solid tumors. Previous experiments showed that tumor lactic acidosis selectively targets the signaling pathway including JNK/c-Jun and p38, resulting in inhibition of IFN- γ production. In contrast, granule exocytosis, which is regulated via the MEK1/ERK pathway, was moderately affected. Based on the contrasting effects on these two essential T cell effector activities, we investigated in more detail the effects of lactic acidosis on the killing process conducted by T cells.

Material and Methods Tumor cells and cytotoxic T cells were co-cultured in lactic acid or regular culture medium and analyzed for effector function by flow cytometry and cell-mediated cytotoxicity assays. Additionally, 'in-channel micropatterning' in combination with artificial intelligence (AI) aided image analysis was used to visualize and analyze T cell cytotoxicity and mobility on a single cell level. Usage of collagen-matrices allowed the observation of T cell activity in a physiological three-dimensional environment. Cell metabolism was analyzed by Seahorse technology.

Results In the presence of lactic acid, IFN- γ production was strongly inhibited, while degranulation was only moderately reduced. Detailed analysis of the different processes involved in T cell cytotoxicity revealed that T cell recognition of tumor cells resulted in less secretion of cytotoxins (perforin, granzyme B and granzyme A). Lytic activity against tumor cells was strongly reduced at low T cell to tumor cell ratio (1:2). This deficiency could be compensated by increasing the T cell to tumor cell ratio (10:1). Using live cell imaging we investigated underlying mechanisms that might explain how higher T cell to target cell ratios might overcome lactic acid inhibition. T cells in lactic acid covered less distance, they moved for longer time periods and made less contacts with tumor cells in comparison to T cells cultured in regular culture medium.

Conclusions Micropatterning and AI based image analysis allows for detailed assessment of the processes involved in T cell-mediated killing such as mobility, speed, directionality and attachment on target cells. Lactic acidosis is hampering T cell killing activity by reducing the T cell's capacity to find its target cell and attach to it. Repeated addition of T cells or neutralization of lactic acidosis in the TME are means to overcome these deficits and hold promise to improve the outcome of T cell-based immunotherapies.

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On Demand Talks: Precision Medicine Meets Immunotherapy (Immuno-Monitoring)

02 DIRECTLY LINKING SINGLE T CELL PHENOTYPE AND FUNCTION TO GENOTYPE

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T cell therapies for cancer treatment are challenging to develop because of the complex mechanisms and cell interactions that underly T cell-mediated tumor killing. Current technologies rely on correlating phenotype, function, and gene expression based on experiments performed on different populations of T cells because no one platform is able to assess cell surface marker expression, cytokine secretion, and tumor cell killing activity of the same T cell and recover this cell for downstream genomic analysis. Here we share two use cases - CAR-T cell functional screening and TCR sequence recovery following functional assay - that demonstrate how the T Cell Analysis Suite on the LightningTM optofluidic platform can be used to directly link T cell phenotype and function (IFN γ secretion and tumor cell killing) to genotype (TCR sequence recovery) at a single-cell level and on the same T cell, enabling deeper and more thorough characterization of how T cells mediate tumor cell death and potentially the development of more efficacious therapies.

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03 HIGH-DIMENSIONAL ANALYSIS OF TUMOR ARCHITECTURE PREDICTS CANCER IMMUNOTHERAPY RESPONSE

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Background Immunotherapies have induced long-lasting remissions in countless advanced-stage cancer patients, but many more patients have not benefitted. Therefore, novel predictive markers are needed to stratify patients before treatment and select those who will most likely benefit from immunotherapy, while avoiding potentially devastating adverse effects and high treatment costs for those who will not. We reasoned that thoroughly characterizing the architecture of the tumor microenvironment (TME) at the single-cell level by highly multiplexed tissue imaging should reveal novel spatial biomarkers of immunotherapy response.

Materials and Methods We used CODEX (CO-Detection by indEXing) highly multiplexed fluorescence microscopy to investigate the TME of cutaneous T cell lymphoma (CTCL) in samples from patients treated with pembrolizumab. 55 protein markers were visualized simultaneously using a tissue microarray of matched pre- and post-treatment skin biopsies from 7 pembrolizumab responders and 7 non-responders. After computational image processing and extraction of single-cell information, cell types were identified by unsupervised clustering followed by supervised curation, and cell-cell distances and 'cellular neighborhoods' were computed. We also performed RNA sequencing on laser-capture microdissected tissue microarray cores to extract cell-type specific gene expression profiles by CIBERSORTx analysis.

Results CODEX enabled the identification and characterization of malignant CD4+ tumor cells and reactive immune cells in the CTCL TME at the single-cell level, resulting in 21 different cell type clusters with spatial information. Cluster frequencies were not significantly different between responders and non-responders pre- and post-treatment. However, advanced computational analysis of the tumor architecture revealed cellular neighborhoods (CNs) that dynamically changed during

pembrolizumab therapy and were correlated with response. Effector-type CNs enriched in tumor-infiltrating CD4+ T cells and dendritic cells were significantly increased after treatment in responders. In contrast, a regulatory T cell-enriched CN was significantly increased in non-responders before and after therapy. Furthermore, a spatial signature of cell-cell distances between tumor cells and effector/regulatory immune cells predicted therapy outcome. In addition, CIBERSORTx analysis revealed that tumor cells in responders, but not in non-responders, increased their expression of immune-activating genes.

Conclusions High-dimensional spatial analysis of CTCL tumors revealed a pre-existing immunosuppressive state in pembrolizumab non-responders. Thorough analysis of the TME therefore enables the discovery of novel spatial biomarkers in a concept that accounts for both cell type information and higher-order tumor architecture. Combining highly multiplexed microscopy with CIBERSORTx allows for the discovery of novel, predictive spatial biomarkers of immunotherapy response and will pave the way for future studies that functionally address these cell types and their interactions.

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On Demand Talk: 'Lost in Translation'

04 MECHANISMS OF LUNG CANCER HYPER-PROGRESSION PROMOTED BY PD-1 IMMUNE CHECKPOINT BLOCKADE

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Background Immune checkpoint blockade (ICB) with antibodies against PD-1 or PD-L1 may provide therapeutic benefits in patients with non-small cell lung cancer (NSCLC). However, most tumours are resistant and cases of disease hyper-progression have also been reported.

Materials and Methods Genetically engineered mouse models of Kras^{G12D}p53^{null} NSCLC were treated with cisplatin along with antibodies against angiopoietin-2/VEGFA, PD-1 and CSF1R. Tumour growth was monitored by micro-computed tomography and the tumour vasculature and immune cell infiltrates were assessed by immunofluorescence staining and flow cytometry.

Results Combined angiopoietin-2/VEGFA blockade by a bispecific antibody (A2V) modulated the vasculature and abated

immunosuppressive macrophages while increasing CD8⁺ effector T cells in the tumours, achieving disease stabilization comparable or superior to cisplatin-based chemotherapy. However, these immunological responses were unexpectedly limited by the addition of a PD-1 antibody, which paradoxically enhanced progression of a fraction of the tumours through a mechanism involving regulatory T cells and macrophages. Elimination of tumour-associated macrophages with a CSF1R-blocking antibody induced NSCLC regression in combination with PD-1 blockade and cisplatin.

Conclusions The immune cell composition of the tumour determines the outcome of PD-1 blockade. In NSCLC, high infiltration of regulatory T cells and immunosuppressive macrophages may account for tumour hyper-progression upon ICB.

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On Demand Talk: Young Researcher Session

05 DECONSTRUCTION OF HAMPERED DENDRITIC CELL DEVELOPMENT BY MICRO-ENVIRONMENTAL CROSSTALK IN AN ORGANOTYPIC HUMAN MELANOMA-IN-SKIN MODEL

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Background Immune suppressive conditions in the melanoma tumor microenvironment (TME) block dendritic cell (DC) development and lead to the accumulation of M2-like macrophages and myeloid-derived suppressor cells (MDSCs). This will effectively hamper T cell priming, recruitment, and effector functions, and so interfere with the efficacy of immunotherapy. Targeting tumor-mediated myeloid suppression represents an interesting therapeutic option to promote the immune attack on tumors. The preclinical human models currently used to study myeloid suppression often fail to reflect the complexity of the TME.

Materials and Methods To study the cross-talk between melanoma and stroma cells and assess its effect on DC differentiation, we therefore established an *in vitro* three-dimensional (3D) reconstructed organotypic human melanoma-in-skin (Mel-RhS) model, allowing the monitoring of tumor growth and progression for up to six weeks.

Results Significantly higher levels of immune suppressive cytokines (IL-10, M-CSF, VEGF, TGF-beta) were detected in the melanoma model, constructed with the BRAF- and PTEN-mutated SK-MEL-28 cell line, as compared to its control (without melanoma cells). Indeed, Mel-RhS culture supernatants interfered with monocyte-to-DC differentiation, leading to the development of M2-like macrophages with a distinct phenotype (CD14⁺CD11a⁺BDCA3⁺CD163⁺CD16⁺PDL1⁺PDL2⁺), as established by polychromatic flowcytometry. Correlation matrix heatmap analysis identified IL-10, TGF-beta and M-