to target multiple antigens is required. We propose a controlled ACT approach, where T cells are armed with synthetic agonistic receptors (SARs) that are conditionally activated only in the presence of a target AML-associated antigen, and a cross-linking bispecific T cell engager (BiTE) specific for both (SAR) T cell and tumour cell.

Materials and Methods A SAR composed of an extracellular EGFRvIII, trans-membrane CD28, and intracellular CD28 and CD3z domains was fused via overlap-extension PCR cloning. T cells were retrovirally transduced to stably express our SAR construct. SAR-specific bispecific T cell engagers (BiTE) that target AML-associated antigens were designed and expressed in Expi293FTM cells and purified by nickel affinity and size exclusion chromatography (SEC). We validated our approach in three human cancer models and patient-derived AML blasts expressing our AML-associated target antigen CD33.

Results CD33-EGFRvIII BiTE, monovalently selective for our SAR, induced conditional antigen-dependent activation, proliferation and differentiation of SAR-T cells. Further, SAR T cells bridged to their target cells by BiTE could form functional immunological synapses, resulting in efficient tumor cell lysis with specificity towards CD33-expressing AML cells. SAR-BiTE combination could also mediate specific cytotoxicity against patient-derived AML blasts whilst driving SAR T cell activation. *In vivo*, treatment with SAR-BiTE combination could efficiently eradicate leukemia and enhance survival in an AML xenograft model. Furthermore, we could show selective activation of SAR T cells, as well as a controllable reversibility of said activation upon depletion of the T cell engaging molecule.

Conclusions Here we apply the SAR x BiAb approach in efforts to deliver specific and conditional activation of agonistic receptor-transduced T cells, and targeted tumour cell lysis. The modularity of our platform will allow for a multi-targeting ACT approach with the potential to translate the ACT successes of B cell malignancies to AML. With a lack of truly specific AML antigens, it is invaluable that this approach possesses an intrinsic safety switch via its BiTE facet. Moreover, we are able to circumvent pan-T cell activation due to the specific targeting and activation of SAR T cells.

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L5

RIG-I ACTIVATION ENHANCES MELANOMA IMMUNOGENICITY AND IMPROVES ANTI-TUMOR T CELL RESPONSES IN COMBINATION WITH ANTI-PD-1 IMMUNE CHECKPOINT BLOCKING ANTIBODIES

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Background Clinical efficacy of immune checkpoint blocking (ICB) therapy critically relies on the killing of melanoma cells by CD8⁺ T cells, becoming activated upon recognition of tumor antigens presented by HLA class I (HLA-I) surface

molecules. Patient-derived melanoma cells can escape from cytotoxic T cell effector functions by loss of HLA-I surface expression due to the silencing of HLA-I antigen processing and presentation machinery (APM) genes.

Material and Methods Seeking for a strategy to restore HLA-I expression, we transfected melanoma cells obtained from distinct patient metastasis with synthetic short double stranded RNA (3pRNA), an activating ligand of the cytosolic innate pattern recognition receptor RIG-I. 3pRNA-transfected melanoma cells were analyzed for HLA-I surface expression by FACS analysis and gene expression of HLA-I APM components by qPCR. *In vivo* 3pRNA-transfected tumors were analyzed for HLA-I expression by immunohistochemistry staining. Furthermore, T cell activation after coinubation with 3pRNA-transfected melanoma cells was determined by IFN γ -ELISpot assay. The effect of combined 3pRNA and blocking anti-PD-1 antibody treatment on T cell activation was measured by intracellular cytokine staining and FACS analysis.

Results Activation of RIG-I by 3pRNA increased the expression of HLA-I APM components and strongly enhanced recognition of melanoma cells by autologous CD8⁺ T cells. Based on these findings, we asked whether the combination of 3pRNA and blocking anti-PD-1 antibodies could improve anti-melanoma T cell responses in an anti-PD-1 non-responder patient model. Indeed, T cell activation by 3pRNA-transfected melanoma cells was significantly increased in the presence of anti-PD-1 antibodies. In line with the enhancement of anti-tumor T cell responses, we found an association of elevated RIG-I mRNA levels with prolonged patient survival in TCGA melanoma samples.

Conclusions In summary, this study demonstrates a beneficial effect of RIG-I activation on antigen presentation and T cell recognition of melanoma cells. Improved T cell responses by combined 3pRNA and anti-PD-1 treatment suggests that combinational therapy could be a strategy to overcome T cell resistance in melanoma.

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On Demand Talks

On Demand Talks: Tumor Microenvironment

01

TUMOR LACTIC ACIDOSIS ALTERS DECISIVE T CELL ACTIVITIES

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Background Adoptive T cell therapy is a promising treatment strategy for tumor patients. However, when entering the tumor microenvironment (TME), T cells lose their effector function showing reduced degranulation and cytokine secretion. Besides T cell inhibition through checkpoint pathways (i.e. PD-1/L1, CTLA-4), suppressor cells (i.e. TAM, T_{reg}) and cytokines (i.e. IL-10, TGF, VEGF), various metabolites of the TME also counteract antitumoral activities. Among the latter, lactate and extracellular acidosis are byproducts of the cancer metabolism and commonly observed in high concentrations in

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 Lightning™ 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