translated, with protein synthesis rates less than one tenth of those driven by canonical isoforms, as determined by polysome profiling and full-length CD20 reporter assays. The inhibition was mediated by upstream open reading frames (uORFs) and a putative stem-loop structure-forming nucleotide sequence; removing these elements fully rescued CD20 expression. Notably, the translation-incompetent variants were prevalent in RNA-seq datasets corresponding to BL, DLBCL, and especially CLL samples, where they comprised the majority of CD20 transcripts. In our search of splicing factors responsible for these effects, we identified multiple consensus sites for the RNA-binding protein KHDRBS1 (a.k.a. Sam68), in the 5' UTR of CD20. Moreover, shRNA-mediated knock-down of KHDRBS1 in BL and DLBCL cell lines led to a shift in CD20 RNA splicing towards the canonical translation-competent variants, followed by increased CD20 expression and robust rituximab-mediated cytotoxicity *in vitro*. We achieved similar effects in a panel of BL, DLBCL and CLL cell lines by targeting the inhibitory 5' UTR exon with a splice-switching oligonucleotide (SSO) (a vivo-morpholino dubbed "5ex3"). Overall, our data suggest that CD20 expression and sensitivity to rituximab are diminished in a subset of BL, DLBCL and CLL samples due to the prevalence of translation-incompetent splice variants. We are currently investigating the role of CD20 splicing in both *de novo* and acquired resistance to CD20-directed immunotherapeutics by performing comprehensive RNA-seq analyses of paired diagnostic/relapsed non-Hodgkin lymphoma samples. Our findings will have important implications for overcoming resistance to both antibody- and CART-based immunotherapies.

B059: Longitudinal local and peripheral immunity associated with response to PD-L1 blockade in murine models of breast cancer

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Immune checkpoint inhibitors (ICI) have significantly enhanced patient survival in some cancer types but yield limited success in breast cancer. ICIs activate anti-tumor immunity by overriding the inhibition of tumor infiltrating lymphocytes (TILs). Clinical trials in triple negative breast cancer (TNBC) patients, who are more likely to harbor TILs within tumor stroma, have demonstrated increased progression-free survival (IMpassion130) and pathologic complete response (KEYNOTE-522) to ICI. Consequently, combinations of ICI and chemotherapy have been FDA-approved for metastatic TNBC. However, the therapeutic benefit of ICIs is highly heterogeneous among breast cancer patients; as such, we sought to model ICI response in vivo to evaluate therapeutic resistance and response heterogeneity, as well as ascertain predictive biomarkers for favorable outcomes to ICI in breast cancer.

We used an immunocompetent EMT6 orthotopic mammary tumor model to investigate the efficacy of ICI (anti-PD-L1). Analysis of the primary tumor immune landscape was performed by flow cytometry and single-cell RNA sequencing. Matched longitudinal samples of the tumor microenvironment (collected by fine-needle aspiration) and peripheral blood (PBMC) from mice were profiled by bulk RNA and T-cell receptor (TCR) sequencing to identify systemic genomic alterations and T-cell expansion, respectively.

Single-agent ICI robustly suppressed primary tumor growth ($p = 0.0046$) and extended survival ($p < 0.0001$) beyond the control group in the EMT6 model. The addition of chemotherapy (paclitaxel and/or doxorubicin) demonstrated moderate therapeutic efficacy but failed to enhance ICI benefit. Phenotypic profiling of the tumor microenvironment (TME) revealed increased T cells, dendritic cells, and NK cells in anti-PD-L1 only and chemotherapy combination groups. Despite using a genetically identical tumor model and murine host, we found that PD-L1 blockade induced heterogeneous responses, similar to clinical outcomes in breast cancer patients, ranging from complete response to intrinsic resistance. Analysis of the primary tumor microenvironment showed upregulated signatures of cytotoxic T cell response and activation, specifically inflammatory interferon signaling (both prior to and post ICI administration) that corresponded to favorable response to anti-PD-L1 in individual mice. Longitudinal analysis of the peripheral blood identified modest changes among mice at baseline that progressively deviated by response type (non-responders-vs-responder mice). Moreover, mice harbored enriched myeloid signatures and clonal T cell expansion during therapy corresponding to ICI resistance and response, respectively. Further investigations of matched peripheral blood and the primary tumor microenvironment signatures may identify systemic biomarkers and tumor antigen-specific T cell clones to accurately predict ICI response in patients and uncover mechanisms for sensitizing tumors refractory to ICI.

In conclusion, we identify a heterogeneously ICI-responsive in vivo model that emulates TNBC patient response to combinatorial ICI approaches. We describe host-specific signatures, specifically myeloid cell responses, that correlate with differential responses to immunotherapy, which may serve as a basis for tracking immunotherapy response in peripheral blood from breast cancer patients.

B060: In vitro modeling of CD8 T cell exhaustion identifies novel transcriptional regulator Bhlhe40

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Identifying novel molecular mechanisms of exhausted CD8 T cells (T_{ex}) is a key goal of improving immunotherapy of cancer and other diseases. However, high-throughput interrogation of *in vivo* T_{ex} can be costly and inefficient. *In vitro* models of T_{ex} are easily customizable and quickly generate high cellular yield, offering an opportunity to perform CRISPR screening and other high-throughput assays. We established an *in vitro* model of chronic stimulation and benchmarked key phenotypic, functional, transcriptional, and epigenetic features against *in vivo* T_{ex} generated during chronic LCMV infection. We then leveraged this model to perform pooled

CRISPR screening to investigate transcriptional regulators of T cell exhaustion. These results identified the transcription factor Bhlhe40 as a novel regulator of T_{ex} differentiation. *In vitro* and *in vivo* validation defined a role for Bhlhe40 in regulating a central differentiation checkpoint between progenitor T_{ex} and downstream intermediate and terminally differentiated subsets T_{ex}. Thus, by developing and benchmarking an *in vitro* model of T_{ex}, we demonstrate the utility of mechanistically annotated *in vitro* models of T_{ex}, in combination with high-throughput approaches, as a discovery pipeline to uncover novel T_{ex} biology.

B062: Discrepancies between empirical HLA ligand presentation and in silico prediction: Implications for T cell-based therapies

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The presentation of Human Leukocyte Antigen (HLA) bound peptides is a function of many different variables, including HLA and antigen expression, as well as protein abundance, degradation and transport. Therefore, it is challenging to define all presented HLA ligands from a given protein in the context of a specific HLA allele as some peptides generated from this antigen might be outcompeted by other peptides from more abundant proteins or not presented due to downregulation of HLA.

A powerful solution to define the complete set of HLA ligands from any single protein is the co-overexpression of one HLA allele and one protein of interest in a model cell line with no endogenous HLA expression, e.g. monkey fibroblast COS-7 cells[1]. We optimized this strategy so that only 10 million COS-7 cells co-transfected with mRNA encoding an antigen and HLA allele of interest are sufficient to identify the complete immunopeptidome derived from this antigen. Results from this model are highly reproducible between replicates as well as different protein/HLA pairs. Thus, this model system can overcome many of the above-mentioned limitations, and additionally - considering the very strong signals for peptide detection - ensure that non-detection of HLA ligands is due to non-presentation rather than technical limitations.

We compared the performance of netMHCpan4.1, the most commonly used *in silico* HLA binding prediction algorithm, with data generated using the COS-7 system with subsequent HLA ligand isolation and mass spectrometry analysis as described previously[2,3]. Altogether, we tested 10 different antigens in 7 HLA contexts: A*01:01, A*02:01, A*03:01, B*07:02, B*08:01, B*44:02, C*03:04. Only 24.7 % (95%-CI 22.2%-27.2%) of predicted HLA class I ligands were identified in our overexpression experiments, with high consistency between antigens and HLA alleles. Interestingly, most of the results clustered among the HLA ligands predicted to be the

best netMHC binders resulting in an overlap of predicted and detected HLA ligands for strong binders (%rank≤0.5) of 84.9%. In contrast, for weak binders (0.5<%rank≤2.0), this overlap was considerably lower at only 10.4%; we also frequently detected HLA ligands that were considered non-binders by netMHC. These empirical findings have important implications for the prediction of T cell targets or immune checkpoint therapy response models, which are often based on these predictions and suggest that strict cutoffs are required to avoid biases in these models.

Finally, we exemplify the importance and consistency of our findings with one COS-7 experiment (A*03:01/Wilms Tumor Protein) in which we identified only 25.0% (4/12) of netMHC-predicted HLA ligands. We then performed HLA ligand isolation and targeted mass spectrometry for the 4 previously unreported hits identified in the COS-7 system on one WT1 positive AML cell line (U937) and one primary AML sample. Only two of the four novel identified HLA ligands were detected, which could be a result of the variables mentioned above influencing HLA ligand presentation in more physiological settings.

Overall, these data demonstrate the importance of HLA ligandomics as the gold standard for defining T cell targets in contrast to *in silico* prediction algorithms. We further hypothesize that current binding prediction models could highly benefit from HLA ligand data generated from overexpression models to provide datasets with truly complete ligandomes of the investigated antigens. Ultimately, this might help to better understand the rules for antigen processing and presentation and to improve HLA ligand prediction models.

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B063: Multimodal single cell profiling of CD8+ T cells in NSCLC to predict tumor reactivity

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Personalized cancer immunotherapies such as adoptive cell transfer have shown remarkable results over recent years. Still, a large percentage of patients does not respond to these treatments. One major hurdle is the highly variable, and often unknown, fraction of tumor reactive T cells in the expanded tumor infiltrating lymphocyte (TIL) product. Thus, increasing the number of reactive T cells might improve therapeutic outcome. A major limitation for personalized application is the amount of labor necessary to identify reactive T cells. To this end, previous publications have shown certain levels of enrichment of tumor reactive T cells through the use of exhaustion markers. A more defined phenotypic characterization may be necessary to identify and use tumor reactive T cells with high accuracy and possibly non-exhausted cell states in therapeutic applications as well as in patient classification.

Here, we performed multimodal characterization of TILs that can be separated into two workflows. In the phenotypic workflow, we conducted various single cell sequencing methods, such as scRNA, scVDJ, scATAC & scRNA from the same cell (multiome sequencing), on CD8+ sorted TILs from 10 non-small cell lung cancer (NSCLC). The functional workflow consists of functional reactivity testing of TCRs against autologous tumor tissue (aTT), tumor associated antigens (TAA) or viral peptides. In detail, by introducing synthesized TCRs from TILs into our previously developed TCR testing platform (TnT, Vazquez et al. 2021 under revision at Immunity) via Cas9 integration into the endogenous TRBC locus, we disentangled the TCR from its cell state of origin and provide stable cell lines for various co-cultures.

Multiome sequencing analysis of TILs allowed us to identify gene regulatory networks in dysfunctional and cytotoxic TILs and transcription-factor-motif relationships. Functional testing resulted in enrichment of 10 TCRs after aTT or TAA co-culture and enrichment of 11 TCRs after viral peptide co-culture across 5 patients. Phenotypic analysis of aTT T cell clones compared to viral peptide T cell clones, showed differential gene expression of known markers such as CXCL13 but also novel markers such as KLRB1, TNFRSF18 and CTSH.

In conclusion, our integrated multimodal analysis pipeline exploits state-of-the-art methods in single-cell genomics, computational analysis, genome engineering and functional screening of CD8+ TILs from NSCLC. This allowed for an unbiased TCR reactivity and phenotypic TIL assessment, resulting in the identification of new markers and patient sample integration on the multi-omic level.

B064: Resolving the functional protein nanoscale organization of T cell bispecific antibody (TCB)-induced immunological synapse using LUX-MS

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T cell bispecific antibodies (TCBs) are potent cancer immunotherapeutics that redirect T cells against cancer. Recent studies show that TCBs, by virtue of crosslinking T cells to tumor cells, can induce the formation of immunological synapses that functionally mimic physiological immune synapses. However, despite the functional similarity, TCB-induced synapses show different spatial and temporal architecture, suggesting that different receptor-ligand interactions may occur during this process. To identify cell surface proteins that sustain the TCB-induced synapse, we utilized the recently developed optoproteomic technology LUX-MS, that enables the discovery and assessment of protein-protein interactions in (cis) and between living cells (trans) using light-controlled singlet oxygen generators (SOG). First, we established a co-culture model of naive T cells and MKN45 gastric cancer cells in the presence of Cibisatamab (CEA-TCB) or a TCB construct recognizing the murine CD3 moiety (that cannot bind human CD3), which thus served as negative control (CEA-muCD3). Next, we labeled Cibisatamab with SOG and confirmed comparable functionality to its unconjugated counterpart. To identify an amenable time point for studying synapse formation, we performed FACS-based time-course experiments

in which we identified 14h as an ideal time point for optoproteomic synapse architecture analysis. Our proteogenomic analysis revealed cell surface protein candidates involved in the acute organization of the TCB-induced synapse inbetween gastric cancer cells and primary T cells. Integrative pathway analysis showed that the majority of the identified cell surface proteins belonged to integrin-mediated signaling networks, heterotypic cell-cell adhesion networks and extracellular structure organization. Validation of the functional role of these targets in sustaining TCB-mediated effects are currently ongoing. In conclusion, we successfully established a strategy enabling the time-resolved analysis of the TCB-induced surfaceome synapse at the molecular nanoscale level. The generated data provides a rational path forward to target validated hits synergizing with TCB efficacy, and to decrease the threshold of TCB-induced T cell activation for therapeutic application.

B065: Unraveling tumor-associated neutrophils' origin at single-cell resolution

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Neutrophils dominate the immunological landscape of multiple solid tumors and are responsible for chronic inflammation and cancer development. Consequently, their presence has been associated with poor disease outcomes in cancer patients. This project aims to characterize the specific alterations of granulopoiesis during cancer progression at single-cell resolution, in order to identify cues driving cancer-associated neutrophils. We applied a multiparametric flow cytometry method called Infinity Flow (266 markers, LegendScreen system), followed by single-cell RNAseq, and functional assays in four different orthotopic cancer models (melanoma, lung, breast, and pancreatic cancer) to evaluate if the granulopoiesis alterations are specific to each tumor type. In parallel, we tracked neutrophil dynamics in cancer, from granulopoiesis to circulation and tumor infiltration, using iLy6G^{tdTom} mice, a property mouse model that permits tracking synchronous waves of neutrophils (tdTomato) released from the bone marrow. Multiparametric flow cytometry analysis was also performed to validate candidate markers of tumor-associated neutrophils in bone marrow (BM), blood, and tumors. From the different orthotopic cancer models in this study, the lung carcinoma model showed the strongest induction of granulopoiesis, evidencing expansion of neutrophil progenitors and a large accumulation of neutrophils in blood, spleen, and primary tumors. Multiparametric cytometry identified BM granulocytic lineages from tumor-bearing mice and UMAP projections allowed visualization of a continuum of neutrophil development. We identified the largest changes in the lung cancer model, where PDL1 was upregulated, suggesting a pre-education of neutrophils already in the BM to become tumor-supportive. Further analysis of the developmental path of neutrophils in the BM of tumor-bearing mice suggested that lung cancer induces an alternative pathway of maturation of neutrophils. Thus, cancer-associated alterations to neutrophils can be imprinted early during their development in the BM and these alterations are tumor-type specific. Single-cell RNA seq analyses of BM and lungs from the lung cancer model showed enrichment of immature forms of neutrophils. Healthy lungs exhibit at least two distinct clusters of tissue-specific neutrophils: Cluster 1 featured immune defense functions and represented the majority of the lung neutrophil population, whereas a small second cluster (Cluster 2) featured a pro-angiogenic and immunosuppressive signature. In tumor-bearing lungs Cluster 1 disappeared while Cluster 2 expanded, and additional clusters appeared that associated with BM neutrophils,

including signatures for proNeus, preNeus, and immature neutrophils. Cluster 2 was characterized by high expression of CD14, Cxcl3, Ccl3, Ccl4, and PDL1 expression (among others), and predicted high glycolytic metabolism and immunosuppressive activity. We validated these markers using a 16-color panel for flow cytometry and observed that the population of CD14+ neutrophils could be detected in BM and blood of lung cancer-bearing mice, indicating that tumors induce the generation and systemic circulation of tumor-associated neutrophils. Importantly, functional assays confirmed the increased angiogenic and immunosuppressive function of lung neutrophils. Using our iLy6G^{tdTom} mice we observed not only an extended lifespan of neutrophils in lung cancers but also that CD14 is induced early in the pro-tumoral education of neutrophils, while markers such as PDL1 appeared at later times. We conclude that neutrophils acquire tumor-associated features as they mature in the bone marrow and raise the possibility of targeting granulopoiesis to prevent the onset of pro-tumoral immunity. Further, the identification of markers expressed early in neutrophil development could help clinicians identify patients at risk of developing lung cancer, or predict when this cancer will metastasize.

B066: Longitudinal monitoring of immunotherapy response by visualization of T cell activation markers in tumor bearing mice using immunoPET

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In recent years, cancer immunotherapies (CIT) aiming to activate endogenous T cells have proven to be indispensable alternatives to existing therapies in the treatment of advanced tumor diseases. Thus, for monitoring these novel therapies, characterizing the functional status of T cells, such as activation and exhaustion, could allow for an early and accurate prediction of therapy response. In this study, we investigated the utility of immunoPET, (PET imaging using radiolabeled antibodies), of the T cell surface activation markers CD69 (early-) and OX40 (late expression) for monitoring activated T cells and CIT responses non-invasively in a syngeneic mouse tumor model over 7 days.

Both αCD69 and αOX40 monoclonal antibodies (mAb) were conjugated to the chelator deferoxamine and radiolabeled with zirconium- 89 (^{89}Zr). The radiochemical purities and construct stabilities were assessed by HPLC over 7 days. Murine CD69 and OX40 expression kinetics on activated T cells were assessed by flow cytometry for 7 days. The specific binding of both radiolabeled antibody conjugates *in vitro* was determined using activated murine T cells and CD69 and OX40 expressing cell lines. For *in vivo* evaluation, C57BL/6N mice were injected

with murine MC38 colon carcinoma cells subcutaneously. Tumor-bearing mice were either intravenously injected with ^{89}Zr-Df-αCD69 or ^{89}Zr-Df-αOX40 and were treated with a combinatory CIT composed of αPD-1/4-1BB or isotype control mAb 1 day and 4 days post tracer injection. Serial PET imaging was performed just prior to (baseline, day 0) and on days 1-7 post CIT administration. For *ex vivo* validation, organs were collected, and tissue-associated radioactivity was measured by y-counting after the terminal PET scan. Additionally, CyTOF studies were performed to analyze activation marker expression on immune cell infiltrates in the tumor. Tumor growth ratios (calculated as the volume at the last imaging time point divided by the volume at the baseline scan) of <1.5 were considered responsive to treatment.

Both constructs were stable over time and high radiochemical purities of >95% were consistently achieved for both radiolabeled antibody conjugates with specific binding capacities of >60%. In the αPD-1/α4-1BB mAb treated group, 50% of mice showed significant inhibition in tumor growth when compared to the sham-treated control group. CD69-immunoPET revealed a significant increase in ^{89}Zr-Df-αCD69 tumor uptake in responding (10.23% injected dose per cubic centimeter (%ID/cc) ± 2.19 SD) compared to non-responding (6.88%ID/cc ± 2.11 SD) and control mice (6.74%ID/cc ± 0.79 SD) on day 4 post CIT start. Imaging of the late T cell activation marker OX40 revealed a significantly higher ^{89}Zr-Df-αOX40 uptake in tumors of responding mice 7 days after onset of treatment (11.09%ID/cc ± 1.35 SD; non-responding: 5.76%ID/cc ± 2.37 SD; control: 6.98%ID/cc ± 1.55 SD). Tumor-to-muscle ratios were significantly higher for both tracers in responding mice on days 4 and 7, respectively, whereas organ uptake values in the lymphatic and clearance organs were similar across all groups. *Ex vivo* biodistribution analyses on day 7 confirmed the *in vivo* PET results. CyTOF experiments revealed higher OX40 expression on $CD4^+$ T cells, whereas CD69 was highly expressed on both $CD4^+$ and $CD8^+$, and on NK cells, B cells, and myeloid cells.

In conclusion, immunoPET of both CD69 and OX40 allows for longitudinal monitoring of the T cell activation and detects distinct temporal kinetics for each marker and immune sub-populations associated with their expression patterns. This approach enables the visualization of T cell activation and identification of CIT responders at early stages post CIT initiation, highlighting the potential utility as a new strategy for therapy monitoring and early treatment adjustments.

B067: Spatiotemporal measurements of cytokines, immune cells, and cancer invasion in the tumor microenvironment

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Introduction: Cancer is a complex and heterogeneous disease, an ecosystem architecture that involves far more than just cancer cells. A diverse cast of components in the surrounding microenvironment interact with cancer cells and actively facilitate malignant progression across and within a 3-Dimensional (3D) organization. We have developed a 3D ex vivo culture platform that enables the controlled delivery of media and removal of waste metabolites via perfusion in Liquid-Like Solids (LLS) made from a soft granular microgel bed that acts as both a 3D support medium and provides an open porous network mimicking a capillary-bed. Using

integrated fast scanning laser confocal microscopy we have developed techniques to make in situ spatiotemporal measurements of cytokine concentrations, track immune cells in the tumor microenvironment, and study tumor invasion dynamics in 3D.

Materials and Methods: Glioblastoma tumor microexplants (200-400 μm) were created from resected cancers obtained from properly consented donors. Purified PBMCs were labeled with CellTrace CFSE and co-cultured in 3D Liquid Like Solid (LLS) with Organoid liquid media and supplements. Spatiotemporal cytokine profiles were measured by taking advantage of LLS stability; using 3D printing in LLS we are able to precisely print arrays of ELISA beads or create large random field of ELISA beads across the experiment to measure local concentrations of cytokines in situ. Understanding the bead kinetics (cytokine on-rates and off-rates), coupled with measurements of each bead's fluorescence at a specific time and position from the tumor periphery, allows for fitting of sophisticated spatiotemporal reaction-diffusion models to quantify the tumor's production rate, tumor margin concentrations, and the entire immuno-regulatory micro-environment. 3D imaging of immune cells and cancer cells at 5-minute intervals is used to create sequential time-lapse images and movies that can be analyzed frame-by-frame to track 3D positions, motion, proliferation, action, cell death, and elimination. Data is collected at sufficiently high resolutions and temporal fidelity to track immune cells, quantify tumor evolution-dynamics, and T cell killing. Surface conjugation of the LLS microgels with type 1 collagen (COL1-LLS) enabled cell adhesion to the LLS and facilitated adhesion-dependent opportunistic cancer invasion into accessible spaces independent of proteolytic activity. Cell tracking is enabled through novel Artificial Intelligence (AI) algorithms that were originally developed for astrophysics data processing.

Results and Discussion: 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. Invasive fronts of the micro-tumor protruded into proximal interstitial space and analysis of these invasive paths revealed super-diffusive behavior of these fronts. Off-lattice agent based computational simulations reveal that the interstitial space guided tumor invasion by restricting available paths and this mechanical restriction is responsible for the super-diffusive behavior. Additionally, the COL1 bioconjugation reveals that glioblastoma cancer cells utilize anchorage-dependent migration to explore their surroundings, and geometrical cues guide 3D tumor invasion along the accessible paths independent of proteolytic capability. 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 μm/min. Average CD8+ T cell killing rates were ~3 cancer cells/h, which decreased monotonically after 12 hours to approximately 1 cancer cell/h.

Conclusions: The in vitro immuno-oncology platform with in situ fast scanning fluorescence microscopy was able to quantify spatiotemporal concentrations of cytokines, T Cell motions and activity, and tumor invasion dynamics.

B068: InteractPrint predicts clinically meaningful interactions between cancer epithelial cells and immune cells: Lessons from a single-cell breast cancer atlas

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BACKGROUND: While immunotherapy has revolutionized the treatment of many solid tumors, the efficacy of immunotherapy regimens is comparatively lower in breast cancer. Immunotherapy efficacy is often negatively correlated with intratumor heterogeneity. Novel immunotherapy approaches in breast cancer should leverage how cancer epithelial cell heterogeneity affects immune cells in the tumor microenvironment. However, current definitions of cancer epithelial cell heterogeneity in breast cancer have limited resolution. Single cell RNA-seq (scRNA-seq) provides an unprecedented opportunity to further define cancer epithelial cell heterogeneity and identify how heterogeneity influences interactions with immune cells.

METHODS: We generated a novel scRNA-seq dataset of 236,363 cells from 119 primary breast tumors biopsied from 88 patients taken from 8 publicly available datasets, currently the largest published scRNA-seq dataset in breast cancer. To define cancer epithelial cell heterogeneity, we performed unsupervised clustering and supervised clustering based on molecular subtype and expression of clinical target genes on all cancer epithelial cells. This identified 11 gene elements (GEs), which reflect key molecular features that vary between cancer epithelial cells. Receptor-ligand pairing analysis allowed us to determine how cells that highly express each GE interact with various immune cells. We developed InteractPrint, a score to predict the predominant tumor-interacting immune cells, based on the GE composition of an individual patient tumor.

RESULTS: In our dataset, 17% of samples were HER2+, 41% were HR+, and 42% were TNBC. This dataset was statistically powered to characterize cancer epithelial cell heterogeneity. For each of the 11 GEs, we predicted interactions with immune cells. Experimentally, GEs with predicted NK cell interactions showed sensitivity to NK cell cytotoxicity. In a spatially resolved transcriptomics dataset, GEs with predicted T cell interactions demonstrated colocalization with CD8+ T cells, while those with limited predicted T cell interactions did not. To infer GE-immune interactions at the patient level (GEs define cell-level interactions), we developed InteractPrint. To validate InteractPrint, we assessed the accuracy of the T cell InteractPrint in predicting response to anti-PD-1 therapy. Across two trials and all breast cancer subtypes, T cell InteractPrint demonstrated significant improvement over PD-L1 in predicting response to anti-PD-1 therapy. In an scRNA-seq dataset of samples from patients treated with pembrolizumab, we observed AUC of 85% ($p < 0.005$) for T cell InteractPrint vs. 61% ($p > 0.05$) for PD-L1 in predicting response to anti-PD-1 therapy. In patients treated with paclitaxel + pembrolizumab in the I-SPY 2 trial, we observed AUC of 81% ($p < 0.00001$) for T cell InteractPrint versus 72% ($p = 0.001$) for PD-L1.

CONCLUSIONS: Our results demonstrate considerable cancer epithelial cell heterogeneity across primary breast tumor samples and clinical subtypes. We defined this heterogeneity and leveraged it to predict immune cell interactions within a patient's tumor. We developed T cell

InteractPrint to capture heterogeneous interactions between cancer epithelial cells and CD8+ T cells. T cell InteractPrint is predictive of response to anti-PD-1 immune checkpoint inhibition at higher AUC than PD-L1. This provides a path forward for the interpretation of cancer epithelial cell heterogeneity in a clinically meaningful way.

B069: Space-time mapping identifies concerted multicellular patterns and gene programs in healing wounds and their conservation in cancers

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Tissue repair responses in metazoans are highly coordinated by different cell types over space and time. However, comprehensive single-cell based characterization covering this coordination is lacking. Here, we captured transcriptional states of single cells over space and time during skin wound closure, revealing choreographed gene expression profiles. We identified shared and prominent space-time patterns of cellular and gene expression enrichment: which we call multicellular 'movements' and which spanned multiple cell types. We validated some of the discovered space-time movements using large volume imaging of cleared wounds and demonstrated the value of this analysis to predict gene products made by macrophages or fibroblasts, which activated gene programs in the opposite cell type. Finally, using two different tumor models, we tested the hypothesis that tumors are like 'wounds that never heal' finding conserved wound healing movements in the tumor space, wherein some movements were preferentially used in one tumor versus another.

B070: B7-H3 dimerization activates oncogenic signaling and increases proliferation

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As B7-H3 (*CD276*), a member of the B7-family of immune regulators, has gained considerable traction as an emerging cancer immunotherapy target, new studies have started to re-probe the functional significance of expression of B7-H3 and the molecular mechanisms that support a mostly pro-oncogenic role in tumor development. Although it is well established that tumor-expressed B7-H3 correlates with poor prognosis and advanced disease for many tumor types, the physiological functions remain unclear. No cognate ligand or receptor(s) have been identified, and differential effects on immune cell activation have been reported, thus there are many unanswered questions about the structural functional state of activated B7-H3 and its direct impact on tumorigenesis and immune evasion. Herein, we confirmed an oncogenic role of tumor expressed B7-H3 in gynecological cancers which could be ablated following crispr knockout of *CD276* gene expression and rescued upon re-expression of 4Ig B7-H3. We measured *in vitro* clonogenic growth ($p<0.01$) and *in vivo* tumor volume and survival ($p<0.05$), suggestive of a direct impact on tumorigenesis, as well as direct activation of known oncogenic signaling pathways when B7-H3 was expressed. As previous evidence suggests a role for protein-dimerization of the B7-family of immune regulatory proteins to elicit an immune response, we sought to determine whether B7-H3 dimerized, and if dimerization related to the oncogenic functions of B7-H3. Capitalizing on recent advances in spatiotemporal characterization of protein-protein interactions (PPIs) using non-invasive fluorescence and luminescence-based approaches, we tested the hypothesis that B7-H3 dimerization/multimerization contributes to its

oncogenic signaling, promoting cancer cell survival and immune evasion. Using a recombinase-enhanced bimolecular luciferase complementation (ReBiL 2.0), we cloned the human 4Ig isoform of B7-H3 into the construct to interrogate homodimerization and multimerization. Using a bi-directional doxycycline regulated promoter, 4Ig B7-H3 was expressed with both an n-Luciferase and c-Luciferase fragment and a HA tag linker at near physiologic levels. We observed luciferase bimolecular complementation following B7-H3 expression by bioluminescence imaging ($p<0.001$) and confirmed B7-H3 dimerization using a second non-invasive imaging technique: fluorescence lifetime imaging microscopy (FLIM). Upon 4Ig B7-H3 expression and dimerization, cell proliferation and oncogenic signaling increased. By kinase array analysis, increases in PI3K/AKT signaling, Jak/Stat pathway, and modulators of HIF1α and NF-κβ pathways were observed, all which are known to support increased oncogenic functions, such as increased glycolysis, increased survival and proliferation, as well as cytokine modulation and immune evasion. These studies provide the first direct evidence that B7-H3 utilizes the process of self-association to regulate its function and promote tumorigenesis.

B071: Spatial transcriptomics identifies key gene signatures of tertiary lymphoid structures and immune perivascular niches in human glioblastoma

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Glioblastoma (GBM) is a highly aggressive brain cancer, which is poorly infiltrated by T cells and is thus resistant to T cell-reactivating immunotherapies. Tertiary lymphoid structures (TLS) are ectopic lymphoid aggregates which form in inflamed tissues and are associated with a positive prognosis in numerous cancers. Recently, we discovered that the presence of TLS correlates with increased T cell presence in human GBM, making them attractive targets to improve T cell infiltration. To date however, the role and diversity of lymphoid aggregates in GBM remain to be better clarified.

In this study, we coupled immunofluorescence (IF) staining techniques and spatial transcriptomics to identify, classify and study the composition of lymphoid aggregates in GBM patients. Paraffin sections of human GBM tumors were stained for T cells, B cells and vessels, and various lymphoid clusters 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” depending on their B cell to T cell ratio. Next, we profiled the annotated lymphoid aggregates using the GeoMX® spatial transcriptomics platform. In line with our manual annotation, K-means-based clustering analysis identified five independent clusters: B-TLS, T-TLS, mixed TLS, B-PN and T-PN.

B cell-dominant structures (B-TLS and B-PN) exhibited a classical B follicular signature, expressing higher levels of genes associated with B cells, immunoglobulins, follicular helper T cells and fibroblastic reticular cells. Interestingly, the expression of germinal center markers and major histocompatibility complex (MHC) genes was stronger in B-TLS than in B-PN, which

instead displayed a higher memory signature, indicating that they likely exert different roles in the tumor. T cell-dominant structures (T-TLS and T-PN) exhibited higher levels of genes associated with helper T cells, dendritic cells and macrophages, while mixed TLS displayed a clearer cytotoxic T cell signature. Notably, the expression of MHC molecules was low in T-TLS, but strong in mixed TLS and T-PN, suggesting that they may be more efficient sites of T cell priming.

In conclusion, this study highlights that lymphoid aggregates in human GBM are diverse and likely to exert different functions depending on their nature and their B to T 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.

B073: STAT6 D419N mutation promotes microenvironmental remodelling in a murine model of B-cell non-Hodgkin's lymphoma

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Diffuse large B cell lymphoma (DLBCL) is the most common type of B cell non-Hodgkin's lymphoma, and 40% of patients relapse or progress following treatment [relapsed/ refractory DLBCL (rrDLBCL)]. rrDLBCL is fatal in 90% of patients, and thus, characterizing tumors with mutations enriched at relapse may define novel therapeutic options. Previous work in our lab demonstrated that mutations in the DNA-binding domain of STAT6, hereby $STAT6^{D419N}$, are enriched in rrDLBCL tumor cells. In *in vitro* models, $STAT6^{D419N}$ upregulates expression of similar gene targets as $STAT6^{WT}$ upon IL-4 stimulation, but with increased magnitude. Some of the target genes that are upregulated by $STAT6^{D419N}$ are associated with intercellular interactions, migration, and chemotaxis, thus, we hypothesized that $STAT6^{D419N}$ remodels its tumor microenvironment to recruit cells that provide a growth advantage and/or resistance to therapy. Using the Eμ-myc model of aggressive lymphoma, cells transduced with $STAT6^{WT}$ or $STAT6^{D419N}$ were transplanted into C57BL/6 mice via tail vein injection. Over the course of disease, inguinal and cervical lymph node size was monitored via ultrasound, and mice were sacrificed 8-, 11-, and 14-days post-injection for immunophenotyping by flow cytometry or PhenoCycler imaging. At 8-days post-injection, the tumor size and cellular composition between $STAT6^{WT}$ and $STAT6^{D419N}$ tumors was identical. However, by 11-days post-injection, $STAT6^{D419N}$ tumors were larger, but with a lower percentage of tumor cells, and the increased mass of $STAT6^{D419N}$ tumors was found to be associated with an increase in CD4+ T cells. Additionally, $STAT6^{D419N}$ tumors began to upregulate expression of the checkpoint inhibitor protein PD-L1 earlier in disease progression (ie. day-11) and by day-14, $STAT6^{D419N}$ tumors had increased differentiation of CD4+ cells into immunosuppressive regulatory T cells. Interestingly, patient biopsies from STAT6-mutant rrDLBCL also had a significant increase in CD4+ T cells as compared to patient biopsies which were STAT6-WT, suggesting that this mechanism is relevant in the patient population. Overall, our data suggests that $STAT6^{D419N}$ tumors become progressively more immunosuppressive, thus; our results will have an important impact in

informing therapeutic avenues, specifically immunomodulatory therapies, for patients with STAT6-mutant rrDLBCL.

B074: Deep learning-based quantification of tertiary lymphoid structures predicts outcome in solid tumor patients

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Tertiary lymphoid structures (TLS) are dense accumulations of lymphocytes in inflamed peripheral tissues including cancer. In multiple solid tumors, TLS are associated with improved survival and response to immunotherapy. Thus, TLS quantification in histological images has been proposed as a novel biomarker for patient risk and treatment stratification. Routine pathology analysis is based on hematoxylin and eosin (H&E) staining, which allows robust recognition of most TLS owing to their unique morphology. However, there is no standardized methodology available for objective TLS detection in multiple tissue types.

Here, we developed a novel artificial intelligence-based approach called HookNet for automated and unbiased TLS quantification and maturation assessment in H&E images. The HookNet model integrates contextual information with high-resolution details, which allows defining structures detectable only at lower magnification and at the same time identifies morphological features at high magnification like the presence of germinal centers. We took advantage of the large diagnostic image repository of the Cancer Genome Atlas (TCGA) to train and validate our model using three distinct solid tumor types with known prognostic associations for TLS: clear cell renal cell cancer, muscle invasive bladder cancer and lung squamous cell carcinoma. All diagnostic slides were initially checked for their tissue content and only slides containing tumor with adjacent normal tissue were included in the analysis. TLS and germinal centers were exhaustively manually annotated in all selected slides and used as ground truths for the training and performance validation of the model. Around 20% of slides from each tumor type were included in the training set, around 7% - in the validation set and the rest of the slides were used as an independent test set for the assessment of the model's performance in survival analysis. During each training iteration, false positive predictions were sampled as hard negative annotations used in the next training round.

Both, manual and HookNet-predicted TLS counts revealed significant differences between overall TLS densities among the three tumor types with lung and bladder cancers having the highest and kidney cancer – the lowest average TLS density. Analysis of high-resolution features of the manual annotations showed that inflamed regions containing dense unorganized infiltrates are indistinguishable from TLS. Here, the unique capacity to integrate the broader context from a lower magnification with the high magnification features proved essential to distinguish TLS from such infiltrates by HookNet. When analyzing the independent test set, we saw a high concordance between HookNet-predicted and manual TLS counts in all tumor types with a consistently higher TLS detection sensitivity by HookNet. Furthermore, HookNet and not manual TLS annotations showed a significant reduction in TLS size in tumors where TLS development is hampered. Analysis of matched clinical data demonstrated similar prognostic

associations for HookNet-predicted and manually-defined TLS counts, namely positive prognosis in lung cancer and bladder cancer and negative prognosis in kidney cancer.

In summary, we have developed a deep-learning-based algorithm for automated unbiased TLS detection compatible with different tumor types and sources of H&E images that can be used for large cohort studies.

B075: Simultaneous PET/MRI using 64Cu-NOTA-anti-CD206 and 19F-PFC provides new insights into the role of macrophages in cancer immunotherapy

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Immune checkpoint Inhibitors are novel and promising immunotherapeutics that improve the therapy outcome of many solid cancers, but the clinical response rates are still low (from 20-40%). Hence, uncovering the underlying molecular and cellular mechanisms of treatment success or failure and the interplay of different immune cells is a key step toward understanding antitumoral immune responses. It was recently shown that pH-changes of the tumor microenvironment (TME) impacts the polarization of tumor-associated macrophages (TAMs) during tumor development. Our study aimed to analyze the dynamics of immune-suppressive mannose receptor CD206 expressing TAMs and phagocytes non-invasively *in vivo* by simultaneous positron emission tomography (PET)/magnetic resonance imaging (MRI) in a therapeutic setting.

For this multimodal *in vivo* imaging approach, we have employed a novel cross-reactive (human/murine) CD206-specific radiolabeled Nanobody (^{64}Cu-αCD206) combined with ^{19}F-Perfluorcarbon (^{19}F-PFC) for simultaneous determination of the presence of TAMs and phagocytes via PET/MRI. OVA-MC38 colon adenocarcinoma bearing C57BL/6 mice were treated with bicarbonate ($HCO3^-$, Bic; *ad libitum* resolved in drinking water) and with αPD-L1 mAbs or isotype (Iso) mAbs. ^{64}Cu-αCD206-PET/^{19}F-PFC-MRI was performed 24h/48h after i.v. injection of ^{64}Cu-αCD206 and ^{19}F-PFC, respectively, at two time points: before the onset (baseline) and after two weeks of treatment (follow-up)). The *in vivo* PET/MRI results were cross-correlated by mulitcolor flow cytometry.

First,^{64}Cu-αCD206 yielded a radiochemical purity of 99%, *in vitro* bead- and cell-based studies revealed strong incorporation of ^{19}F-PFC by bone marrow (BM)-derived macrophages but not by the tumor cells or T cells. In addition, a shift from physiological to acidic pH (7.4-6.8),

mimicking acidic pH within the TME, did not affected the *in vitro* ^{19}F-PFC-uptake of BM-derived macrophages. We also analyzed the *in vivo* dynamics of TAMs by PET/MRI during combined immunotherapy consisting of αPD-L1 mAbs and pH-neutralization (Bic) over two weeks (baseline and two weeks of treatment). To determine the anatomical localization of the TAMs in the tumor and in the lymphatic organs for image analysis, we co-registered the ^{64}Cu-αCD206-PET- and ^{19}F-PFC-MR signal with T2-weighted MR images. The signal of ^{64}Cu-αCD206 in the OVA-MC38 tumor increased during the two weeks of Bic + Iso mAbs-treatment (tumor/muscle (T/M) ratio: 4.7±0.7 (SEM) to 13.9±1.7), suggesting an increase of suppressive TAMs in the TME. Surprisingly, the addition of PD-L1 blockade (Bic+αPD-L1, n=7) exhibited a similar effect (T/M ratio: 3.6±0.7 to 14.1±1.1). In sharp contrast, the ^{19}F-PFC signal within MC38 tumors dropped in both groups during two weeks of treatment (Bic: 94.9±10.0 arbitary units (a.u.) to 45.3±1.9 a.u., Bic+PD-L1: 83.3±7.4 a.u. to 43.3±1.4 a.u.). Moreover, *in vivo* ^{19}F-PFC MRI for both experimental groups (e.g., spleen and muscle) showed consistent and stable uptake (spleen: Bic: 86.6 ±14.0 to 63.9±8.0 a.u., Bic+αPD-L1: 60.9±3.1 to 56.8±4.0 a.u.; muscle: Bic: 45.6±2.3 to 39.0±3.5 a.u., Bic+αPD-L1: 39.6±3.3 to 36.7±4.9 a.u.). *Ex vivo* flow cytometry of F4/80$^+$ TAMs isolated from OVA-MC38 tumors revealed an increase in CD206$^+$ expression (Bic: 8.74±1.41 to 14.83±3.98, Bic+PD-L1: 3.71±0.56 to 24.07±4.23 %of F4/80$^+$ TAMs) in both experimental groups.

To conclude, we detected dynamic changes, more specifically an increase in the ^{64}Cu-NOTA-anti-CD206 Nb PET- and decrease of ^{19}F-PFC MRI-uptake, accompanied by an increasing CD206$^+$ TAM population in the TME of OVA-MC38 bearing mice after pH neutralization independent of the blockade of the PD-1/PD-L1 axis. These findings emphasize the translational use of the cross-reactive ^{64}Cu-NOTA-anti-CD206 Nb in the clinical setting as non-invasive *in vivo* imaging of the temporal dynamics of suppressive TAMs might serve as an early biomarker for tumor progression.

B076: Longitudinal monitoring of CD8 T cell migration dynamics in response to cancer immunotherapy

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CD8$^+$ cytotoxic T cells (CTLs) are the main actors in antitumoral immunity. Several studies demonstrated their prognostic value for immunotherapy response in solid tumors. ImmunoPET provides the opportunity to non-invasively decode immune cell motility, distribution, and homing. Engineered scFv-C_H3-antibody fragments (minibodies) have optimized distribution and clearance properties particularly for PET imaging, capable of both patient stratification and guiding immunotherapies. Currently, the radiolabeled human CD8 targeting minibody ^{89}Zr-Df-Crefmirlimab is being evaluated in a phase II clinical study (NCT05013099). In this project, we investigated whether its mouse CD8-directed counterpart is applicable to longitudinally monitor

the CTL migration and homing dynamics during immune checkpoint blockade (ICB) in a syngeneic tumor mouse model.
The desferrioxamine conjugated murine CD8 targeting minibody IAB42 (DFO-mCD8-Mb) was radiolabeled with ^{89}Zr. Labeling efficiency was assessed via HPLC and TLC. The immunoreactive fraction of ^{89}Zr-DFO-mCD8-Mb and its cellular internalization were tested on freshly isolated murine CTLs. MC38 murine colon adenocarcinoma cells were injected subcutaneously in C57BL/6 mice. When the tumors reached a diameter of ~6-8 mm, mice were treated either with 500 µg of αPD-L1/αLAG-3 mAbs, or 100 µg of αPD-1 mAbs. To block lymphocyte migration from the lymphoid organs, 20 µg of Fingolimod (FTY720) was repetitively administered intraperitoneally. 2 MBq/10 µg of ^{89}Zr-DFO-mCD8-Mb was injected intravenously one day before therapy initiation. Simultaneous PET/MR scans were performed 24 h post-injection (baseline, day 0) and 1-7 days post-treatment to follow T cell migration into the tumor and lymphatic organs. Mice were sacrificed after the last imaging time point for *ex vivo* organ biodistribution analysis and immunohistochemistry. Tumor growth ratios (volume d7/d0) <1.5 were considered treatment responders.
The radiolabeling efficiency of ^{89}Zr-DFO-mCD8-Mb was constantly >90.0%. The immunoreactive fraction, tested on CTLs, yielded 95.4±0.7%. Rapid tracer incorporation into CTLs was achieved with 32.3±0.3% of the ^{89}Zr-DFO-mCD8-Mb being internalized within 4 h of incubation. Based on the *in vivo* MC38 tumor growth rates, 7 mice out of 15 were identified as responsive to αPD-L1/αLAG-3 treatment. ^{89}Zr-DFO-mCD8-Mb uptake in the tumor of responding mice (2.58±0.34 %ID/ml) was two-fold higher compared to the uptake in the non-responding group (1.31±0.23 %ID/ml) seven days after ICB onset, which was confirmed by *ex vivo* biodistribution analysis. Interestingly, the longitudinal *in vivo* tumor uptake graphs of both groups separated already at an early time point of 2 days post ICB, suggesting that longitudinal imaging of CTL migration allows for early response evaluation. A second cohort of experimental tumor-bearing mice was treated with a suboptimal ICB dose of αPD-1 mAbs to mimic the clinical situation of a huge variety of treatment responses. In line with the first experiment, we were able to differentiate therapy responses as early as 2 days after treatment initiation by PET. Importantly, FTY720 treatment resulted in significantly lower ^{89}Zr-DFO-mCD8-Mb tumor accumulation compared to the responding mice, proving that the PET-uptake is derived from immigrating CTLs into the tumor. We further identified the spleen with increased tracer uptake by *ex vivo* biodistribution analysis as a potential source for anti-tumoral CTLs that migrate to the tumor during effective ICB.
The internalized ^{89}Zr-DFO-mCD8-Mb and the long half-life of ^{89}Zr enable the longitudinal monitoring of the migration of endogenous CTLs in response to ICT. Furthermore, our data emphasize the feasibility to non-invasively determine the individual outcome to immunotherapy at early time points

B077: scFLUX: a web-based metabolic flux and variation predictor for single cell data
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A key challenge in studying metabolic variation of in tumor microenvironment (TME) is to assess the metabolic reaction of different reactions, i.e., the metabolic fluxome, in different cells in a TME. We have recently developed a novel computational method, namely scFEA (single

cell Flux Estimation Analysis), to infer cell-wise metabolic flux from single cell RNA-sequencing (scRNA-seq) data. ScFEA is empowered by a comprehensively reorganized human metabolic map, a novel probabilistic model to integrate flux balance assumption with scRNA-seq data, and a novel graph neural network architecture based solution, which enables an accurate estimation of cell-wise metabolic flux by tackling the challenges in handling the complex metabolic network and the non-linear dependency between transcripts level and reaction rate. We further presented scFLUX.org (http://scflux.org/), which provides an online platform for prediction of metabolic fluxome and variations using transcriptomics data, on individual cell level. To the best of our knowledge, scFLUX is the first and only web-based tool dedicated to predicting individual sample-/cell- metabolic flux of different metabolic networks and variations of metabolites using transcriptomics data. We have optimized scFLUX to estimate cell-wise fluxome of central metabolic pathway and the whole metabolic map of human and mouse, which has been demonstrated as a powerful tool to study metabolic shifts in cancer, stromal, and immune cells in TME.

Glutaminolysis has been considered as a major carbon and energy source that promote the proliferation of cancer cells. Recent studies reported immune cells trend to use more glucose while cancer cells use more glutaminolysis for ATP production. However, the observations made on cell line or limited mouse orthotopic models may not reflect the general metabolic shifts in real human cancer tissue. We reconstructed subcellular-compartment-specific metabolic network and applied scFLUX to characterize the flux and distribution of glucose and glutamine metabolism in TME, including production of lactate (glycolysis), TCA cycle, nucleic acids synthesis, glutaminolysis, glutathione and amino acids synthesis, in multiple cancer types and normal tissue, by using TCGA, CCLE cell line and GTEx transcriptomics data, and 14 sets of scRNA-seq data of human cancer and mouse orthotopic tumors. Our analysis confirms the increased influx in glucose uptake and upper part of glycolysis and decreased upper part of TCA cycle in cancer cells. Increased lactate production and second half of TCA cycle were only seen in certain cancer types. More interestingly, we did not see cancer tissues have highly shifted flux from glutamine to TCA cycle comparing to normal tissue samples in human cancer. In addition, cancer cells have highest glutaminolysis rate compared to stromal and immune cells in tumor microenvironment (TME). And we did not observe stromal and immune cells have higher glucose consumption than cancer cells. Further analysis illustrates immune cells may have higher potential in glucose uptake (by glucose transporters), while their downstream consumption of glucose is much lower than cancer cells. A systems biology model of metabolic shifts and competition in TME is further developed.

B078: Neoadjuvant immunotherapy prevents metastatic relapse in MSS Colorectal cancer
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The effects of immunotherapy have been often investigated at individual timepoints during tumor progression but an understanding of these responses during the evolution of the disease is lacking.

Our study primarily focuses on characterizing the tumor microenvironment of residual disseminated tumor cells, which regenerate metastatic disease in distant organs after primary

CRC resection. To do so, we established a human-like CRC mouse model that, following surgical resection of the primary tumor, undergoes metastatic relapse. Using this model, we discovered that at the onset of metastasis, pre-existing T cell immunity against the primary CRC attacks disseminated cancer cells as they reach the liver. Metastatic cells, however, constrain T clonal expansion by upregulating PD-L1. During this phase, neoadjuvant checkpoint immunotherapy given before surgical removal of the primary tumor is sufficient to eradicate micrometastases. Yet, this curative effect is restricted to a narrow temporal window as a result of a rapidly evolving TME.

Adjuvant immune-checkpoint therapy is currently part of the standard of care for MSI/mismatch repair-deficient CRC patients, but it does not exert therapeutic benefits in MSS/mismatch repair-proficient metastatic CRCs. Besides the lower neoantigen burden of MSS CRCs, data in experimental models and patients suggest that the TME of MSS CRCs excludes and limits the activity of T-cells. Our findings reveal that residual metastatic cells lodged in foreign organs lack TME and are susceptible to the attack of the adaptive immune system upon immunotherapy treatment. This window of vulnerability could be exploited to prevent metastatic relapse. Our results back up current efforts to use neoadjuvant immunotherapy in early-stage MSS CRC patients. This and other therapeutic strategies capable of eliminating DTCs may prevent disease relapse if applied before the metastatic disease is overt.

B079: Divergent transcriptional immune cell states in colorectal primary tumors and liver metastases

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While mismatch repair sufficient colorectal cancer (CRC) metastases generally do not respond to immune checkpoint inhibitors of the PD-1/PD-L1 axis, radiological responses have been reported in mismatch repair sufficient primary colorectal tumors. To understand these different response patterns, previous studies focussed on comparing cell types and their proportional changes in CRC primaries and metastases. However, differences in the transcriptional states within cell types and their biological relevance remain unclear. Here, we generated single cell RNA sequencing data from tumor-infiltrating leukocytes in synchronously-resected primaries and liver metastases from 27 CRC patients. We define cell state changes within each cell type by analyzing the fold change of cells from primary and metastatic tumors within cellular neighborhoods of the k-nearest neighbors graph using the miloR package. While several cell types were almost exclusive to either liver metastases (NK/NKT-cells) or primary tumors (plasma cells, germinal center/memory/naïve B cells, mast cells, ILC3) other cell types were present in both primaries and metastases but showed disparate gene expression states (CD4 T cells, CD8 T cells, macrophages, dendritic cells). We retrieved the transcriptional programs defining these disparate cell states using SPECTRA. SPECTRA is a factor analysis method that defines general and cell type specific gene programs and their activation by adapting user-provided annotations of these programs to the data under analysis. Comparing cell states

enriched in metastases vs. primary tumors, SPECTRA revealed higher TH17 program expression in metastasis CD4 T cell states, features of tumor-reactivity in metastasis CD8 T cell states, and traits of TNF-alpha signaling in metastasis TAM/DC states. Hence, our study uncovered divergent cell states defined by their expression of continuous gene programs in CRC primaries and liver metastases and provides a blueprint to interpret cell state changes within cell types in single cell RNA sequencing data.

B080: Spatial meta-transcriptomics reveal associations of intratumor bacteria burden with lung cancer cells showing a distinct oncogenic signature

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The lung intratumor microbiome influences lung cancer tumorigenesis and treatment responses, but detailed data on the extent, location, and effects of microbes within lung tumors is missing, information needed for improved prognosis and treatment. To address this gap, we developed a novel spatial meta-transcriptomic method simultaneously detecting the expression level of 1,811 host genes and three microbe targets (bacteria, fungi, and CMV). After rigorous validation, we analyzed the spatial meta-transcriptomic profiles of tumor cells, T cells, macrophages, other immune cells, and stroma in surgically resected tumor samples from12 patients with early-stage lung cancer. We discovered that bacterial burden was significantly higher in tumor cells compared to T cells, macrophages, other immune cells, and stroma. This burden increased from tumor-adjacent normal lung and tertiary lymphoid structures to tumor cells to the airways, suggesting that lung intratumor bacteria derive from the latter route of entry. Expression of oncogenic β-catenin was strongly correlated with bacterial burden, as were tumor histological subtypes and environmental factors. Hence, intratumor bacteria were enriched with tumor cells and associated with multiple oncogenic pathways, supporting a rationale for reducing the local intratumor microbiome in lung cancer for patient benefit. This research is supported by the Intramural Research Programs of NCI and NIAID.

B081: Temporally-resolved scRNA-seq atlas of PyMT mouse lungs reveals tumor-mediated reprogramming of both infiltrating and tissue-resident myeloid cells during metastatic progression.

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Cancer metastasis involves a complex interplay between metastatic ‘seeds’ (i.e., disseminated tumor cells) and the microenvironmental ‘soil’ (i.e., stromal cells and infiltrating/tissue-resident immune cells) at distant organ sites. Most efforts to treat metastasis have focused on the ‘seed’ (e.g., therapies aiming to selectively kill metastatic tumor cells), but have had limited success. As a result, treating metastatic cancer by targeting the microenvironmental ‘soil’ has garnered

renewed interest in recent years, with particular emphasis placed on the immune system. Indeed, the immune system is thought to represent the 'Achilles heel' of metastatic progression, as tumor-mediated immune reprogramming is both necessary for metastasis and represents a potential axis of vulnerability which can be exploited by novel immunotherapies.

Despite this potential, systematic analyses of how distant organ sites are reprogrammed during the course of metastatic progression have not been described. To address this unmet need, we used sample-multiplexed single-cell RNA-sequencing (scRNA-seq) to build a temporally-resolved 'cell atlas' of lungs isolated from the polyomavirus middle T antigen (PyMT) mouse model of metastatic breast cancer. Specifically, we generated and analyzed scRNA-seq data representing >300,000 lung immune and stromal cells from 33 mice (11 time-points in triplicate) spanning the extremes of metastatic progression – namely, before primary breast tumor detection (6 and 7 weeks of age), during pre-metastatic niche and micro-metastatic lesion formation in the lung (8-13 weeks), and when lungs become inundated with macro-metastatic lesions (14 and 15 weeks).

These analyses reassuringly-revealed a multitude of literature-supported shifts in the lung immune compartment (e.g., transient uptick in the proportion of infiltrating neutrophils, arrival of myeloid-derived suppressor cells, depletion of lymphocytes and dendritic cells, suppression of T-cell cytotoxic responses, etc.) and yielded a series of unexpected insights. For instance, we identified a coherent transcriptional profile (e.g., increased CD14 expression, cytokine secretion, NF-kB signaling, etc.) that mirrors previous observations in splenic and primary tumor myeloid-derived suppressor cells (MDSCs; Alshetaiwi et al., 2020, Science Immunology; Veglia et al., 2021, Journal of Experimental Medicine) but which is expressed by both infiltrating and tissue-resident myeloid cells. This MDSC signature was not seen in lungs isolated from tumor-free mice, was detected prior to micro-metastatic lesion formation in PyMT mice, and scaled with metastatic progression – supporting its role in promoting lung metastasis.

While preliminary, the detection of the MDSC signature in tissue-resident myeloid cells in PyMT lungs suggest that the mechanistic underpinnings of the MDSC signature may extend beyond altered neutrophil differentiation in the spleen (as shown by Alshetaiwi and colleagues) or reprogramming of myeloid cells proximal to the primary tumor (as shown by Veglia and colleagues) and include system-level communication networks which directly influence the lung microenvironment (and perhaps other distant organ sites). Considered collectively, this longitudinal scRNA-seq atlas will empower future efforts in the field of metastatic cancer immunology as it systematically outlines shifts in lung immune and stromal cell gene expression states, cell population composition, and intercellular communication patterns in a mouse model where primary tumors and metastases arise spontaneously and in the presence of a fully-intact immune system.

B082: Understanding 3D neutrophil migration using PCA-based cell morphological analysis

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Neutrophils are fast-moving innate immune cells that play important roles in the initiation and progression of multiple cancers. Although the ability of neutrophils to migrate in 3D is essential to mediate their immunological activity, relatively little is known about the mechanics of this process. Neutrophils rapidly deform and contort themselves to navigate the fibrous extracellular matrix, resulting in complex and dynamic morphology. Cellular morphology encodes information about the cell intrinsic mechanisms and environmental factors that define it. Recent analytical advances using spherical harmonic transformation and principal component analysis (PCA) have improved our ability to understand 3D cellular morphology. This new 3D shape analysis allows us to identify and visualize the primary ways in which 3D cell shape varies by reconstructing the shapes of average cells that represent each mode of variation. I probed the limitations of this new methodology by performing simulations using simple shapes with known dimensions of shape variation and determined that the method is highly sensitive to object alignment and resolution. To capture the shapes of migrating neutrophils, I fluorescently labeled the membranes and nuclei of dHL-60 neutrophil-like cells and imaged both labels at high resolution during 3D cell migration in collagen gels with instant structured illumination microscopy. In this data, I found that basic metrics of cell and nuclear shape, including surface area, volume, and elongation, are highly correlated with one another. To determine the primary modes in which dHL-60 shape varies, I performed PCA-based 3D shape analysis using dHL-60s cells aligned to their trajectories. Migration speed was most correlated with the top PC, which represented cell elongation along the axis of trajectory and alignment of the cell's long axis to the axis of trajectory. Collective shape analysis of CRIPSRi knockdown HL-60 cell lines for two candidate genes from a 3D motility screen, FMNL1 and CORO1A, along with control cells, revealed significant differences in a number of PCs that represented cell elongation as well as cell and nuclear volume. This work demonstrates that 3D shape analysis can be a useful tool in understanding the mechanics of neutrophil migration in 3D, but also has great potential as a platform for phenotypic comparison.

B083: SNS-101, a monoclonal antibody that is highly selective for VISTA in the tumor microenvironment, demonstrates favorable pharmacokinetic and cytokine release characteristics and potentiates anti-PD-1 responsiveness

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Introduction: VISTA (V-domain Ig suppressor of T-cell activation) is an immune checkpoint, which suppresses T-cell activation and is highly expressed on myeloid cells, including macrophages and neutrophils. 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 three major factors: 1) lack of clarity on the identity of the critical counter-receptor responsible for T-cell suppression; 2) high clearance via target-mediated drug disposition (TMDD) by $VISTA^+$ neutrophils and monocytes at physiologic pH and; 3) cellular activation and cytokine release syndrome (CRS) at sub-therapeutic doses by engagement of VISTA in the blood.

Objective: To prevent TMDD and mitigate potential CRS we developed SNS-101, a human monoclonal IgG1 antibody specific for the protonated, active form of VISTA, and interrogated its inhibitory potential of VISTA interacting with proposed binding partners at low (6.0) and physiological pH (7.4). CRS was tested *in vitro* and *in vivo* in a humanized mouse model. The pharmacokinetic (PK) profile and anti-tumor efficacy in combination with anti-PD1 of SNS-101 was assessed in syngeneic tumor models utilizing target-expressing human VISTA knock-in (KI) mice.

Experimental Procedures: VISTA binding to PSGL-1, VSIG-8, Syndecan-2, LRIG-1 and VSIG-3 was determined at pH 6.0 and pH 7.4 followed by competition with SNS-101. CRS risk was evaluated in an *ex-vivo*system using circulating fresh human whole blood over a dose range between 1µg/mL and 100µg/mL and *in vivo* in human $CD34^+$ cord blood cells reconstituted BRGSF mice. PK studies were conducted in human VISTA-KI mice and compared to wild type (target-null) mice. Anti-tumor efficacy was assessed in VISTA-KI mice implanted with the syngeneic tumor models, MC38 and 1956.

Summary: Among the candidate binding partners, VISTA binds strongest to PSGL-1 at pH 6.0, but not at pH 7.4 whereas VSIG-3, VSIG-8 and LRIG-1 interactions are generally weak at both pH 6.0 and 7.4. SNS-101 inhibits the interaction between VISTA and PSGL-1 and all other potential partners with IC_{50}'s below 7nM. Epitope mapping corroborates SNS-101's blockade of the PSGL-1:VISTA protein interface. *Ex-vivo* CRS assays (IL-2, IL-6, IFN-γ, TNF-α) indicate no significant cytokine induction by SNS-101 compared to the clinical stage, non-pH-selective anti-VISTA antibody JNJ-61610588 (now CI-8993). An *in vivo* CRS model demonstrates that CI-8993 induces IL-6, IL-10, CCL-2 CCL-5, CXCL-8 CXCL-10, IFN-γ, TNF-α, and IL-1RA in a dose dependent-manner, whereas SNS-101 only very weakly induced CCL-5. PK studies show linear elimination kinetics in target-bearing VISTA-KI mice vs. target-null mice and indicate selective binding and target-mediated clearance in the TME of tumor-bearing VISTA-KI mice. Anti-tumor efficacy studies demonstrate that SNS-101 enhanced anti-PD-1 response in MC-38 and re-sensitized anti-PD-1 resistant 1956 sarcoma tumors, resulting in tumor rejection.

Conclusions: Our results demonstrate that PSGL-1 interacts pH-dependently with VISTA and that SNS-101 potently inhibits this interaction. *In vitro* and *in vivo* CRS assays indicate that SNS-101 has a significantly lower risk of inducing CRS than non-pH-dependent VISTA antibodies. PK studies demonstrate that rapid clearance by TMDD has similarly been eliminated. In multiple syngeneic tumor models, SNS-101 demonstrates significant enhancement of anti-tumor effects in combination with anti-PD-1 antibodies. Taken together, these data demonstrate that SNS-101's exquisite selectivity for active, protonated VISTA abrogates TMDD and lowers CRS risk, while preserving the ability to significantly enhance the anti-tumor effects of PD-1 blockade.

B084: Human anti-human Epidermal Growth Factor Receptor polyclonal antibodies block ligand binding and increase survival of non-small cell lung carcinoma xenograft mice

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Human polyclonal antibodies (pAbs) generated from the Diversitab™ transchromosomic bovine platform are being evaluated in preclinical studies and human clinical trials for infectious disease, autoimmune disorders, and transplant-rejection indications. Using this platform technology, specific tumor targeted pAbs to treat human cancers are also under development. The Epithelial Growth Factor Receptor (EGFR) mediates downstream cell signaling pathways that control fundamental cellular functions. Although several anti-EGFR monoclonal antibodies, such as cetuximab, are approved for clinical use to treat EGFR-positive non-small cell lung cancer (NSCLC), colorectal cancer, and head and neck cancer, tumors which initially respond to these monoclonal antibodies eventually become resistant. Because better immunotherapies are needed, we produced a human anti-EGFR pAb (SAB-162E) and herein report pre-clinical *in vitro* and *in vivo* results. Consistent with the ability of pAbs to bind multiple epitopes on protein targets, SAB-162E bound EGFR at higher levels than cetuximab as measured by both ELISA analysis with recombinant proteins and a cell surface binding assay. Binding to the EGFR truncation mutant, EGFRvIII, was analyzed by ELISA, and SAB-162E had a higher level of binding to EGFRvIII compared to cetuximab. SAB-162E demonstrated complete blockade of EGF ligand binding to EGFR-positive cells and altered EGFR downstream signaling pathways by decreasing the phosphorylation status of Erk1/2, Akt and p38. Functionally, this signaling blockade led to a decrease in cellular proliferation. SAB-162E decreased *in vitro* cellular migration of a human NSCLC cell line containing a wild-type EGFR signaling pathway. Additionally, SAB-162E effector functions were investigated including complement dependent cytotoxicity (CDC) and antibody dependent cellular cytotoxicity (ADCC). SAB-162E activated CDC on *EGFR*-expressing cells, while cetuximab did not. To measure ADCC activity, a reporter assay with FcgRIIIa Jurkat effector cells was used and demonstrated that SAB-162E initiated ADCC. Lastly, using a human NSCLC cell line-derived xenograft (CDX) model in immunodeficient mice, treatment with SAB-162E decreased tumor growth and almost tripled survival time. These findings provide evidence that SAB-162E represents a promising first-in-class human pAb therapeutic for EGFR-positive cancers and the potential use of the Diversitab™ platform to produce oncology therapeutics.

B085: Prevalence of intratumoral and circulating extrafollicular memory B cells is associated with disease progression in cancer patients

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Many patients with recurrent/metastatic (R/M) cancer fail to produce a durable response to immunotherapy and other cancer treatments. Thus, there is a need to identify new biomarkers that can help predict risk of recurrence and monitor disease progression. Tertiary lymphoid structures (TLS) were recently shown to predict response to immune checkpoint inhibitors (ICI) and survival in cancer. Further, the presence of germinal centers (GCs) within TLS can predict the risk of recurrence in cancer patients. Additionally, memory B cells (MBCs) which are key products of GCs, correlate with survival in several solid tumors and predict response to ICI. This highlights the biomarker potential of MBCs and TLS. However, it is unclear if intratumoral MBCs are propagated from GCs within TLS in patients or via a GC independent maturation pathway (extrafollicular; EF), and how this impacts their anti-tumor function. We addressed these questions by assessing the prevalence, phenotype, and function of GC and EF associated MBC populations in tumors and peripheral blood (PBL) of patients with locally advanced (LA) or R/M head and neck squamous cell carcinoma (HNSCC) as well as patients with metastatic melanoma and lung cancer. We also report on the spatial location of GC and EF associated MBCs in HNSCC using multispectral immunofluorescence imaging.

Using spectral flow cytometry and in vitro small-scale functional assays, we observed an expansion of EF associated double negative (DN) MBC subsets: Tbet $^{+/-}$ CD11c^{-} CD21^{-} (DN3) and Tbet^{+} CD11c^{+} CD21^{-} (DN2) in the PBL of patients with HNSCC, melanoma, and lung cancer. DN3 EF MBCs are hyporesponsive to antigen stimulation, poor antibody producers and fail to differentiate into antibody secreting cells (ASC) while GC derived MBC subsets retain these functions. Circulating DN3 and DN2 share a B cell exhaustion-like program which includes: Tbet, Tox, CD85J, CD72 and LAIR1 in cancer patients. Higher frequency of intratumoral DN3 EF MBCs in HNSCC is observed in HPV- patients and is associated with advanced tumor stage. Further, higher numbers of circulating DN3 EF MBCs were predictive of melanoma patients who progressed on anti-PD1 therapy. These findings support further clinical assessment of drivers of EF vs GC B cell responses in cancer. Additionally, the potential of EF and GC B cell responses to be used as biomarkers to monitor disease progression following treatment should be further investigated.

B086: NX-1607, a small molecule inhibitor of the CBL-B E3 ubiquitin ligase, promotes favorable local and systemic changes in innate and adaptive immune cells in multiple mouse tumor models

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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 target cell killing.

Here we characterize the antitumor and immune effects of NX-1607, a potent, specific and orally bioavailable intramolecular glue inhibitor of CBL-B, in three individual mouse tumor models: the subcutaneous CT26 colon carcinoma, the metastatic 4T1 triple negative breast tumor and the A20 syngeneic B cell lymphoma. We show that oral administration of NX-1607 induced significant tumor growth inhibition in all three tumors. In the 4T1 tumor model, when administered in a neo-adjuvant setting, NX-1607 therapy induced durable complete responses (CRs) in 13 out of 24 animals surviving at 140 days, compared with 2 out of 24 surviving animals for the control. Mice treated with NX-1607 demonstrated immunological memory, with 92% of mice with CRs successfully rejecting tumor growth upon rechallenge with 4T1 tumor cells.

Immunophenotyping studies of tumor infiltrating lymphocytes (TILs) showed an increase in antigen-specific CD8+ T cells in 4T1 tumor-bearing mice treated with NX-1607. Tumor infiltrating antigen-specific CD8+ T cells displayed lower expression of the exhaustion markers TIM3 and LAG-3 in mice treated with NX-1607. Within the CT26 tumor, NX-1607 treatments doubled the ratio of CD8+ to Treg TILs and significantly increased the frequency of the cytotoxic marker Granzyme B within NK cell subsets.

Profiling of circulating cells from mice treated with NX-1607 showed increased antigen-experienced PD-1+ CD8+ T cells and an increased percentage of circulating CD8+ T cells co-expressing activation markers (CD44 and 4-1BB/CD137) and memory markers (CD44, CD127 and CD27). Moreover, we showed that following NX-1607 treatment, the observed increase in antigen-experienced CD8+ T cells in the blood correlated with antitumor response (day 19 post treatment, $p < 0.001$).

Collectively, these studies provide insight into the *in vivo* activity of this novel inhibitor of CBL-B, demonstrating that NX-1607 displays single agent antitumor activity in multiple preclinical tumor models and functions to enhance innate and adaptive immune responses that may help overcome a suppressive tumor microenvironment. These findings also deliver experimental support for clinical development of this novel CBL-B E3 ligase inhibitor, NX-1607. We have initiated a clinical trial with NX-1607 in patients with advanced solid tumors NX-1607-101 (NCT05107674).

B087: Polymorphonuclear Myeloid Derived Suppressor Cells Die by Ferroptosis in the Tumor Microenvironment

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Myeloid-derived suppressor cells (MDSC) in the tumor microenvironment (TME) function as an immunosuppressive shield that protects the tumor from the host's immune system and considered a barrier to effective immunotherapy. Here, we focused on polymorphonuclear (PMN)-MDSCs, the most prevalent MDSCs in the TME, to identify mechanisms regulating their maintenance, turnover, and accumulation. Using four mouse models of cancer including autochthonous pancreatic adenocarcinoma from KPC genetically engineered mice (KrasG12D/p53R172H, PdxCre), we found that PMN-MDSCs spontaneously die by ferroptosis, a non-apoptotic form of regulated cell death triggered by the discoordination of regulatory redox mechanisms culminating in massive peroxidation of polyunsaturated phospholipids. Only PMN-MDSCs within the TME were observed to spontaneously undergo ferroptosis. Further, ferroptosis-related gene expression in $CD11b^{+}L6C^{low}Ly6G^{+}$ PMN-MDSC isolated from bone marrow, spleen, and tumor also demonstrated tumor-specific ferroptosis across tumor models. In humans, whole transcriptomic analysis of PMN-MDSC sorted from tumors and matched blood of lung cancer patients vs blood of healthy donors revealed up-regulation of genes involved in the regulation of ferroptosis in tumor PMN-MDSC. Ferroptosis gene signatures correlated with the PMN-MDSC signatures in pancreatic cancer patients and was associated with worse overall survival. Thus, ferroptosis is an unappreciated, prominent pathway of cell death of PMN-MDSCs in cancer linked to clinical outcome in patients with pancreatic cancer.

B088: Pharmacological reprogramming of dysfunctional tumor-reactive infiltrating lymphocytes (TILs) isolated from glioblastoma enhances generation of progenitor central memory CD8+ T cells retaining a high potential for a personalized cell therapy

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Glioblastoma (GBM) is one of the deadliest and most hard-to-treat cancers. Despite the first classification of GBM as an immunologically "cold" tumor, recent studies have revealed the presence of tumor-infiltrating lymphocytes (TILs) in the tumor microenvironment (TME), providing a glimmer of hope for the development of cell therapy strategies.

We have optimized a protocol for isolating tumor-reactive TILs from Cavitron Ultrasonic Surgical Aspirator (CUSA) emulsion. Tumor-reactive TILs were identified using the expression of CD137 as a marker. CD8+ and CD4+ TILs isolated from 40 GBM themselves expressed appreciable levels of CD137 ($p<0.0001$ vs. peripheral blood mononuclear cells), supporting the possibility of isolating the tumor-reactive TIL subset. CD137+ TILs were enriched to a median purity of 84%, using immunomagnetic separation. After enrichment, tumor-reactive TILs were activated and expanded in the presence of TexMACS, OKT3, irradiated feeder, IL-15, IL-7, and IL-21.

CD137+ TILs successfully expanded in 70% of cases. In these cases, TILs showed an exponential expansion between 14 and 21 days (from $1.0x10^7 \pm 4.5x10^6$ to $2.2x10^8 \pm 2.9x10^7$ TILs). Enrichment of CD8+ TILs was measured from day 10 (37.2 ± 6.7%) to day 21 (88.2 ±

5.2%) when precisely TILs acquired an effector function. The antitumor activity was evaluated in a co-culture assay using matched primary GBM cells (E:T = 3:1). Tumor-reactive TILs showed a strong effector ability, secreting higher levels of IFNγ compared to CD137neg counterparts (2700.0 ± 96.0 vs. 80.7 ± 12.0 pg/ml/1X10^5 TILs, respectively).

Pharmacological inhibition of Akt was performed using two different dosages of Akt1-2 inhibitor (0.3 and 0.7 uM). This treatment induced an early enrichment of CD8+ T cells, starting from day 10. The proliferation ability of treated CD8+ TILs was reduced in a dose-dependent manner immediately after the first four days of stimulation, maintaining a low rate of expansion up to day 16. Notably, TILs preserved a progenitor phenotype by the expression of TCF1 and persisted in a central memory phase expressing high levels of CD62L without any effector activity when specifically stimulated. This phenotype lasted over time even after five stimulations delaying the terminal effector differentiation.

These preliminary results support that the pharmacological inhibition of Akt signaling during ex vivo expansion can drive TIL-dysfunction reprogramming by enhancing the antitumor central memory subset and potentially improving their antitumor efficacy once reinfused back into patients.

B089: pHLIP-targeted delivery of STINGa to tumor stroma and myeloid immune cells powers tumor eradication and the development of immune memory

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The progression of immune-excluded “cold” tumors is associated with the formation of dense stroma consisting of acidic cancer-associated fibroblasts (CAFs) and tumor-associated macrophages (TAMs), generating immuno-suppressive signals that impair cytotoxic T-cell function. Targeting of STINGa by pHLIP to CAFs, TAMs, myeloid derived suppressor cells (mMDSCs) and dendritic cells (DCs) and the resulting activation of cytokines triggers complete eradication of CT26 tumors in Balb/c mice (90% incidence rate) and the mice remain tumor-free for 60 days after a single pHLIP-STINGa administration. Mice given the same dose of untargeted STINGa develop tumors within 20-35 days. The tumor stroma was completely destroyed after a single pHLIP-STINGa injection. The number of CAFs, which constitute 25% of all tumor cells, was reduced by 98%, and intratumor hemorrhage was observed. Cytokine signaling (increase in level of TNF-a and IL-6 within TME), destruction of the tumor stroma, and reduction of intra-tumoral interstitial fluid pressure are expected to lead to the recruitment of T-cells and their free penetration into the tumor mass. We also assessed the level of acidity in the treated tumors and showed that the acidity within the tumor mass was significantly reduced compared to the control group. Thus, not only did T-cells have access to the tumor core, but also the T-cell cytotoxic activity was not impaired by an acidic environment.

When mice, which remained tumor-free after a single injection of pHLIP-STINGa on day 1, were inoculated with CT26 cells on day 61, 15 out of 18 animals (83%) failed to develop tumors for another 40 days (totaling 100 days after single IP/IV injection of pHLIP-STINGa). Thus, a single pHLIP-STINGa injection promoted the development of strong T-cell immune memory.

The importance of the T-cells was confirmed in a study performed on athymic nude mice lacking T-cells, where pHLIP-STINGa showed no significant therapeutic efficacy on CT26 tumors. Intra-tumoral hemorrhage was observed suggesting that the tumor stroma could be destroyed, but the absence of T-cells resulted in an absence of tumor eradication.

Along with our studies of small (100 mm^3) tumors, we also examined the efficacy of pHLIP-STINGa for the eradication of large (400-700 mm^3) CT26 tumors. A single IP injection of pHLIP-STINGa into mice bearing large CT26 tumors led to tumor eradication in 7 out of 10 treated animals, with complete eradication in 20-40 days. Large tumors have more cancer cells (80% cancer cells vs 35% cancer cells in small tumors), but also have a higher proportion of myeloid cells (87%) and a lower proportion of $CD3^+$ cells (7%). Thus, successful treatment of large tumors may indicate a path for the eradication of immunosuppressive tumors.

We note that a single injection of pHLIP-STINGa could be combined with immune checkpoint inhibitors, T-cell engagers, CAR T-cell therapies or ADCs to enhance the access and activity of these therapeutics to the tumor.

The pHLIP technology potentially allows the transformation of immuno-activating agents into potent therapeutics, since pHLIP can target and deliver these agents to cancer cells, tumor stroma and myeloid cells. As opposed to delivery to specific receptors on the surface of particular cells, pHLIP offers targeting of the majority of metabolically active cells within the TME. Since the TME is complex, such general targeting and delivery leads to a significant synergistic effect. A single injection of pHLIP-STINGa induces the production of cytokines, obliterates the tumor stroma and increases the tumor pH, resulting in the eradication of tumors and the development of immune memory.

B090: Preclinical development of CodaLytic™, a novel codon-modified influenza virus, as a potent immunotherapeutic agent

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Oncolytic viruses (OVs) of multiple species have been demonstrated to induce beneficial changes in the tumor microenvironment (TME), increasing immune cell infiltration and activating stimulatory immune responses, which ultimately support induction of an anti-tumor immune response. The majority of engineered OVs achieve tumor specificity by either gene deletion or mutations and are then armed with immunomodulatory transgenes to promote anti-tumor immune responses. Codon/codon pair modification using Codagenix's SAVE platform is an alternative approach that retains the full natural immunostimulatory capacity of a viral species while leveraging innate immune sensing and apoptosis defects in cancer cells together with receptor overexpression for tumor selectivity.

CodaLytic is a novel virotherapeutic derived from influenza virus strain A/California/07/2009, that is synthetically engineered to contain over 600 silent mutations in hemagglutinin and neuraminidase genes. Efficacy and survival after intratumoral injection of 10^8 PFU three times a

week for up to 4 weeks as a monotherapy and in combination with anti-PD-1 checkpoint blockade were determined in EMT6 triple-negative breast cancer, B16F10 melanoma and MC38 colon carcinoma mouse models. Pharmacodynamic changes in the TME after treatment were characterized using flow cytometry and transcriptomics. Anti-tumor immune memory was assessed by interferon-γ ELISpot in splenocytes of long-term survivors and by tumor re-challenge.

CodaLytic monotherapy of orthotopic EMT6 tumors led to significant tumor growth inhibition by 76%, translating into a significant survival benefit with a 66% cure rate. Tumor growth was inhibited by 69% in subcutaneous B16F10 tumors, which was further improved upon by combination with PD-1 blockade to 86%. Intravenous re-challenge of EMT6 long-term survivors led to a 27-fold reduction in lung nodule formation and a tumor-specific interferon-γ recall response was observed in their splenocytes. Anti-tumor efficacy after CodaLytic treatment was accompanied by a change in the composition of the tumor immune infiltrate with increases in T, B, NK and cross-presenting dendritic cells and increased gene expression of pathways and genes related to T cell effector function, dendritic cell activation, antigen presentation and chemoattraction as determined by NanoString analysis. In MC38 tumors, combination treatment with an anti-PD-1 checkpoint inhibitor further enhanced $CD8^+$ T cell infiltration to 24.8% (vs 11.2% with PD-1 alone and 16.4% with CodaLytic monotherapy), the majority of which showed evidence of cytotoxicity by Granzyme B positivity. Both CD3+ and CD8+ T cell infiltration correlated with tumor volumes ($p < 0.001$). In this experiment, only combination treatment of CodaLytic and anti-PD-1 was able to reduce tumor growth significantly (63% tumor growth inhibition, $p < 0.05$ vs vehicle control), while neither monotherapy was efficacious.

Taken together, these preclinical data demonstrate CodaLytic induces broad innate and adaptive immune changes in the TME that translate into potent anti-tumor efficacy and prolonged survival. At the background of preclinical toxicology data and demonstrated clinical safety of this attenuated influenza virus after intranasal administration in healthy individuals, CodaLytic emerges as a promising immunotherapeutic with the potential to deepen or broaden responses to checkpoint inhibitors.

B091: Formation of intratumoral MAdCAM-1+ high endothelial venules is key for LIGHT-mediated anti-tumor immunity against murine glioma

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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 just 7%. This is due to a number of treatment barriers including the extensive abnormal vasculature and the immunosuppressive tumor microenvironment, which impede the infiltration of immune cells. Tertiary lymphoid structures (TLS) are ectopic lymphoid clusters that can form in inflammatory conditions, and have been associated with a positive prognosis in various cancer types. As such, we aimed to utilize lymphoneogenic cytokines to promote the development of TLS and anti-tumour immune responses in murine glioma.

To explore the effects of lymphoneogenic cytokines on glioma progression *in vivo*, C57BL/6 mice were orthopically injected with the control CT2A murine glioma cell line or CT2A cells which had been engineered to overexpress CXCL13, CCL21, LTαβ or LIGHT. Overexpression of CXCL13 or CCL21 did not provide any survival benefit compared to control, whereas mice bearing LTαβ- or LIGHT-overexpressing tumors exhibited prolonged survival, with a proportion of mice completely clearing their tumors. Upon immunofluorescent staining, clusters of $CD45^+$ immune cells resembling TLS were found in the meninges and ventricles. Interestingly, these clusters had a higher CD3:B220 ratio in the mice implanted with CT2A-LTαβ or CT2A-LIGHT cells, suggesting that these cytokines may influence the composition of glioma-associated TLS. Furthermore, LTαβ and LIGHT induced an increase in the formation of intratumoral MAdCAM-1^+ high endothelial venules (HEVs), a specialized type of blood vessel which is usually found in secondary lymphoid organs and is involved in the extravasation of immune cells into the tissue. Accordingly, the HEVs were associated with a higher number of $CD3^+$ T cells compared to normal blood vessels, indicating that LTαβ and LIGHT could increase the infiltration of $CD3^+$ T cells into the tumor area by inducing intratumoral HEV formation.

Although both LTαβ and LIGHT were promising candidates, we selected LIGHT for further evaluation due to its ability to interact with both stromal and immune cells. To determine whether intratumoral HEVs are responsible for the survival benefit provided by LIGHT, we utilized a MAdCAM-$1^{-/-}$ mouse line to abolish the formation of MAdCAM-1^+ HEVs in the tumor area. 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. This suggests that formation of HEVs within the tumor area has an important role in LIGHT-mediated tumor clearance, but is not the only mechanism at play.

In conclusion, this study demonstrates that intratumoral MAdCAM-1^+ HEVs have a central role in LIGHT-mediated anti-tumor immunity. 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.

B092: 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 non-genetic mechanisms of tumor immunogenicity. In more detail, we showed that SMARCB1 loss-of-function induces the expression of endogenous retroviruses, triggering IFN production

through activation of the dsRNA-sensing pathway in the tumor, and inducing RT immunogenicity despite their inherent genetic similarity to self; positioning RT as an interesting model system for studying epigenetically driven tumors. By FACS, scRNAseq and TCRseq analysis of the tumor-infiltrating immune cells, we showed 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. However, this treatment was not strong enough to eliminate the tumor in all cases. Deeper characterization of the myeloid infiltrate indicated that tumor-associated macrophages (TAMs) are 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), which induced a significant delay of tumor growth. Using FACS, scRNAseq and immunofluorescence we showed that poly(I:C) treatment: i) increases tumor infiltration of neutrophils and inflammatory monocytes; ii) decreases the number of protumoral macrophages; iii) induces the production of iNOS in peritumoral macrophages which support tumor infiltration by immune cells; iv) induces the accumulation of dendritic cells in tumor-draining lymph nodes (cDC1); and v) induces the expansion of precursor exhausted CD8+ T cells (T_{PEX}), which have been described as the main population responding to anti-PD-1. As expected, combination treatment of poly(I:C) with anti-PD1 induces durable complete tumor rejection and full memory against tumor rechallenge. Altogether, our study suggests that combined targeting of myeloid and T cells represent a promising immunotherapy strategy which we are now translating to RT patients.

B093: 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 (T_{RM}) contribute a significant portion of the T cell arsenal providing long-term protection from reinfection and tumorigenesis. The regulation of metabolic programming that accompanies T_{RM} adaptation to diverse tissue environments permitting differentiation, survival, and sustained function is not fully established. Understanding how T_{RM} acclimate to specific tissues may provide key insights into the ability to promote host protection from reinfection as well as tumor growth as tumor-infiltrating lymphocytes (TIL) with T_{RM} characteristics possess enhanced antitumor functions

and often predict responses to immunotherapy and favorable prognosis. Here, we reveal tissue-specific metabolic adaptations of T_{RM} and highlight the cholesterol biosynthetic pathway, and its upstream regulator Srebp2, to be of particular relevance for small intestine (SI) T_{RM} formation and the antitumor capacity of TIL. Unexpectedly, this tissue-specific adaptation was supported by the production of non-steroidal metabolites, such as ubiquinone, rather than cholesterol alone. Leveraging this information, we show that genetic and pharmacologic inhibition of squalene synthase (Fdft1), which prevents cholesterol production and promotes accumulation of upstream intermediates, enhances memory formation upon acute viral infection and boosts the antitumor function of CD8 T cells in mice. Together, this study profiles metabolic adaptations of T_{RM} and illustrates how these pathways can be co-opted to potentiate CD8 T cell memory formation in the context of acute infections and revamp CD8 T cell functions in the context of tumors.

B094: NG-796A, a T-SIGn viral vector designed to produce IL-12 and IL-15 within the tumor microenvironment: critical role for co-encoding the sushi domain of the IL-15Rα chain

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Enadenotucirev (EnAd) is a chimeric oncolytic (OV) adenovirus that was selected by screening recombinant viruses having high potency and selectivity for tumor cells over normal cells, as well as being able to function in whole blood to enable intravenous dosing and thus application to cancer patients with metastatic disease (most other OVs are incompatible with systemic dosing and must be dosed intratumorally). The smaller genome of EnAd (due to E3 and E4 deletion mutations) enabled the generation of a vector for creating transgene-bearing variants (T-SIGn vectors) where individual or combinations of therapeutic transgenes are inserted downstream of the virus major late promoter which ensures expression of the transgene-encoded proteins where the virus can replicate, which is selectively in tumor cells. Thus, after systemic dosing with a T-SIGn vector, transgenes are selectively expressed locally in the tumor microenvironment (TME) where they are needed to function, minimizing systemic exposure and thus decreasing the risk of side effects in response to treatment.

In the initial stages of developing the next candidate for our pipeline, we screened a number of different recombinant cytokines for their ability to drive the production of IFNγ in primary cultures of human tumor cells prepared from multiple surgically-excised patient tumor samples. IL-12 and IL-15 were found to act with potent synergy in these studies as well as further experiments using peripheral blood derived T-cells and NK cells from healthy volunteers. We therefore created a series of T-SIGn vectors expressing single-chain human IL-12 and/or IL-15 transgenes and evaluated their ability to infect A549 tumor cells and produce their protein payloads. High levels of functionally active IL-12 were readily produced, but levels of IL-15 were consistently low or undetectable. Using different leader sequences, different 2A ribosome skipping sequence or changing the position of the IL-15 transgene in the vector transgene cassette all failed to rectify this lack of production.

We hypothesized that the IL-15 protein might need a “chaperone” to enable efficient secretion from virus infected cells and reasoned that, since the IL-15Rα chain is physiologically co-

produced with IL-15 (e.g. by antigen presenting cells) and serves to "trans-present" this cytokine to target cells, this receptor protein may also be required for IL-15 secretion. Initial studies indeed showed that encoding a membrane anchored version of IL-15Rα could enable some production of IL-15, with subsequent designs showing that a secreted version of just the small (66 amino acids) IL-15-binding region of the IL-15Rα protein (the sushi domain) was optimal for T-SIGn vector-driven secretion of high levels of IL-15 by infected tumor cells. Different T-SIGn vectors encoding IL-12, IL-15 and the sushi domain transgenes were shown to re-capitulate the potent induction of IFNγ production by T/NK cells and primary tumor cell cultures that was initially seen using recombinant cytokines. Mechanistic studies using a recombinant sushi domain protein and CMV expression plasmids encoding transgene cassettes containing IL-12 and IL-15 with or without the sushi domain transgene demonstrated that expression of the sushi domain within the tumor cell was required for effective IL-15 production; addition of even high levels of recombinant sushi domain protein was unable to increase detectable IL-15 levels. We conclude from these studies that, at least in the context of production from a viral vector, IL-15 requires a cellular "chaperone" to enable effective secretion and the sushi domain of the IL-15Rα can fulfil this role.

Finally, we also incorporated a fourth transgene - the chemokine CCL21 to increase recruitment of dendritic cells into the TME. This vector, NG-796A, was selected as a candidate for progression into formal preclinical development studies and manufacturing activities in readiness for clinical testing.

B095: Characterizing the Expression and Functional Profile of NKG7 in Solid Tumors

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The development of cancer immunotherapy, which aims to aim to reinstate or enhance cytotoxic and inflammatory functions, requires a comprehensive understanding of the mechanisms underlying anti-tumor immunity. Natural killer cell granule protein 7 (NKG7) has been shown to regulate inflammatory and cytotoxic immunity in mice. Additionally, *NKG7* has been shown to be transcriptionally increased in circulating cytotoxic T cells from patients that respond to immunotherapy compared to pre-treatment but decreased in non-responders.

Analyses of the expression and function of NKG7—at a tissue, cellular, and molecular level—within solid tumors, however, remain unexplored. This study aims to assess the utility of NKG7 as a defining marker of effector and cytotoxic cells, and further investigate the role of NKG7 in anti-tumor responses in patients treated with immunotherapy.

Analyses of median *NKG7* transcript expression in cohorts of patients treated either with tyrosine kinase inhibitor (TKI) therapy or TKI in combination with immunotherapy, indicates that *NKG7* is predictive of survival benefit in the context of immunotherapy. We subsequently profiled the

spatial distribution and co-expression of NKG7 with other cytotoxic molecules in patient samples using analyses of spatial transcriptomic data, high-dimensional flow cytometry and CO-Detection by indexing (CODEX) ultra-high plex immunofluorescence imaging. Genetically editing of primary cells were used to define the function of NKG7 in cytotoxic and inflammatory pathways and to explore the potential benefits of increased NKG7 expression in the context of adoptive cell therapy and chimeric antigen receptor (CAR)-T and NK cells.

The knowledge gained from this study will further our understanding of the mechanisms underlying anti-tumor responses and contribute to the improvement of immunotherapeutics for the treatment of solid tumors.

B096: Targeting stem cell-like phenotypes to avoid breast cancer immunotherapy resistance

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Ligand-dependent corepressor (LCOR) mediates normal and malignant breast stem cell differentiation. Cancer stem cells (CSCs) generate phenotypic heterogeneity and drive therapy resistance, yet their role in immunotherapy is poorly understood. Here we show that immune-checkpoint blockade (ICB) therapy selects for $LCOR^{low}$ CSCs with reduced antigen processing/presentation machinery (APM) driving immune escape and ICB resistance in triple-negative breast cancer (TNBC). We unveil an unexpected function of LCOR as a master transcriptional activator of APM genes binding to IFN-stimulated response elements (ISREs) in an IFN signaling-independent manner. Through genetic modification of LCOR expression, we demonstrate its central role in modulation of tumor immunogenicity and ICB responsiveness. In TNBC, LCOR associates with ICB clinical response. Importantly, Lcor–mRNA therapy in combination with anti-PD-L1 overcame resistance and eradicated breast cancer metastasis in preclinical models. Collectively, these data support LCOR as a promising target for enhancement of ICB efficacy in TNBC, by boosting of tumor APM independently of IFN. Therefore, we are developing a first in class mRNA therapy with nanoparticle delivery to increase tumor antigen presentation across all tumor cells within the tumor population, to render them visible to immune detection, as an essential partner for immune-checkpoint blockade therapy in breast cancer and other cancer types.

B097: AI-driven 3D structure-based neoantigen prediction and its implication on immuno-oncology therapy

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Tumor specific T cell epitopes, also referred as neoantigens, arise from somatic mutations in cancer cells and arguably represent promising targets for personalized cancer immunotherapy. However, only small fraction of somatic mutation-bearing peptides are presented on major histocompatibility complex (MHC) proteins recognizable by T cell receptors (TCRs) and finally stimulate cytotoxic T lymphocyte response against tumor. Prediction of MHC binding peptides

(neoantigens) that lead to complexation with TCR is a key to cancer vaccine or T cell immunotherapy development; yet, currently available neoantigen prediction algorithms focus only on peptide-MHC interaction and do not address TCR recognition.

NEO-ARS™, a 3D structural modeling approach of peptide-MHC class I (pMHC) complex from known crystal structures followed by physics-informed deep learning, was used to predict neoantigens. Neoantigen candidates were then simulated and ranked by binding energy and solvent accessibility of TCR contact residues. Prediction performance of NEO-ARS™ was assessed by conducting prospective studies (i.e., *in vitro* pMHC binding assays; recruitment of healthy donors to test T cell response of NEO-ARS™-positive shared neoantigen candidates by an *ex vivo* IFN-γ ELISpot assay; recruitment of cancer patients to predict and test T cell response of private neoantigens) and by utilizing external retrospective datasets.

In vitro pMHC binding assay results with 92 top-ranked shared neoantigen candidates verified that NEO-ARS™ prediction is highly correlated to real pMHC binding: Positive Predictive Values (PPVs) of HLA-A*02:01 was 96%. The prospective healthy donor *ex vivo* studies showed that the majority of top-ranked predicted neoantigen candidates elicited acceptable immunogenicity: 10/11, 6/11, 8/10, 11/12, 11/11 peptides for HLA-A*02:01, 02:06, 11:01, 24:02, 33:03, respectively, showed T cell response. Preliminary results of the prospective cancer patient *ex vivo* study showed that NEO-ARS™-positive private neoantigen candidates also showed T cell response. Prediction performance evaluation with external datasets showed NEO-ARS™ outperformed netMHCpan-4.0 in multiple performance metrics including AUC values and PPVs.

High degree of correlation between experimental T cell response data and NEO-ARS™ predicted neoantigen candidates was shown by both prospective healthy donor and cancer patient *ex vivo* immunogenicity tests as well as evaluation using external datasets. We envision that NEO-ARS™ will open new avenues for development of personalized cancer vaccine or cell immunotherapy.

B098: Immunotherapeutic activity of OX425 against PD-1 resistant HR+HER2- breast cancer

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Background. Hormone receptor $(HR)^+$ breast cancer is a cold tumor that responds poorly to immune checkpoint blockers targeting PD-1 [1,2], calling for the development of therapeutic strategies that inflame the HR^+ tumor microenvironment to restore PD-1 sensitivity. OX425 is a second-generation poly(ADP)-ribose polymerase 1 (PARP1)-targeting decoy oligodeoxynucleotide (ODN) that drives PARP1 hyperactivation coupled to exhaustion of the DNA damage response, ultimately killing cancer (but not normal) cells as a function of metabolic breakdown [3]. PARP1-targeting decoy ODNs have been shown to mediate multiple immunostimulatory effects, standing out as promising combinatorial partners for PD-1 blockers in cold tumors [3].

Methods. We harnessed a unique endogenous mouse model that recapitulates key immunobiological features of human HR^+HER2^- breast cancer, as driven by subcutaneous, slow-release medroxyprogesterone acetate (MPA) pellets combined with 7,12-dimethylbenz[a]anthracene (DMBA) gavage [4], to investigate the therapeutic efficacy of OX425 delivered intraperitoneally 1X or 2X per week at 100 (5mg/kg) or 500 µg/mouse (25mg/kg - Maximum Tolerated Dose), optionally combined with a mouse PD-1 inhibitor (delivered intraperitoneally in 3 doses of 200 µg/mouse 3 days apart from each other). Tumor growth, mouse-adapted RECIST score assessments, progression-free survival, overall survival and other clinically relevant parameters were monitored until ethical endpoint.

Results. OX425 at the highest dose (500 µg/mouse 2X per week) was associated with weight loss across treated mice (irrespective of PD-1 blockage) and premature mortality in 10% of the mice, calling for dose reduction to 100 µg/mouse 2X per week. At all other administration schedules, OX425 was well tolerated, effective at controlling tumor growth and extending overall survival in mice bearing MPA/DMBA-driven carcinomas (which are intrinsically resistant to PD-1, similar to HR^+ breast cancer in women) [1,2,4]. Blocking PD-1 increased the therapeutic activity of OX425 when delivered 2X per week at 100 µg/mouse as it inhibited the development of secondary tumors.

Conclusions. OX425 at doses < 500 µg/mice 2X per week is well tolerated in mice and mediates single-agent immunotherapeutic activity in models of PD-1-resistant HR^+HER2^- breast cancer, with a potential for synergy with PD-1 blockade. Further investigation of the immunostimulatory and therapeutic properties of OX425 is warranted.

Conflicts of interest. JW is a full-time employee of Onxeo. LG has been holding research contracts with Lytix Biopharma and Promontory, has received consulting/advisory honoraria from Boehringer Ingelheim, AstraZeneca, OmniSEQ, Onxeo, The Longevity Labs, Inzen, Sotio, Promontory, Noxopharm, EduCom, and the Luke Heller TECPR2 Foundation, and holds Promontory stock options.

Ethics approval. This study was approved by Weill Cornell Medicine IACUC.

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B099: Dissecting the interplay between senescence and T cell biology

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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. Notably, senescence is often described as a singular program, yet it can have immunostimulatory or immunosuppressive effects depending on the senescence-inducing stimulus.

We have recently shown in preclinical models of pancreatic ductal adenocarcinoma (PDAC) that RAS signaling inhibition by combinatorial blockade of MEK and CDK4/6 results in therapy-induced senescence that favors T cell infiltration and potentiates the effect of PD-1 blockade. In order to dissect the molecular components required for an immunostimulatory form of senescence, we have developed a model of PDAC that allows for different modalities of senescence induction, such as restoration of tumor suppressors and various forms of therapy. We are currently systematically characterizing how these different forms of senescence affect the cancer cell compartment and the tumor immune and non-immune microenvironment.

Although senescence-inducing therapies only trigger senescence in a (sometimes relatively small) fraction of cancer cells, these therapies can lead to substantial, immune mediated, tumor regressions. We hypothesize that induction of senescence can favor bystander elimination of non-senescent cells. In order to investigate this effect, we have developed a genetic strategy to induce senescence in a defined fraction of cancer cells in a tumor, and to express different T cell epitopes in senescent and non-senescent cells. This will allow to evaluate how T cells specific for senescent and non-senescent cells will behave in response to induction of senescence in a fraction of cancer cells.

B100: BT-001, an oncolytic vaccinia virus armed with a Treg-depleting anti-CTLA-4 antibody and GM-CSF to target the tumor microenvironment

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Background

Immune checkpoint blockade (ICB) is a clinically proven concept to treat cancer. Still, a majority of cancer patients including those with poorly immune infiltrated "cold" tumors are resistant to currently available ICB therapies. CTLA-4 is one of few clinically validated targets for ICB, but toxicities linked to efficacy in approved αCTLA-4 regimens have restricted their use and precluded full therapeutic dosing. At a mechanistic level, accumulating preclinical and clinical data indicate dual mechanisms for αCTLA-4; immune checkpoint blockade and Treg depletion are both thought to contribute to efficacy and toxicity in available, systemic, αCTLA-4 regimens. Accordingly, strategies to deliver highly effective, yet safe, αCTLA-4 therapies have been lacking. Here we assess and identify spatially restricted exposure to a novel strongly Treg depleting, checkpoint blocking, vectorized αCTLA-4, as a highly efficacious and potentially safe strategy to target CTLA-4.

Methods

A novel human IgG1 CTLA-4 antibody (4-E03) was identified using function-first screening for mAbs and targets associated with superior Treg depleting activity. A tumor-selective oncolytic Vaccinia vector was then engineered to encode this novel, strongly Treg-depleting, checkpoint-blocking, αCTLA-4 antibody, and GM-CSF (VV_{GM}-αCTLA4). Viruses encoding matching mouse surrogate payloads were additionally generated, enabling proof-of-concept studies in syngeneic immune competent mouse tumor models.

Results

The identified 4-E03 antibody showed significantly stronger Treg depletion, but equipotent checkpoint blockade, compared with clinically validated αCTLA-4 ipilimumab against CTLA-4 expressing Treg cells in a humanized mouse model *in vivo*. Intratumoral (i.t.) administration of VV_{GM}-αCTLA4 (mBT-001) achieved tumor-restricted CTLA-4 receptor saturation and Treg-depletion, which elicited antigen cross-presentation and stronger systemic expansion of tumor-specific $CD8^+$ T cells and antitumor immunity compared with systemic αCTLA-4 antibody therapy. Efficacy correlated with FcγR-mediated intratumoral Treg-depletion. Remarkably, in several syngeneic mouse models, mBT-001 given i.t. induced a strong anti-tumoral response, synergized with αPD-1 to reject tumors and induced an abscopal effect in a two-tumor model.

Conclusion

Our findings demonstrate in vivo proof-of-concept for tumor delivery by Vaccinia virus of GM-CSF and strongly Treg-depleting, immune checkpoint blocking, vectorized αCTLA-4. This vector-based delivery is a highly effective and safe strategy to target CTLA-4 which overcomes current limitations of approved αCTLA-4 regimens. A clinical trial evaluating i.t. VV_{GM}-

αhCTLA4 (BT-001) alone and in combination with αPD-1 in metastatic or advanced solid tumors is ongoing (see NCT04725331).

B101: EGFR-Ta:RNA: Targeted delivery of a PRR agonist induces a multimodal anti-tumor response in EGFR-overexpressing cancers

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Aberrant activation of epidermal growth factor receptor (EGFR) signaling pathways is associated with tumor cell growth and progression, characterized by angiogenesis, invasion, and metastasis. EGFR overexpression occurs in various types of solid cancers and correlates with poor outcome. Thus, EGFR has become the target of a number of cancer therapies. However, the clinical benefit from EGFR-targeted therapies is usually short-lived, due to the development of resistance. Here, we describe a novel EGFR-targeted therapy for solid cancers that triggers multiple signaling pathways, leading to selective tumor cell killing and anti-tumor immune cell activation. The Ta:RNA technology platform consists of targeted nanoparticle drugs wherein synthetic dsRNA, polyinosinic polycytidylic acid (pIC), a Pattern Recognition Receptor (PRR) agonist that provokes cell killing and anti-tumor immune responses, is formulated together with a non-viral vector. The non-viral vector is composed of linear polyethyleneimine (LPEI) and polyethylene glycol (PEG), linked to a tumor-targeting moiety. The targeting moiety directs the nanoparticles to tumors that overexpress the target receptor, and receptor-mediated internalization delivers the pIC into the cancer cell.

Our EGFR-targeted Ta:RNA polyplex (EGFR-Ta:RNA) induced cytotoxicity and proinflammatory cytokine (RANTES/CCL5, IP-10) secretion selectively in EGFR-overexpressing cancer cells, *in vitro*. Medium from EGFR-overexpressing cancer cells treated with EGFR-Ta:RNA polyplex enhanced the activation of human PBMCs, and this was further potentiated by combination with anti-PD-1 antibody treatment, as demonstrated by increased INFγ secretion. Systemic administration of EGFR-Ta:RNA polyplex led to potent anti-tumor activity in an aggressive syngeneic experimental lung metastasis mouse model overexpressing human EGFR (RENCA-hEGFR). While this model was completely refractory to anti-PD-1 antibody treatment as a single therapy, the combination of EGFR-Ta:RNA with an anti-PD-1 antibody exerted a profound effect and further increased the survival of the mice as compared to EGFR-TA:RNA alone. Thus, the EGFR-Ta:RNA polyplex delivers a PRR agonist in a tumor-targeted manner to invoke potent multimodal anti-tumor activity, inducing tumor cytotoxicity and anti-tumor immunity, as well as opening avenues to potentiate the therapeutic activity of immunomodulatory antibodies.

B102: Cancer-associated fibroblasts as a negative predictor of response to maveropepimut-S, a novel immune educating therapy, in patients with advanced, recurrent ovarian cancer in the DeCidE1 phase 2 trial

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Background: The tumor microenvironment (TME) plays a significant role in shaping the interaction between tumor cells and the immune system. Stromal cells can influence the immune contexture of the TME through cytokine secretion and up- or down-regulation of several key pathways. Within the TME, cancer-associated fibroblasts (CAFs) are known to influence various aspects of the immune system. Recent studies show a role for CAFs in immune evasion and poor responsiveness to cancer immunotherapy through modulation of key pathways (e.g. EGFR, Wnt/β-catenin, Hippo, TGF-β, JAK/STAT). Such an influence on the TME would negatively impact the efficacy of immunotherapies. Maveropepimut-S (MVP-S, formerly DPX-Survivac) is comprised of immunogenic T cell peptides from the cancer antigen, Survivin, as well as a universal T helper peptide, A16L, and the innate immune activator, polydIdC, packed within the proprietary DPX lipid-in-oil delivery platform. Herein are presented translational data from the DeCidE[1] phase 2 trial showing a negative correlation between CAFs and immune cell infiltration that may provide key insight into mechanisms of resistance to MVP-S treatment.

Methods: Baseline and on-treatment tumor biopsies were collected from advanced, recurrent ovarian cancer patients treated with MVP-S primed with immune-modulating low-dose cyclophosphamide (DeCidE1 trial, NCT02785250). Multiplex-immunohistochemistry (mIHC, Akoya Biosciences) and RNAseq (Personalis Inc.) were used to analyze tumor immune microenvironment affected pathways.

Results: RNAseq analysis showed CAF-associated genes (e.g. , ACTA2, FAP, CXCL12, FGF, and PDGF) are more highly expressed in the pre-treatment samples of patients with progressive disease (PD) than those with stable disease (SD) or partial response (PR). Moreover, pathway enrichment analyses showed upregulation of CAF-associated pathways (e.g. TGF-β, EGFR, HIPPO, JAK/STAT, WNT) in patients with PD compared to PR.

Fibroblast and CAF gene signatures (TIROSH[1], Xcell[2], and EPIC[3]) also demonstrated higher baseline enrichment scores for patients with PD versus those with SD or PR. Further analysis revealed that the on-treatment sample of patients with PD showed increased CAF signature scores whereas these scores were decreased in patients with PR. Moreover, CAF signature scores showed a negative correlation with tumor immune cell infiltration assessed using the RNAseq immune score (sum of B cell, T helper, cytotoxic T cell, macrophage, and NK cell scores). To further assess the impact of CAF on immune cell infiltration, high CAF score patients (above median) were compared to low CAF score patients. The low CAF score patients showed higher infiltration of total lymphocytes, cytotoxic T cells, B cells, and macrophages assessed by mIHC in the baseline and on-treatment tumor samples.

Collectively, these data show that CAF contributes to an immune unfavorable TME and may represent a resistance mechanism and negative predictor of response to MVP-S treatment.

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B103: Response to combination immunotherapy in a mouse model of pancreatic cancer is a function of baseline T cell content

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Findings from the recently reported phase 2 PRINCE trial for patients with metastatic pancreatic cancer suggest potential clinical benefit with certain combinations of chemotherapy and immunotherapy. Overall survival following gemcitabine/nab-paclitaxel and either PD-1 blocking antibody or CD40 agonistic antibody (aCD40) correlated with the prevalence of distinct T cell subsets in circulation at baseline and with the extent of certain tumor infiltrating T cells (Padron et al., *Nature Medicine*, 2022). Here, we modeled the therapeutic effect of aCD40/PD-1/CTLA-4 therapy in the absence of chemotherapy in mice bearing tumors derived from the autochthonous pancreatic ductal adenocarcinoma (PDAC) KPC model and studied the dependency of tumor regression on the degree of tumor infiltrating T cells. We used a panel of KPC tumor cell clones that exhibit reproducible and transplantable levels of T cell infiltration ranging between high, intermediate, and low amounts. Mice were subcutaneously injected with these clonal PDAC lines and treated 14-21 days later with either aCD40 alone or aCD40/PD-1/CTLA-4 (all i.p., n=5-8 mice per cohort of T cell high, intermediate and low clones). Systemic immune dynamics were surveyed by bleeding mice on day 0, 7, and 14 of treatment and analyzing for immune cell activation and exhaustion markers. In mice bearing T cell high and intermediate tumors, aCD40/PD-1/CTLA-4 induced higher rates of complete tumor regressions (71% for T cell high; 25% for T cell intermediate) and prolonged survival ($P < 0.001$ for T cell high; $P = 0.013$ for T cell intermediate) compared to untreated controls. Treatment with aCD40 alone was effective in mice with T cell intermediate tumors, leading to regressions in 29% of mice. In mice bearing T cell low tumors, however, tumor regressions after aCD40/PD-1/CTLA-4 were less frequent (12.5%), although survival was improved significantly following the treatment ($P <0.001$). aCD40 alone improved overall survival of T cell low tumor-bearing mice ($P = 0.026$) without inducing tumor regressions. Regardless of T cell high, intermediate, or low tumors, aCD40/PD-

1/CTLA-4 increased the proportions of circulating activated and exhausted $CD4^+$ and $CD8^+$ T cells. aCD40/PD-1/CTLA-4 also increased T cell infiltration into T cell low tumors, but this enhanced infiltration did not correlate with complete regressions. Thus, using tumor clones derived from the same autochthonous genetic model, we show that response to combination immunotherapy is a function of baseline T cell content, with 'hot' tumors responding the best and 'cold' tumors responding the worst. Additional tumor cell-intrinsic factors in the T cell low tumor microenvironment such as those revealed in other mouse experiments and in the human samples from the PRINCE study likely restrict a robust anti-tumor immune response even when T cells have been recruited.

B104: Attenuation of YAP1 enhances anti PD-L1 efficacy in Urothelial carcinoma of bladder

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Cancer stem cells (CSCs) have been known to be associated with drug resistance and immune evasion. However, the mechanism of immune evasion remains to be elucidated in UCB. YAP1 mediates cancer stemness by regulating the IL-6/ STAT3 and COX2/PGE2 associated signaling pathway in different solid tumors including urothelial carcinoma of bladder (UCB). Our results using suggest that YAP1 expression induces different genes in facilitating cancer stemness such as SOX2, ALDH1, NOTCH1, FOXA2 and GJB1. Genetic and pharmacologic modulations of YAP1 in mouse UCB cell lines suggest that YAP1 induces cellular stemness properties including sphere formation ability. *In vivo* studies also determined that YAP1 expression is correlated with the infiltration of MDSCs and CD8+ T cells in the tumor microenvironment (TME). Whole transcriptome sequencing of multiple human UCB cell line revealed that YAP1 expression leads to the upregulation of tumor promoting interleukins and chemokines. Additionally, PCR based array also revealed that YAP1 positively correlates with the expression of different CXCR1/CXCR2 associated ligands such as CXCL2, CXCL3, CXCL6 and CXCL10. YAP1 expression was also found to polarize the naive macrophages into M2 type. Our data further indicates that YAP1 inhibition promotes the adaptive immunity of the tumor bearing mice through differential secretion of extracellular vesicles and YAP1 induced IL-6/ STAT3 signaling pathway that may regulate the expression of CXCLs in the UCB cells. Further *in vitro* and *in vivo* experiments suggest that YAP1 attenuation increased the tumor immunogenicity including CD8+ T cells activity. Therapeutically, combination of YAP1 inhibitor with anti PD-L1 enhances the efficacy of anti-PDL. In summary, our findings suggest that YAP1 play a major role in immune evasion by regulating the IL-6/ STAT3 and COX2/PGE2 associated signaling pathway in urothelial carcinoma of bladder (UCB) and offer a preclinical rational to combine YAP1 inhibitor with immune checkpoint blocker (ICB) to improve the clinical management of UCB.

B105: Enhancing anti-cancer immunotherapy by disruption of the non-receptor tyrosine kinase Fes

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Introduction:
Cancer immunotherapy is an emerging pillar of treatment, but it still faces many barriers due to the immunosuppressive nature of cancer. Cancer immunotherapy relies on the activation of both innate and adaptive anti-cancer immune responses. One way of stimulating these responses, known as immunogenic cell death (ICD), causes the release of various tumour-associated antigens, and damage associated molecular patterns (DAMPs). These DAMPs function to recruit and activate antigen-presenting cells (APCs) by binding to specific receptors known as pattern recognition receptors (PRRs), which subsequently lead to the production of pro-inflammatory cytokines (colloquially known as Signal 3), required for adaptive immune cell activation (e.g., Cytotoxic T Lymphocytes [CTLs]). The non-receptor tyrosine kinase Fes, abundantly expressed in antigen-presenting cells (APCs), dampens innate immune responses by inhibiting downstream components of the PRR signaling cascade. In non-cancer contexts, our lab has shown that negative regulation of APCs by Fes may guard against consequences of overactive innate immunity, such as endotoxic shock. However, we hypothesize that this same inhibitory effect on APC function may also serve as a barrier 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 production, resulting in greater CTL activation, and therefore improved tumour control.

Methodology:
Using bone marrow derived macrophages from wildtype (WT) or *fes*$^{-/-}$ mice, we assessed PRR signal transduction cascades regulated by Fes using immunoblot analysis following stimulation with Toll-like receptor 4 agonist, lipopolysaccharide. Using syngeneic orthotropic engraftment models of triple negative breast cancer (EO771) and melanoma (B16-F10) in wildtype and *fes*$^{-/-}$ mice, we treated mice with DMSO or doxorubicin and assessed tumour growth and survival. Tumours and spleens were then harvested and immune profiles analyzed by flow cytometry. In separate experiments, EO771 and B16-F10 tumor bearing WT and *fes*$^{-/-}$ were treated with doxorubicin and either IgG isotype control antibody or anti-PD-1 antibody.

Results:
We show that *fes*$^{-/-}$ APCs display stronger PRR signaling *in vitro* compared to wildtype following PRR agonist stimulation. *In vivo*, we show increased tumour control and survival in *fes*$^{-/-}$ mice compared to wildtype mice, further enhanced by stimulating ICD with doxorubicin. We also demonstrate an increase in CTL activation and PD-1 positivity, increased further by doxorubicin, and a higher degree of activated and PD-1-positive NK cells in *fes*$^{-/-}$ mice indicating a potential novel role for Fes in regulating activation of both CTLs and NK cells. Additionally, we found a shift in tumour-associated macrophages from predominately an M2-polarized pro-

tumourigenic phenotype to a predominantly M1-polarized anti-tumourigenic phenotype in *fes*$^{-/-}$ mice compared to WT. Finally, when treated with anti-PD-1 antibody, *fes*$^{-/-}$ mice demonstrated greater tumour control and survival than WT mice.

Conclusion:
Loss of Fes results in a hyper-inflammatory phenotype in BMDMs. This effect translates into improved doxorubicin responses in *fes*$^{-/-}$ mice resulting in improved tumour control, survival, and a shift to an anti-tumourigenic microenvironment compared to wildtype mice. Fes deficiency also improves the efficacy of anti-PD-1 antibody therapy. These results combined with the observed improved overall- and disease-free survival of breast, melanoma, and ovarian cancer patients supports Fes as a potential novel therapeutic target in enhancing anti-cancer immunotherapy.

B106: Therapeutic blocking of VEGF binding to neuropilin-2 inhibits PD-L1 to activate anti-tumor immunity in prostate cancer

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Prostate cancers are largely unresponsive to immune checkpoint inhibitors and there is strong evidence that PD-L1 expression itself must be inhibited to activate anti-tumor immunity. Here, we report that neuropilin-2 (NRP2), which functions as a VEGF receptor on tumor cells, is an attractive target to activate anti-tumor immunity in prostate cancer because we demonstrate that VEGF/NRP2 signaling sustains PD-L1 expression. NRP2 depletion increased T cell activation *in vitro* and immune-mediated tumor elimination *in vivo* using a humanized mouse model. Inhibition of the binding of VEGF to NRP2 with using a mouse specific anti-NRP2mAb in a syngeneic model of prostate cancer that is resistant to checkpoint inhibition resulted in significant necrosis and tumor regression compared to control IgG. This therapy also decreased tumor PD-L1 expression and increased immune cell infiltration. We also observed that the *NRP2* and *VEGF-A* genes are amplified in neuroendocrine prostate cancer (NEPC) and that NRP2^{high} PD-L1^{high} population in metastatic PC patients had a significantly lower AR and higher NEPC scores than other populations. Therapeutic inhibition of VEGF binding to human NRP2 with a high affinity humanized mAb, which is suitable for clinical use, in organoids derived from NEPC patients also diminished PD-L1 expression and caused a significant increase in immune-mediated tumor cell killing consistent with the animal studies. These findings provide justification for the initiation of clinical trials using this novel function-blocking NRP2 mAb in prostate cancer, especially for patients with more advanced cancers, where blocking NRP2-VEGF signaling shows potential in mitigating the morbidity and mortality associated with the development of NEPC.

B107: AI/ML-driven discovery of Collagen Triple Helix Repeat-Containing 1, CTHRC1, a novel proteoglycan for disruption of stromal barriers and enhancement of immunotherapy responses in desmoplastic tumors

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Background: While checkpoint inhibitors (CPIs) such as anti-CTLA-4 and anti-PD-1/L1 have demonstrated efficacy in many solid tumor indications, those with high stromal presence have been difficult to treat. We aimed to use a proprietary machine learning/artificial intelligence platform to identify novel stromal targets that, upon targeting, will relieve this immunosuppressive barrier and increase CPI responsiveness in difficult-to-treat indications.

Methods: Based on bioinformatic analysis using a large single-cell RNA atlas, we assessed cancer-associated fibroblasts (CAFs) / fibroblastic cells in cancer tissue for the identification of novel targets, including proteoglycans. Antibodies were generated by immunization of humanized mice. Lead antibodies demonstrated potent *in vitro* activity in inhibiting cell adhesion and reducing survival of pancreatic cancer cells in CAF conditioned media. We assessed efficacy and PD (flow cytometry and IHC) in the EMT6 orthotopic tumor model in immunocompetent mice.

Results: Analysis of a large single-cell RNA atlas spanning many solid tumors identified CTHRC1 as one of the most upregulated CAF targets in the genome. CTHRC1 was also highly associated with CAFs from immune-excluded and desert-like tumor samples. Finally, we also find that CTHRC1 is highly expressed by both cancer cells and CAFs within the tumor, in key indications such as breast, ovarian, and pancreatic, with the potential for Fc-mediated depletion of both tumor and fibroblast cells. Profiling by scRNAseq of syngeneic tumor cells identified EMT6 breast cancer model as representative of human tumor, mirroring both CAF and tumor CTHRC1 expression. Assessment of efficacy demonstrated monotherapy activity with strong combination activity and enhanced survival when we combined anti-CTHRC1 mAbs with anti-PD-1.

Conclusions: We have identified CTHRC1 as a novel proteoglycan expressed by both CAFs and tumor cells that appears to be an ideal target for inhibiting of stromal barrier function with therapeutic monoclonal antibodies that may also serve as ideal for targeting payloads to the tumor microenvironment. These data demonstrate the power of large scRNA atlases for novel target ID and show the potential of breaking down stromal barriers in opening up tumor microenvironments to immune attack.

B108: Dendritic cell intrinsic androgen receptor signaling reduces dendritic cell function and anti-tumor immunity

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Sex differences, defined by distinct chromosome content and sex-determined hormone levels, impact immune responses as witnessed by the high incidence of autoimmunity in females compared to males (11:1 ratio). In general, men have a higher risk of developing cancer with increased mortality compared to women[1]. In contrast, women account for almost 80% of the autoimmune cases reported[2] identifying male sex as protective in autoimmunity. Notably, cancer immunotherapy is designed to generate an autoimmune-like response against an individual's cancer. Given the striking sexual dimorphism in autoimmunity, there is a clear and pressing need to investigate the impact of sex hormones on the effectiveness and function of cancer immunotherapy. Testosterone, the dominant male androgen, is immune suppressive; limiting T cell proliferation and function and enhancing differentiation of inflammatory monocytes. Further several recent publications have demonstrated that blockade of sex hormones in tumor-bearing mice results in improved adaptive immune responses via direct modulation of $CD8^+$ T cell-intrinsic androgen receptor signaling resulting in increased IFNγ expression and improved overall anti-tumor immunity[3-5]. And while single-cell RNA sequencing data suggests that increased androgen receptor signaling correlates with decreased antigen presentation in patient tumors[3], whether androgen inhibition and androgen receptor signaling directly impacts dendritic cells, professional antigen presenting cells required for antigen-specific T cell responses, has yet to be explored. Here we demonstrate using a B16 melanoma model that dendritic cells, both bone marrow-derived and those sorted from murine tissue, express androgen receptor. Further by inhibiting androgen receptor signaling using the clinically relevant inhibitor, enzalutamide, we find that dendritic cells have increased phagocytic potential and higher expression of dendritic cell maturation markers. Strikingly, we find that inhibition of androgen receptor signaling results in increases in antigen processing and antigen presentation as evidenced by improved $CD8^+$ T cell proliferation. Our data provides insight into the regulation of dendritic cells within the tumor microenvironment and how suppressing inhibitory hormone molecules can lead to more effective T cell responses. These studies have the potential to improve our understanding of the sexual dimorphic immune responses observed in cancer and may facilitate the generation of more productive dendritic cell targeted immunotherapies.

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B109: Microbiome-modulated migratory group 2 innate lymphoid cells induce tertiary lymphoid structures in cancer and inflammation.

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Tertiary lymphoid structures (TLSs) are immune cell aggregates that form ectopically in inflamed tissues such as cancer. TLSs arise when inflammation induced-tissue damage stimulates TLS inducer cells to express lymphotoxin, the canonical lymphoneogenic protein, to recruit dendritic cells and B cells to prime antigen-specific T cells at inflammatory sites. In inflammatory bowel diseases, TLSs restrict pathogenic gut microbes. In cancer, TLSs boost anti-tumor immunity, correlate with improved prognosis, and predict higher response to immune checkpoint inhibitors. Yet, though TLSs critically modulate the host immunity to cancer and inflammation, the molecules and cells that initiate TLSs remain undefined.

To identify molecules that induce TLSs, we searched for the most significantly correlated genes to three TLS transcriptional signatures in bulk transcriptomes of human cancer. Surprisingly, interleukin-33 (IL33), an alarmin released by damaged tissues was one of the most highly correlated genes in pancreatic ductal adenocarcinoma (PDAC), melanoma, and breast cancer ($P < 0.001$ in all), introducing IL33 as a potential novel molecule that induces TLS.

To probe this finding, we examined if host IL33 generated TLS in mouse models of inflammation and PDAC. In a model of dextran sulfate sodium (DSS)-induced inflammatory colitis that induces colonic TLSs, *Il33*$^{-/-}$ mice had significantly fewer colonic TLSs ($P = 0.009$) and worsened survival compared to DSS-treated WT mice ($P = 0.0001$). Similarly, αLtbr, a TLS-inducing lymphotoxin receptor agonist, induced ~6-fold greater TLS densities in tumors of wild type (WT, $P < 0.001$), but strikingly not in *Il33*$^{-/-}$ PDAC mice. Thus, IL33 generates TLSs in inflammation and cancer.

We next examined if IL33 induced TLS by binding its receptor ST2. Truncated recombinant IL33 (rIL33) containing only the ST2-binding domain increased intratumoral TLS density (~10-fold, $P \leq 0.01$-0.001) in 6 PDAC mouse models, suppressed PDAC growth, and prolonged survival, further highlighting IL33 as a novel TLS-inducing alarmin.

Group 2 innate lymphoid cells 2 (ILC2s) are canonical IL33-responsive $ST2^{Hi}$ immune cells at barrier surfaces that coordinate tissue repair. We found rIL33 highly expanded (~420-fold; P = 0.0007) $ST2^{+}$ inflammatory ILC2s (iILC2s) that express lymphotoxin in tumors. Furthermore, rIL33 induced intratumoral TLSs in WT but not *Rora*$^{fl/fl}$;*Il7r*^{cre} mice (ILC2-deficient) PDAC mice. Consistently, transferred WT, but not lymphotoxin-deficient iILC2s accumulated within tumors, induced TLS, and suppressed PDAC growth, identifying iILC2s as novel TLS inducer cells.

Unlike most ILC2s that reside in tissues, iILC2s migrate from the terminal ileum (TI) to inflammatory sites. Migration is modulated by deleterious gut microbes (dysbiosis) that also suppress anti-tumor immunity. To examine if iILC2s migrated to PDACs from the TI, we found rIL33 increased iILC2 frequencies in the TI, and in parabiotic mice, that rIL33 induced iILC2s to migrate hematogenously to PDACs in different tissues (pancreas, skin). Notably, PDAC dysbiosis suppressed iILC2s as microbial ablation increased iILC2 frequencies in the TI, and fecal transplantation in germ-free mice lowered intestinal iILC2s. Finally, in parabiotic mice, ablating dysbiosis lowered donor-derived iILC2 frequencies in recipient blood and TI. Thus, dysbiosis suppresses iILC2 frequencies in circulation and gut reservoirs.

In summary, we identify IL33 and ILC2s as new molecules and cells that induce TLS. We find that iILC2s are TLS inducer cells, migrate to inflamed tissues, and are modulated by gut microbes. Furthermore, we introduce rIL33 as a novel ILC2-targeted immunotherapy that increases TLSs to treat cancer and other inflammatory diseases. More broadly, we identify that a previously unrecognized gut-ILC2 axis initiates ectopic lymphoneogenesis in inflamed tissues.

B110: IL-21 promotes NK cell cytotoxicity and stemness through metabolic modulation for treatment of MHC I-deficient tumors

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Natural killer (NK) cells are highly cytotoxic lymphocytes that could eliminate infections and cancerous cells independent of the major histocompatibility complex (MHC) restriction. NK cell-based cancer immunotherapy is currently actively pursued as it is effective in eradicating MHC-deficient cancer cells complementing current immunotherapies that are mostly rely on T cells mediated killing. However, similar to T cells, the responses to NK cell-based immunotherapy is greatly limited by the dysfunction of NK cells in the tumor microenvironment (TME). Immune metabolism has been reported to be a crucial factor that regulates differentiation and exhaustion of lymphocytes including NK cells. Here, we produced an IL-21 and IgG1Fc fusion protein (IL-21/Fc) with extended half-life. In vivo administered IL-21/Fc enhanced cytotoxic function of NK cells through granzyme B and interferon-γ production. In addition, IL-21/Fc increased $CD27^{+}$ NK cell population and the stemness of NK cells in the TME. When combined with IL-15 superagonist (IL-15SA), IL-21/Fc induced potent antitumor activity in multiple subcutaneous MHC-I deficient tumor models including B16F10_*β2m*$^{-/-}$, CT-26_*β2m*$^{-/-}$, and RAM-S tumor models and durable protection against tumor rechallenge. Mechanistically,

we found IL-21/Fc promoted the glycolytic metabolism in a LDHA dependent manner, which was critical for enhanced NK cells effector function and stemness. Our results establish a new approach to counter NK cell dysfunction through metabolic reprogramming, which may overcome a major barrier in the current NK cell-based immunotherapy in the clinic and increase the response rate.

B111: Sodium chloride prevents exhaustion and enhances cancer immunotherapy of CD8+ T cells

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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, thus limiting the effectiveness of cancer immunotherapy. Recent evidence has shown that ionic alterations may underlie pathological conditions due to altered immune functionality, such as in experimental models of autoimmune diseases, yet the role of ionic metabolism in anti-tumor immunity is less clear. We show that NaCl utilization by CD8+ T cells prevents exhaustion while promoting potent effector differentiation and anti-tumor immunity in multiple experimental systems. High salt diet (HSD) administration prior, but not after tumor implantation reduced tumor growth in the MC38 colon cancer model in mice in a CD8+ T cell-dependent manner. Single cell RNA sequencing of CD45+ cells from tumors revealed the reduced presence of *Tox*^{high} *Eomes*^{high} terminally exhausted CD8+ T cells and the accumulation of highly activated CD8+ T cells characterized by the co-existence of stem-like (*Tcf7, Lef1, Foxo1*) and effector (*Tbx21*, *Stat4*, *NF-kB*) gene programs in mice fed with HSD. Accordingly, human CD8+ T cells activated by CD3/CD28 and suboptimal doses of IL-2 upregulated several effector genes while maintaining the expression of stem-like genes when cultured in hypertonic conditions, proliferated more vigorously following transfer in highly immunodeficient hosts, and showed enhanced rejection of established xenogeneic melanomas compared to control cells. In vitro, Na+ uptake by activated CD8+ T cells was inhibited by the presence of tumor cell lines. In line with this finding, transcriptional programs related to NaCl utilization and NaCl-induced T-cell reprogramming were missing in CD39+ CD8+ terminally exhausted T cells from human tumors, but were highly enriched in Ki-67+ CD8+ proliferating T cells reinvigorated by PD-1 blockade in cancer patients. Overall, these data underline the central role of NaCl metabolism in promoting stem-like CD8+ T cells with potent effector functions, and prompt to favor NaCl metabolism to counteract dysfunction and enhance anti-tumor immunity.

B112: Role of PI3Kγ inhibition in preventing progression of inflammation towards fibrosis and tumorigenesis in various animal models

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Anti-inflammatory, immunosuppressive macrophages play significant roles in promoting both tumorigenesis and fibrosis. Wound-healing type macrophages accumulate in sites of tissue injury, such as the cirrhotic liver, where they can induce oxidative damage and secrete immunosuppressive and pro-fibrotic factors that lead to oncogenesis. These innate immune cells are also major cellular producers of PDGFB and TGF-β1, which stimulate fibroblasts to secrete

extracellular matrix proteins, such as collagen, and which also promote metastatic progression of neoplastic diseases. Chronic liver and pancreatic inflammation are significant risk factors for the development of hepatocellular carcinoma (HCC) and pancreatic ductal adenocarcinoma (PDAC), respectively. We have determined that the myeloid cell specific PI3K isoform PI3Kγ promotes the accumulation of myeloid cells and their immune suppressive polarization within the tumor microenvironment (TME), and that this kinase thereby promotes tumor growth in animal models of cancer, including PDAC. Genetic and pharmacological inhibition of PI3Kγ suppresses tumor growth and reduces fibrosis that is associated with PDAC and other tumors. Targeting immune suppressive myeloid cells represent an emerging approach within the field of cancer immunotherapy and fibrosis treatment; indeed, inhibition of PI3Kγ by the therapeutic Eganelisib has shown beneficial effects in clinical trials for melanoma, head and neck carcinoma, urothelial carcinoma and triple negative breast carcinoma. In this project, we are exploring whether PI3Kγ inhibition can prevent cancer development by suppressing damaging chronic hepatic and pancreatic inflammation, associated fibrosis, and progression towards tumorigenesis using models of HCC and PDAC. We found that PI3Kγ inhibition prevented inflammation and fibrosis in mouse models of chronic pancreatitis and liver inflammation. Studies are underway to determine if PI3Kγ inhibition can also prevent the development of pancreatic and liver carcinoma that results from chronic inflammation and fibrosis. Our studies thus far suggest the potential role of PI3Kγ inhibition in preventing the inflammation that leads towards fibrosis and carcinogenesis. The results of these studies could be applied to the future design of clinical trials with clinical PI3Kγ inhibitors.

B113: Anti-CTLA-4 therapy promotes CD8 T-cell infiltration in anti-PD-1 refractory melanoma with T-cell desert and exclusion phenotypes

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Background: CTLA-4 blockade allows T cells to expand, circulate and infiltrate tumors, which may overcome resistance to PD-1 blockade therapy mediated by lack of a pre-existing intratumoral T cell infiltrate. Thus, we hypothesized that anti-CTLA-4 may reverse primary resistance to PD-1 blockade therapy in patients with metastatic melanoma. Here, integrate manual and digital pathologic assessment of melanoma biopsies with paired bulk sequencing to determine whether the baseline immune status of anti-PD-1 refractory melanoma is associated with CD8 T-cell infiltration following anti-CTLA-4 alone or in combination with anti-PD-1.

Methods: Baseline tumor specimens (N=43) and on-therapy (N=49; paired, N=37) were collected from patients enrolled in SWOG S1616 (NCT03033576, N=94), a multi-center phase II 2:1 randomized trial evaluating the combination of nivolumab and ipilimumab or ipilimumab single agent in patients with anti-PD-1-refractory melanoma. Single-stain immunohistochemistry (IHC) was performed on sequential sections of tumor biopsies for S100 (melanoma) and CD8 (T-cell) protein expression. Each sample was manually annotated by an expert dermatopathologist and concurrently processed by an automated pipeline for the annotation of tumor regions. CD8 T-cells were quantified within the tumor and the tumor boundary to classify

tumors as infiltrated, excluded, or desert. Sequential sections were used for RNA sequencing (RNAseq; baseline, N=27; on-therapy, N=19; paired, N=17) for bulk transcriptome analysis.

Results: Our automated pipeline for detection of tumor regions using S100 protein staining reliably detected 87% of areas manually annotated by expert dermatopathologists; automated detection tumor regions performed more inaccurately in tumors with high levels of pigmentation (1% of cases), on-therapy specimens exhibiting high quantities of macrophages and melanosis (14%), or specimens with no tumor cellularity (2%). Of the baseline tumor specimens, 49% of tumors exhibited CD8 T-cell infiltration, 27% showed exclusion of CD8 T-cells, and 24% were desert. Infiltrated tumors exhibited increased IFNG and T-cell receptor (TCR) signaling pathway expression by RNAseq (FDR<0.05) at baseline, compared to desert tumors; however, only CD8 T-cell exclusion and desert tumors showed increased expression of these processes over the course of treatment, comparing paired on-therapy to baseline biopsies. When analysis was performed within each study arm, IFNG and TCR signaling were significantly upregulated in on-therapy biopsies treated with combination therapy (FDR<0.05); anti-CTLA-4 therapy did not result in changes to these processes.

Conclusions: The landscape of anti-PD-1 refractory melanoma is heterogeneous, and recent studies have attempted to stratify patients by histopathologic, gene expression, and genetic features. Our results support the mechanism by with anti-CTLA-4 facilitates trafficking to the tumor site. Importantly, analysis in on-therapy biopsies showed increased expression of T-cell-related processes over the course of treatment in both CD8 T-cell desert and excluded tumors, suggesting that anti-CTLA-4-mediated infiltration is not contingent upon baseline immune status. Infiltrated tumors also showed increased T-cell activation patterns on-therapy, but only in the context of anti-CTLA-4/anti-PD-1 monotherapy.

B114: Nuclear EGFR as a regulator of inflammation and the tumor microenvironment

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Triple Negative Breast Cancer (TNBC) is one of the most aggressive and metastatic subtypes of hormone receptor negative breast cancers and displays an immune suppressive microenvironment. Most patients with TNBC overexpress the Epidermal Growth Factor Receptor (EGFR), yet its role in driving the disease remains unclear, as EGFR-targeted antibodies and tyrosine kinase inhibitors fail to work effectively in these patients. A possible explanation for therapeutic resistance is the role of intracellular EGFR, where retrograde trafficked EGFR can function directly as a transcriptional cofactor, regulating the expression of genes that promote tumor growth and metastasis. One subset of these genes is those that induce an immune suppressive microenvironment, and our goal is to understand how targeting nuclear EGFR may impact these oncogenic drivers. We recently developed a therapeutic (cSNX1.3) that blocks the accumulation of nuclear EGFR and effectively induces regression of EGFR-dependent breast cancer in an immune intact mouse model. One phenotype in this model is an associated activation of the immune microenvironment, including decreased PDL-1 expression. We hypothesize that nuclear EGFR is suppressing the expression of inflammatory cytokines and recruitment of cytotoxic lymphocytes, thereby allowing immune evasion and ultimately progression of the disease. RNA-Seq analysis of cSNX1.3 treated tumors found alterations in

cytokine production and the IL-1 signaling pathway in addition to an upregulation of the proinflammatory genes IL-6 and IL-8. NanoString analysis further demonstrated impacts on the TLR family of genes as well as a greater abundance of cytotoxic cells present in the drug treated tumors. Future studies will investigate the potential for combination therapy between cSNX1.3 and checkpoint inhibitors to both activate the tumor immune microenvironment and promote optimal therapeutic efficacy.

B115: Tumor-intrinsic STING pathway promotes abscopal effects post primary tumor cryoablation

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Metastatic tumors have shown to be a challenge for cancer treatment. Identifying an approach to boost systemic anti-tumor immunity could help improve the efficacy of treatment. Tumor cryoablation, a medical procedure that utilizes ultra-cold temperatures to destroy local tumor tissues *in situ*, has the potential to induce immunogenic cell death (ICD) that initiates tumor-specific immune responses. Here, we show that using local cryoablation on an *in vivo* model of syngeneic rhabdomyosarcoma (RMS), an aggressive sarcoma affecting children and young adults, was able to induce both the regression of primary tumor and the inhibition of lung metastases. We found that the efficacy of cryoablation was primarily T cell-dependent. We also report that the tumor-intrinsic STING pathway contributed to the reduction of both the treated tumor and the untreated, distal tumor post tumor cryoablation via the abscopal effect. Our results suggest that the tumor-intrinsic STING pathway was not only a contributor but also an essential component underlying the effectiveness of cryoablation, and this approach may help guide future immunotherapy to improve cancer treatment outcomes.

B116: A non-canonical role for CCL2 in T cell migration: improving autologous monocytes and dual-interferons as a therapy for ovarian cancer.

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Patients with recurrent, platinum-resistant ovarian cancer have limited effective treatment options, and immunotherapies—such as immune checkpoint inhibitors and chimeric antigen receptor T cells—have unfortunately been ineffective in this disease, due to the immunosuppressive tumor microenvironment and low mutational burden. As such, additional therapeutic strategies are needed to improve the survival of these women. Our group pioneered an approach manipulating innate immunity where autologous monocytes, interferon-γ, and interferon-α2b (AMIGA: autologous monocytes and interferon-γ and -α) are given intraperitoneally to women with recurrent, platinum-resistant ovarian cancer. Of the 18 patients enrolled in the Phase 1 clinical trial, two patients had partial responses and four patients had stable disease. Of these, both responders and two of the four patients with stable disease

experienced clinical benefit for five or more months, and were thus labelled as long-term responders (LTRs). Through bulk-cell RNA-sequencing, the peripheral blood mononuclear cells (PBMCs) of these LTRs showed increased levels of T cell activation and migration genes compared to non-responders (NRs). By utilizing cell type enrichment on this dataset, the gene signatures of effector T and B cells were also found to be increased in the PBMCs of LTRs relative to NRs. Interestingly, monocyte and macrophage gene signatures were higher in NRs, suggesting possible egress of monocytes from the peritoneal cavity into circulation. In light of this, we assessed patient malignant ascites for relevant chemokines and found CCL2, a known monocyte and T cell trafficking molecule, had increased in response to treatment. Through *in vitro* 2D and 3D co-cultures, we then validated CCL2 significantly increased in the presence of ovarian cancer cells and our novel therapy, and that this increase correlated with increased T cell and monocyte migration. Furthermore, by targeting the CCL2-CCR2 signaling axis *in vitro*, we were able to significantly alter the migration of the aforementioned cell types. Future studies will evaluate the impact of manipulating CCL2 *in vivo* on T cell migration and function, as well as monocyte retention, in combination with our novel immunotherapy. Herein, we describe a potential mechanism to simultaneously increase the presence of both effector monocytes and T cells in hopes of improving the clinical efficacy of AMIGA, a novel innate-based immunotherapy. By understanding how AMIGA contributes to T cell migration and function in the ovarian cancer tumor microenvironment, we will next seek to combine innate- and adaptive-based immunotherapies to improve the treatment options and, ultimately, survival of women with ovarian cancer.

B117: Concomitant inhibition of tyrosine phosphatases TC-PTP and PTP1B as a synergistic pathway to PD-1 blockade

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CD8 T cells are the main effectors of the adaptive immunity against tumours, giving them an essential role in cancer immunosurveillance. In recent decades increased understanding of the modulatory pathways controlling CD8 T cell effector function led to the formulation of the successful checkpoint inhibitor-based immunotherapies. However, their success is limited to a small number of tumour types, motivating the search for novel targets. T cell protein tyrosine phosphatase (TC-PTP) and protein tyrosine phosphatase 1B (PTP1B) are 2 protein tyrosine phosphatases known to participate in the regulation of pathways involved in CD8 T cell activation, as JAK/STAT and Src family kinases. Work from our laboratory established TC-PTP as a main systemic immune modulator, moreover, recent publications specifically place TC-PTP deficiency in improving CD8 T cell effector qualities by reducing exhaustion. Given that TC-PTP and PTP1B share extensive sequence homology and functional redundancy have been documented in embryos and other immune cell types, we investigated the potential effects of conditionally targeted the genetic deletion of either or both phosphatases in mouse CD8 T cells. Our results demonstrate that hemizygous deletion of PTP1B in a TC-PTP deficient background heightens the enhanced effector phenotype already observed in TC-PTP deficient CD8 T cells, an effect that was reproducible with the use of small-molecule inhibitors which target

simultaneously both phosphatases. Moreover, we found that in combination with PD-1 blockade the systemic use of these inhibitors enhanced the antitumoral effects of PD-1 blockade alone. Hence, our results suggest that small molecule inhibitors with dual specificity for PTP1B and TC-PTP are a powerful therapeutic tool for potentiating CTL cytotoxic responses.

B118: Intratumoral neurotransmitter signaling controls T cell exhaustion and anti-tumor responses

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Background: Immune checkpoint inhibition via CTLA-4, PD-1, and PD-L1 blocking has provided an effective and long-term durable cancer immunotherapy for many patients with diverse diseases, however, most patients do not respond to the therapy. Although pre-clinical studies of additional cell surface receptors (LAG-3, TIGIT, and TIM-3) have been promising, other additional inhibitory pathways that contribute to immunosuppression have yet to be delineated.

Neurotransmitters (NTs) are small signaling molecules, generally secreted by neurons to facilitate communication between these cells and their targets. NTs are detectable in serum and present in the tissue and can be sensed by immune cells. The effects of NTs on immune cells have been immunosuppressive due to multiple distinct mechanisms. However, the role of NTs in the tumor microenvironment has not been studied, and their presence in the tumor microenvironment (TME) has not been reported.

Hypothesis: We hypothesize that intratumoral neurotransmitters provide additional immunosuppression and can be targeted pharmacologically to improve anti-tumor responses.

Methods and Results: We have detected dopamine and norepinephrine in TME of a murine melanoma model by mass cytometry and showed that dopamine beta-hydroxylase (DBH, the enzyme that converts dopamine to norepinephrine) is expressed in a subset of human T cells in human melanoma biopsies by scRNAseq. High DBH expression correlated with increased expression of exhaustion markers. Additionally, the presence of these cells was confirmed in murine models. Subsets of immune cells isolated from murine melanoma TME could convert dopamine to norepinephrine ex vivo, indicating that the DBH enzyme in immune cells is functionally active.

We have additionally shown that the inhibition of the DBH enzyme or inhibition of the adrenergic receptors that norepinephrine will act on improves tumor control in animals that have received immune checkpoint blockade compared to controls.

Conclusions: This study attributes a novel function to immune cells in TME: the production of neurotransmitters. We demonstrate that intratumoral neurotransmitter signaling acts as an immunosuppressive pathway and is addressable with FDA-approved compounds to modulate to improve the efficacy of immune checkpoint blockade. Future studies will focus on the molecular

mechanisms that lead to Dbh expression on select immune cells and how this pathway can be expanded to other cancers.

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B119: Bacterial anti-phage immunity as a discovery platform for novel signaling pathways in human 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 to be 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. One of the first examples of this cross-kingdom conservation was the discovery that bacteria encode cGAS-like enzymes that are activated in response to viral infection and produce a cyclic nucleotide signal that activates downstream receptors and drives an antiviral response, similar to the cGAS-STING pathway in eukaryotes. In prokaryotes, this antiviral system is named ‘cyclic oligonucleotide based antiphage signaling system’ (CBASS), and there are currently no known mechanisms used by viruses to evade CBASS defense. Here, we show that bacteriophages encode anti-CBASS (Acb) proteins that counteract defense by specifically degrading cyclic nucleotide signals that activate host immunity. Using a biochemical screen of 32 Escherichia coli phages, we discover Acb1 from phage T4 as a founding member of anti-CBASS immune evasion proteins. Crystal structures of Acb1 in complex with 3′3′-cyclic GMP–AMP reveal a mechanism of cleavage that closely mirrors the mechanism used by poxin enzymes in vaccinia virus to degrade 2’3’-cGAMP and evade cGAS-STING immunity. However, in contrast to the exquisite specificity of poxin enzymes for 2’3’-cGAMP, Acb1 cleaves a diverse array of cyclic nucleotide signals, suggesting that it may be a useful tool to discover novel cyclic nucleotide signals used in eukaryotic immunity. These findings demonstrate that mechanisms of viral evasion of innate immunity are conserved across kingdoms of life, and highlight the utility of studying prokaryotic defense systems in developing tools to discover new features of eukaryotic innate immunity.

B120: 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 molecule and deliver a label or payload—i.e., target—is fundamental in both research and clinical settings. However, existing methods are often limited, failing to identify rare cell populations or distinguish between subsets with overlapping surface marker expression. We are designing a new type of targeting system to enhance sensitivity while also achieving greater precision. By using an enzyme as the core of our approach, we introduce additional parameters—enzyme kinetics, substrate binding affinity, enzyme: substrate ratio—that can be tuned to optimize activity while minimizing off-target interactions. Enzymes' catalytic nature also affords inherent signal amplification to improve sensitivity over conventional targeting methods. We are working to create activatable versions of our system that take advantage of local environment-specific features or incorporate a two-marker requirement for greater selectivity. Our system is designed with modularity in mind, with the ligand and cargo both readily exchanged to target distinct sub-populations of immune and tumor cells for a range of applications, from detection and tracking to selective ablation to delivering nucleic acids for gene therapy.

B121: Development of high-throughput DIANA for screening of CD73 inhibitors

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In recent years, it became apparent that immune checkpoint therapy has its limits, and the need to find new targets for immunotherapy has emerged. One of these promising targets is adenosine. In the tumor, general hypoxic conditions and cellular stress lead to increased levels of extracellular ATP that is catabolized to adenosine and phosphate by two membrane ectonucleotidases CD39 and CD73. Adenosine then binds to A2 receptors on the surface of various immune cells leading to suppression of their response. Adenosine also contributes to the development of resistance to immune checkpoint blockade therapies. Preventing the release of adenosine by blocking the activity of CD73 using small-molecule inhibitors or monoclonal antibodies has been shown to decrease the tumor burden in preclinical models.

Development of CD73 small-molecule inhibitors has mostly focused on rational design and structure-activity relationship (SAR) studies using non-hydrolyzable ADP analogs, such as adenosine (α,β)-methylenediphosphate (AMPCP), as lead structures. Thus far, there has not been an easy-to-perform, high-throughput assay that would directly and with high sensitivity been able to evaluate large chemical libraries for CD73 inhibitors. To find novel lead molecules, we set to develop CD73 DIANA (DNA-linked inhibitor antibody assay), that combines sandwich ELISA with detection by quantitative PCR. In DIANA, the immobilized protein is incubated with a mixture of a tested compound and a detection probe, that consists of a known small-molecule inhibitor covalently attached to reporter DNA. The tested compound and the probe compete over the binding to the protein, which is monitored by qPCR. In addition, due to the

high sensitivity of qPCR, DIANA allows working with small reaction volumes leading to very low consumption of reagents.

We performed high-throughput screening of the IOCB chemical library, a proprietary collection of thousands of compounds synthetized at the Institute of Organic Chemistry and Biochemistry of the Czech Academy of Sciences. We identified several hits, two of which were confirmed to inhibit the enzymatic activity of CD73 in an orthogonal assay. These hits served as leading structures for a small SAR study. The resulting compounds were evaluated in an enzymatic assay using purified CD73 or CD73-expressing cells. We identified a new, potent CD73 inhibitor with low nanomolar Ki values.

CD73 DIANA represents a new highly-sensitive method for a high-throughput screening of chemical libraries. The discovery of new ligands of CD73 enables further elucidation of the biochemical characteristics of the enzyme as well as clinical development of the discovered compounds.

B123: Piezo1 expression in both CD4+ and CD8+ T-cells is essential for optimal anti-tumor responses

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The presence of functional effector T-cells in the tumor microenvironment (TME) is critical for successful anti-tumor responses and is a strong prognostic factor in cancer. T-cell anti-tumor function is dependent on their ability to differentiate from a naïve state, infiltrate into the tumor site, and exert cytotoxic functions. The factors dictating whether a particular T-cell can successfully undergo these processes during tumor challenge are not completely understood.

Piezo1 is a mechanosensitive cation channel with high expression on $CD4^+$ and $CD8^+$ T-cells. Previous studies have demonstrated that optimizes T-cell activation *in vitro* and restrains the $CD4^+$ regulatory T-cell (T_{reg}) pool. However, little is known about the role of $CD4^+$ and $CD8^+$ T-cell during tumor challenge.

We hypothesized that disruption of T-cell Piezo1 impairs anti-tumor immunity *in vivo* by hindering inflammatory T-cell responses. We generated mice with T-cell specific Piezo1 deletion (P1ko) and challenged them using tumor models characterized by T-cell mediated rejection. P1ko mice had the most aggressive tumors, with higher growth rates and unresponsiveness to immune-mediated therapeutic interventions. Remarkably, the tumor draining lymph nodes (tDLN) of P1ko mice demonstrate a dramatic increase in the number of both $CD4^+$ and $CD8^+$ T-cells, with a corresponding drop in the CD4:CD8 ratio compared to WT mice that correlated inversely with tumor size. Adoptive transfer of antigen-specific T-cells into tumor-bearing immunodeficient mice revealed reduced $CD8^+IFN\gamma^+$ T-cells and a somewhat increased T_{reg} pool in the TME. Together, these results suggest multifaceted roles for Piezo1 during T-cell priming that result in optimized functional $CD4^+$ and $CD8^+$ effector T-cell pools during the anti-tumor response.

B125: 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. Using single-cell RNA sequencing of mouse thymic myeloid cells and lineage tracing experiments our data suggests that aDCs in the thymus are of dual origin differentiating either from $XCR1^+$ DC1 (aDC1) or $SIRP\alpha^+$ 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 IFNλR 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.

B126: Tissue-resident memory CD8+ T cells infiltrating human renal cell carcinoma tumors are highly heterogeneous, display tumor reactivity and associate with better survival

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Tissue-resident memory $CD8^+$ T (Trm) cells mediate potent innate and adaptive immune responses. Moreover, tumor-infiltration with Trm cells has been associated with a more favorable prognosis in several solid cancers. However, the tumor reactivity and clinical relevance of tumor-infiltrating Trm cells in patients with renal cell carcinoma (RCC) has not been studied.

First, we analyzed by flow cytometry the phenotype of $CD8^+$ T cells in freshly resected RCC tumors. Based on the expression of Trm cell markers CD69 and CD103, we observed relatively high frequencies of $CD69^+CD103^-$ and $CD69^+CD103^+$ memory $CD8^+$ T cells, which express higher levels of tumor reactivity- and tissue residency-associated receptors, including PD-1, 4-1BB and CD49a. Moreover, the $CD69^+CD103^-$ subset expressed higher levels of exhaustion-associated markers TOX-1, TIM-3 and CD39. Interestingly, both subsets specifically recognized autologous tumor cells but not non-malignant renal tissue. RNA sequencing analyses demonstrated that the $CD69^+CD103^+$ subset highly expresses a residency signature (e.g. Hobbit, CD49a, CXCR6, CXCL13) and effector program (e.g. IL-2, TNF, FOS, JUN), whereas the $CD69^+CD103^-$ subset has an intermediate expression of residency signature and high expression of genes associated with terminally differentiated program (e.g.TOX, TIM-3, CD39). On the other hand, CD69-CD103- $CD8^+$ T cells are enriched in circulating genes (e.g. KLF2, CD62L, S1PR1) with a progenitor phenotype (high expression of TCF1, low expression of TOX and inhibitory receptors). Interestingly, we found by single cell RNA sequencing a high heterogeneity within these subsets, with several subpopulations enriched in Trm cell-associated gene signature, including proliferating, progenitor and terminally differentiated Trm cells. All these Trm subpopulation gene signatures were associated with a better survival in RCC patients.. These data evidence the presence of tumor-reactive Trm cells with distinct functional states infiltrating human RCC tumors associated with a better survival of RCC patients.

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B127: Chlorin e6-based photodynamic therapy enhances abscopal antitumor effects via blockade of PD-1/PD-L1 immunological-checkpoint

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Photodynamic therapy (PDT) has become a prominent technique for cancer treatment due to its inherent advantages, such as a high degree of selectivity, less invasiveness, and minimal systemic toxicity. Through PDT, anti-tumor responses to both primary and distant cancers are connected by immunogenic cell death (ICD). ICD dramatically increases the immunogenicity of dying cancer cells, causing controlled immune system activation. Such immune responses generate abscopal effect, a phenomenon where local irradiation induces distant contralateral non-treated tumors to regress. In this study, we intended to create a model to investigate immunogenic factors that influence the abscopal effect caused by Chlorin e6 (a photosensitizer)-PDT. C57BL/6 mice were procured from Orient Bio (Seongnam, Korea). Mouse models of bilateral melanoma tumors or pancreatic cancer models were established by injecting B16F10 ($2x10^5$) or Panc02 ($8x10^5$) subcutaneously into the right flank of mice (irradiated tumor) and left flank (non-irradiated tumor) into the C57BL/6 mice. Tumors from effective PDT and abscopal, effective PDT but non-effective abscopal and further non-effective PDT but effective abscopal group of mice were selected for tumor extraction. When the mean tumor volume reached 90 mm^3, the mice were randomly assigned to various treatment groups ($n \geq 5$ per group). In the irradiation group, PDT was conducted by injecting 100 μL of Chlorin e6 (Ce6) into the tail vein at a dose of 2.5 mg/kg, with a drug-PDT interval of 1 h and LED 660-nm illumination at 100 J/cm^2. After 28 days, all mice were sacrificed and their tumors were collected for further studies.

Flow cytometry was used to examine tumor infiltrating T cells (TILs) directly using CD4/CD5, CD3, CD8/CD3, CD25/CD4, CD45, CD103/CD8, CD39/CD8, CD11c, F4/80, NK1.1, CD86, CD68 and CD206. The results showed significantly elevated percentage of CD8+ T cells at 4 μM in both 24 and 48 h of coculture incubation in comparison to control, Ce6-only and light only treated cells. Also, with Ce6-PDT and PD-1/PDL1 blockage, the cytotoxic function of $CD39^{+}CD8^{+}$ T cells was impaired in the anti-tumor immune response while the infiltration of $CD39^{+}CD8^{+}$ T cells is improved. Finally, synergistic CD8+ T cells' ability to fight tumors is partially recovered when CD39 is inhibited. Thus, PD-1/PD-L1 blockade in Ce6-PDT-treated tumor cells was associated with increased tumor cell apoptosis and enhanced frequency $CD8^{+}$ T cells through cytokine, IL-2 and suppression of $CD39^{+}$ T cell activity. Synergistic Ce6-PDT and PD-1/PDL-1 checkpoint blockade therapy accomplishes primary and abscopal tumor inhibition. This study extended the use of Chlorin e6-associated photodynamic therapy to the treatment of both local and metastatic illness by examining the combined effect of photodynamic therapy and immunotherapy using a bilateral subcutaneous melanoma mouse model that offers the chance to increase abscopal response rates. [This work was supported by grants from the Korea Medical Device Development Fund grant funded by the Korea government (the Ministry of Science and ICT, the Ministry of Trade, Industry and Energy, the Ministry of Health & Welfare, the Ministry of Food and Drug Safety) (NTIS Number: 1711135018, RS-2020-KD000106)].

B128: Investigating the immunomodulatory effects of histotripsy ablation in spontaneously occurring canine osteosarcoma: A comparative human oncology model

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Osteosarcoma (OS) is the most common malignant bone tumor in both canine and pediatric cancer patients, accounting for greater than 80% and 55% of primary bone tumor diagnoses, respectively. Spontaneously occurring canine OS shares numerous biological and genetic similarities with human OS and is far more prevalent, allowing the canine to serve as an informative comparative oncology model for the human. The primary cause of death in OS patients is metastatic disease; thus, the major hurdle to improving survival expectations continues to be metastasis. The novel, non-thermal, non-invasive, focused ultrasound ablation modality, histotripsy, has been reported in preclinical investigations to induce immunomodulation. Potentially stimulating an anti-tumor immune response which may mitigate metastatic disease burden. Our group has previously established the feasibility of delivering histotripsy ablation treatments to primary bone tumors in canine OS patients. Therefore, the objective of this study was to pioneer an investigation into the immunological outcomes associated with histotripsy ablation of primary OS tumors in clinical trial enrolled canine OS patients. We hypothesized that histotripsy ablation will result in local and systemic immunomodulation and stimulate an anti-tumor immune response. After obtaining owner consent, canine OS patients (n=20) who had not received any immunomodulatory or tumor

directed therapeutics nor had radiographic evidence of pulmonary metastatic disease, were enrolled into a treat, and resect histotripsy ablation clinical trial. A 2 cm spherical portion of the primary tumor was targeted during histotripsy ablation treatment and at either 24 hours, 3-, or 5-days post histotripsy treatment patients underwent standard of care limb amputation surgery. Treated and untreated regions of the tumor were harvested at the time of surgery for immune cell phenotyping via flow cytometry, gene expression analysis, and histological evaluation. Peripheral blood samples were collected prior to histotripsy treatment, time of surgery, and 2 weeks post-surgery for immune cell phenotyping. Successful ablation of the targeted tumor region was achieved in all enrolled patients and characterized microscopically by loss of cellular architecture and lytic necrosis. When comparing treated and untreated regions of tumor, patient dependent changes in immune cell populations (CD5+ CD4+, CD5+ CD8+, and CD11b+ CD14+ cells) were observed most notably at 3- and 5- DPT. Evaluation of immune and inflammatory genetic signatures revealed a fold change of $\leq$ -2 or $\geq$ 2 in 49 genes relating to inflammatory and immune signaling pathways including natural killer cell mediated cell cytotoxicity. Systemically, there was greater monocyte expression of CD80 at 24 hours post histotripsy ablation compared to pretreatment and monocyte populations from healthy control dogs. Collectively, our results indicate that histotripsy ablation has the potential to induce immunomodulation in spontaneously occurring canine OS and continued investigation is underway. The results of this study have also created the foundation for cross species (canine, human, and murine) in-vitro studies to allow for further elucidation of the histotripsy induced immune response in OS and translation relevance for the advancement of histotripsy ablation for human OS patients.

B129: cGAMP receptors from bacteria enable development of a programmable biosensor

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Cyclic GMP–AMP (cGAMP) is a universal signaling molecule utilized by innate immune systems of mammals, invertebrates, and bacteria. In mammals, cGAMP is produced by cyclic GMP–AMP synthase (cGAS) in response to cytosolic double-stranded DNA, which is indicative of infection, organelle damage, or cancer. cGAMP activates the Stimulator of Interferon Genes (STING) which activates antitumor signaling pathways. The cGAS-STING axis is often dysregulated during these pathologies; thus, it is a target for immunotherapy. However, a major hurdle in understanding the basic biology of this pathway is the lack of high-throughput molecular tools for rapid quantification and detection of cGAMP levels. In bacteria, cGAMP signaling executes antiviral immunity against bacteriophages via ancestral cGAS-STING pathways which are the progenitors of their eukaryotic counterparts. Unlike vertebrates, bacterial cGAMP receptors are often fused to highly diverse effector modules such as enzymes that execute diverse reactions in response to low nanomolar to subnanomolar ligand binding. We hypothesized that this feature may allow for engineering of cGAMP-responsive enzymes via domain-swapping to create high-fidelity biosensors for rapid detection of cGAMP in diverse samples. Here we describe the discovery of novel cGAMP receptors in bacteria that are fused to diverse effector modules. Further, we demonstrate proof-of-principle that these receptor-effector pairs can be swapped to create functional chimeric ligand-activated enzymes that respond to nanomolar levels of cGAMP. We postulate that this system can function as a new platform for the development of customized cGAMP-responsive biotechnology with applications in biosensor development, cargo delivery, and synthetic biology for cancer immunotherapy.

B130: The role of different microbiota in metastatic brain tumors

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Background: Metastatic brain tumors are associated with significant morbidity and mortality. The current limited understanding of the mechanisms underlying brain metastasis has hindered the development of efficient diagnostics and therapeutics for this disease. The microbiota has emerged as a novel hallmark of cancer, with a prominent role in tumorigenesis, tumor immunity, and response to treatment. However, the role of different microbial communities in tumor metastasis, and in particular brain metastasis, is poorly understood. We hypothesize that distinct microbial communities can alter the immune microenvironment in the brain and affect brain metastasis development.

Methods: To evaluate the role of different microbial communities in brain metastasis, matched stool, saliva, tumor, and plasma samples were collected prospectively from patients with metastatic brain tumors who underwent surgical tumor resection at the University of Texas MD Anderson Cancer Center. Stool and saliva samples were collected using the OMNIgene microbiome collection and stabilization kits (DNAgenotek, Kit # OM-200 and OM-505, respectively). Tumor samples were flash frozen in sterile conditions. Stool and saliva samples from 30 patients and tumor and plasma from 15 patients were sequenced via metagenomic shotgun and 16S rRNA sequencing, respectively. Microbiome profiling was conducted through established computational pipelines reported previously by our group. To further explore the mechanistic role of the gut microbiota in brain metastasis, we depleted gut microbiota in conventionally raised mice using a broad-spectrum non-absorbable antibiotic regimen. Melanoma tumor cells were subsequently injected intracranially to evaluate the effect of gut microbiota depletion and associated immune changes on tumor growth. Tumor growth was measured through in vivo bioluminescent imaging and histology. Peripheral and tumor immune profiling was conducted through flow cytometry and immunohistochemistry.

Results: In all types of microbiota evaluated in this study, distinct signatures were identified to be associated with brain metastasis compared to other types of brain tumors. Interestingly, we demonstrated an overlap between the tumor microbiome and the oral microbiome but not with the gut microbiome. These findings suggest a direct contribution of the oral microbiome and the potential indirect contribution of the gut microbiome to the development of brain metastasis. Our mechanistic studies on the role of gut microbiota in brain metastasis demonstrated that depletion of the gut microbiota in mice decreased tumor growth in the brain. Evaluation of the peripheral and tumor immune profiles suggested the underlying mechanisms to involve alterations in the circulating cytokine profiles and an increase in anti-tumor T cell activity.

Conclusion: Our clinical studies suggest the association of distinct microbial communities with brain metastasis. Our pre-clinical findings demonstrate that the absence of gut microbiota can modulate the regulation of T cell activity to induce an anti-tumor response in the brain. Further studies, currently in progress, will determine the individual and collective role of different microbial communities in the development of brain metastasis.

B131: Transcriptional mechanism of Aire through the P300/CBP axis

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The mammalian adaptive immune system relies on a series of intricate selection process on immune repertoires to enforce a robust discrimination between self and non-self. Transcription factor Aire is essential to ensure a substantial fraction of peripheral tissue antigens (PTAs) to be expressed on medullary thymic epithelial cells (mTECs), which then selects for autoreactive T cells to undergo clonal deletion or regulatory T cell (Treg) differentiation. However, mechanistically how Aire recognizes and transactivates the target genes remain unclear. The conserved C-terminal tail of Aire ($Aire^{CTT}$) is required and sufficient to activate gene expression. Using two orthogonal and complementary approaches, one through molecular interaction-based biochemical analysis and the other through genetic screen-based functional analysis, I have identified the histone acetyltransferases (HAT) P300/CBP as direct interacting functional partners for $Aire^{CTT}$. Nascent transcriptomic analysis show that the expression of Aire-induced genes is dependent on the HAT activity of P300/CBP. Additionally, the formation of transcriptionally active hub within Aire foci requires P300/CBP. Our study suggests a model that Aire directly recruits CBP/P300 and utilizes their acetylation activity to create a 3-dimensional hub for transcriptional activation of a large collection of genes.

B132: PD-1 signaling and lipid metabolism in Peripheral T-cell Lymphoma

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Background: Despite modern combination therapies, patients with peripheral T-cell lymphoma (PTCL) continue to experience high rates of refractory disease and early relapse. The *PDCD1* gene, which encodes the programmed cell death protein 1 (PD-1) immune checkpoint receptor, is a key tumor suppressor in T-cells and is inactivated in the malignant cells of 20%–30% of patients across most aggressive PTCL subtypes. The highest frequencies of *PDCD1* deletions have been detected in patients with advanced disease stages and are associated with a poor prognosis. Clinical trials have revealed multiple cases of PTCL hyperprogression or *de novo* development after anti-PD-1 checkpoint inhibitor treatment. While all these clinical observations highlight the critical importance of inhibitory PD-1 signaling in T-cell malignancies, the tumor-suppressive mechanisms of PD-1 remain unknown.

Objectives: Oncogenic reprogramming of the lipidome is a key driver of tumorigenesis (*Snaebjornsson et al*. Cell Metab 2020) and many tumors activate *de novo* synthesis of cholesterol and other lipids (*Wang et al.* Front Oncol 2020). Importantly, targeting enzymes associated with lipogenesis has been shown to inhibit tumor growth (*Liu et al.* Cancer Lett 2017, *Xue et al.* Front Oncol 2020) but experimental data on PTCL is missing. We performed global RNA-seq analysis in primary *PDCD1*-mutant and wild-type human PTCLs and detected transcriptional upregulation of key lipogenic enzymes such as HMGCR and SREBP2 and

postulate that PD-1 could be an important regulator of oncogenic lipid metabolism.

Methods: To quantify cellular and plasma membrane cholesterol levels in cells derived from *PDCD1*-mutant and wild-type PTCL xenograft and genetically engineered mouse models, we will employ three techniques: Filipin III staining, cholesterol oxidation-based assays and biotinylation-based plasma membrane lipid raft quantification (*Lee et al.* Nature 2020). Expression of cholesterol biosynthetic enzymes, cholesterol transporters and cholesterol catabolic enzymes will be determined by rtPCR and verified by Western blot or flow cytometry. PD-1-dependent LDLR activity will be determined with fluorescently labeled LDL and flow cytometry (*Stephan and Yurachek.* J Lipid Res 1993). To evaluate PD-1 regulated effects upon perturbation of cholesterol homeostasis, we will investigate viability, proliferation, and colony formation rates of PTCL cells isolated from transgenic mice and xenograft models in presence of cholesterol biosynthesis inhibitors cholesterol transport inhibitors, cholesterol-depleting reagents, Sterol regulatory element binding proteins inhibitors and cholesterol efflux inducers. These experiments will be performed in standard cell culture media and LDL-free media to evaluate dependency on LDL import in the different conditions and genotypes.

Results: We expect to detect enhanced expression of proteins for cholesterol biosynthesis and LDL import in PTCLs with PD-1 pathway inactivation and that this will result in an increase in total and plasma membrane cholesterol and higher LDL uptake rates. Based on our preliminary data, we also hypothesize that elevated levels of cholesterol-rich lipid rafts will induce increased apoptosis sensitivity upon treatment with cholesterol-depleting agents. Thus, we predict that LDL depletion in combination with inhibition of cholesterol biosynthesis will reduce survival and proliferation particularly in *PDCD1*-mutant PTCLs.

Conclusions: Based on our preliminary data, we anticipate that cholesterol metabolism is differentially regulated in PTCL based on their genetic PD-1 status and selective perturbation of their cholesterol homeostasis could serve as a novel therapeutic strategy.