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GEN1042 (DuoBody®-CD40x4-1BB) in combination with PD-1 blockade reverses T-cell exhaustion in vitro

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GEN1042 (DuoBody®-CD40x4-1BB) is a novel, investigational, agonistic, bispecific antibody that primes and (re-)activates tumor-specific immune responses through targeting and conditional activation of CD40 and 4-1BB on immune cells. Previously presented in vitro data has shown that GEN1042 exhibits conditional CD40 and 4-1BB agonist activity, leading to dendritic-cell maturation and enhanced T-cell activation and effector functions. A potential mechanism of resistance to checkpoint inhibitors or costimulatory receptor agonists may be the exhaustion of T cells in the solid tumor microenvironment. Through the use of a multi-omics approach, we evaluated whether GEN1042 is able to reverse T-cell exhaustion in vitro. Publicly available single-cell RNA sequencing datasets (scRNAseq) were harmonized across several solid-tumor indications (including treatment-naive and anti-PD-1 and/or anti-CTLA-4 pretreated samples). To describe target prevalence, these datasets were then analyzed for expression of CD40, 4-1BB, and PD-1 on various immune-cell subsets based on their transcriptomic signatures. In vitro mixed lymphocyte reaction (MLR) functional assays were performed, in which unstimulated healthy donor CD3+ T cells or CD3+ T cells exhausted through repeated stimulation with anti-CD3/CD28 beads were co-cultured with allogeneic lipopolysaccharide (LPS)-matured dendritic cells. These cultures were analyzed for cell surface protein expression and cytokine secretion. Combination treatment with GEN1042 and an anti-PD-1 antibody potentiated IFNγ, TNFα, and IL-2 production when compared with either compound alone in the unstimulated T cell/mDC MLR assay. In the T-cell exhaustion MLR assay, T cells exhibited hyporesponsiveness for both proliferation and cytokine secretion upon restimulation with anti-CD3/CD28 beads and showed increased expression of inhibitory receptors (eg, TIM-3, LAG-3). The combined functional effect of concurrent treatment with GEN1042 and an anti-PD-1 antibody induced IL-2 at similar levels to levels observed for GEN1042 alone and further potentiated IFNγ secretion compared with single-agent activity. These data suggest that GEN1042 in combination with an anti-PD-1 antibody can amplify the magnitude of the immune response through re-establishing the functional activity of dysfunctional T cells. GEN1042 is currently being studied in patients with advanced solid tumors in a phase 1/2 clinical trial (NCT04083599).

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The transcriptional landscape of tumor-infiltrated leukocytes upon OX40 agonist antibody treatment

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Antigen-presenting cells (APCs) present antigen to T lymphocytes, together with co-stimulatory signal, to elicit a specific immune response. By engagement of OX40L with OX40, APCs provide co-stimulatory signal to regulatory and activated T cells. In clinical trials, OX40 agonist antibodies (αOX40) have been used to enhance specific anti-tumoral immune response. Whereas monotherapy did not induce clinical benefit, combination with Immune checkpoint blockade (ICB) (like anti-PD1 and anti-CTLA4) resulted in reduced tumor growth, suggesting a synergistic effect between αOX40 agonist and ICB. Here, we aim to deeper our understanding of the immediate effects of αOX40 agonist therapy on tumor-infiltrating leukocytes, allowing to propose more efficient potential therapeutic combinations. Mice bearing established B16 tumors were administered twice with OX40 agonist antibody or its isotype control. 24 hours after the second injection, tumors were excised and digested, leukocytes were isolated and stained with fluorescent antibodies for immune phenotyping. Live leukocytes were sorted, single-cells libraries were processed by 10× Genomics Chromium controller, and sequenced for further informatics analysis. As expected, no change in tumor growth was observed after two OX40 agonist injections. From the scRNAseq analysis, we found that while OX40 agonist treatment mostly affected T-cells, which up-regulated the OX40 pathway, subsequent changes could already be observed across other immune subsets. CD8+ T-cells activation was accompanied by the upregulation of the transcriptional programs of cytotoxicity, co-stimulation and co-inhibition. Regulatory T-cells (Tregs) expressed a greater suppressive phenotype. An indirect effect of the treatment was observed in several subpopulations of dendritic cells and macrophages, which shifted towards a more pro-inflammatory state, antigen processing and presentations and increased chemotaxis. Finally, our data points to specific compensatory mechanisms which regulate T-cell activation such as Ctla4 and Havcr2 (Tim3). Put together, our findings provide further understanding for αOX40 treatment and rationale for new combination therapy in ongoing and future clinical trials.

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Molecular mimicry and cross-reactive CD8$^+$ T cell responses to cancer testis antigens (CTA) and homologous microbiota-derived epitopes

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The development of cancer immunotherapeutic strategies relies on the identification and validation of optimal target antigens, which should be specific to the tumor as well as able to elicit a swift and potent immune response. In the quest for such target antigens, the human microbiota is a natural source of non-self antigens, which may be expressed by host's cells in the context of the HLA class I molecules. We have recently shown the molecular mimicry between TAAs and microbiota-derived epitopes which might have a significant impact in controlling tumor growth and improving the clinical outcome in cancer. Indeed, cross-reactive $CD8^+$ T cells may be primed by microbiota and swiftly activated, with more potent anti-tumor effect.
To this aim, a library of >100 peptide-MHC combinations was used to generate DNA-barcode labelled multimers. The peptide-MHC library was generated using 9-mers epitopes derived from Cancer Testis Antigens (CTA) from the *Cancer Antigenic Peptide Database,* and *Bacteroidetes/Firmicutes*-derived peptides sharing a high level of sequence and structural homology. $CD8^+$ T cells from peripheral blood of HLA-A*02:01 healthy individuals (n=10) and patients with Hepatocellular Carcinoma, Lung cancer and metastatic Colon Cancer (n=16), were screened for reactivity.
A total of 66 unique peptide-MHC recognized by $CD8^+$ T cells across all groups were identified by such a large-scale multimer screening. Out of these, 21 epitopes from Microbiota have not previously been described as immunological targets. Reactivity against TAAs was observed in both healthy subjects and cancer patients. Furthermore, a subsequent analysis by pMHC tetramer staining validated the presence in three subjects of a $CD8^+$ T cell population able to cross-react against the CTA MAGE-A1 and paired microbiota epitopes.
Our results show that several predicted microbiota-derived antigens are recognized by T cells in healthy subjects as well as cancer patients in a context of MHC class-I presentation, confirming that extracellular microorganisms are able to elicit $CD8^+$ T cells. Reactivity against CTA was observed not only in cancer patients but also in healthy subjects, suggesting that exposure to bacterial antigens may prime the immune system against TAAs. Such a possibility is confirmed by the identification of $CD8^+$ T cell populations able to cross-react against MAGE-A1 and paired microbiota epitopes.
Therefore, the natural T cell memory elicited by microbiota may turn out to be a preventive anti-cancer immunity. Moreover, the microbiota-derived antigens may be included in preventive/therapeutic off-the-shelf cancer vaccines with a more potent anti-tumor efficacy compared to those based on TAAs.

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$CD8^+$ tumor-resident memory T cells have a pivotal role in cancer immunotherapy

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$CD8^+$ resident memory T (T_{RM}) cells expressing the CD103 integrin accumulate in human lung tumors and are associated with a favorable prognosis. We previously demonstrated that a high density of $CD103^+CD8^+$ T cells in tumors is a biomarker of response to immune checkpoint blockade (ICB) immunotherapy. However, a large proportion of tumors is only weakly infiltrated by $CD8^+$ T_{RM} cells, and the reason of this T_{RM} desert is not explained. Therefore, defining the molecular signals that give rise and maintain T_{RM} cells within the tumor microenvironment (TME) and the mechanisms that potentiate their antitumor functions are important challenges. Our data obtained in pre-clinical mouse melanoma models demonstrate that knocking-out CD103 impairs the antitumor T-cell

response. As opposed to highly differentiated effector $CD8^+$ T cells which express high levels of PD-1, Tim-3 and Nrp-1 inhibitory receptors, these $CD8^+CD103^+$ T_{RM} exhibit a non-exhausted phenotype. Moreover, immunization of tumor-bearing mice with a peptide vaccine results in a dramatic decrease in the proportion of $CD8^+CD103^+$ T_{RM}. Algorithm analyses applied to spectral cytometry data of tumor-infiltrating lymphocytes (TIL) support the conclusion that a T_{RM} subset gives rise to effector T cells upon activation with the cancer vaccine. Using in vivo models, we deciphered mechanisms inducing decrease of CD103 expression on $CD8^+$ T cells leading to T_{RM} differentiation shift. In particular, we highlight the role of TGF-β in $CD8^+CD103^+$ T_{RM} formation. These data suggest that tumor $CD8^+CD103^+$ T_{RM} are capable of mounting a potent antitumor immune response and that a fine balance between different signals is essential to induce their differentiation and thereby optimize current cancer immunotherapy strategies.

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Abstract has been withdrawn

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Bladder immune responses upon intravesical Ty21a instillations in non-muscle invasive bladder cancer patients

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Background and Objectives: Although bacillus Calmette-Guerin (BCG) therapy is the standard treatment to treat non-muscle invasive bladder cancer (NMIBC), repeated BCG treatments are associated with significant side effects and treatment failure. Moreover, BCG manufacturing shortage often occurred. Overall, this underlies the necessity for alternative or complementary new treatments. We therefore investigated another bacterial vaccine, the highly attenuated Salmonella enterica serovar Typhi strain, Ty21a, which has showed pre-clinical evidences for its safe intravesical use, as well as for inducing T-cell- and dendritic cell (DC)-mediated tumor regression in mice. In a recent a Phase I trial, we demonstrated the good safety profile of intravesical instillations of Ty21a in NMIBC patients and here, we assessed its immunogenicity.
Methods: 14 patients with low grade NMIBC were prospectively included to undergo 6 weekly instillations of Ty21a after transurethral resection of the bladder tumor. Urinary cytokine concentrations were analyzed by Luminex® and immune cell infiltration by flow cytometry along Ty21a intravesical instillations.
Results: Ty21a, as compared to BCG, increased fewer inflammatory cytokines, though it also induced a local Th1 environment. Although a significant increase in total urine infiltrating cells was observed between pre and postTy21a samples, the urinary cell infiltration was similar among pre or postTy21a samples, and perhaps even tends to decrease after the fourth instillation, suggesting that repeated Ty21a instillations do not promote the accumulation of urinary immune cells. Nevertheless, similarly

to BCG instillations, most of locally recruited cells were neutrophils. Monocytes, T cells, DCs and NK cells were also significantly increased, but only during the first instillations, suggesting that, in contrast to the BCG therapy, a lower number of Ty21a instillations might be required to achieve a maximal immune cell infiltration. Interestingly, Ty21a, but not BCG, induced the infiltration of urinary DCs, including conventional and cross-presenting DCs, which were associated to therapeutic efficacy in the mouse model.
Conclusion: Although limited by the relatively small population included and the low number of urinary cells recovered after the fourth Ty21a treatment, our study showed that Ty21a immunotherapy of NMIBC patients is able to induce immune responses with possible anti-tumor potentials.

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Intra-tumoral delivery of mRNA encoding IL-12-fused to diabodies targeting CSF1R and PD-L1 exert potent anti-tumor efficacy while restraining systemic exposure to IL-12.

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Interleukin 12 (IL-12) is a potent weapon for cancer immunotherapy. However, its systemic delivery as a recombinant protein has shown unacceptable toxicity in the early clinical trials. Ongoing clinical trials are evaluating the intra-tumoral injection of IL-12-encoding mRNA or DNA to prevent such side effects. In this study, we sought to improve this strategy by further favoring IL-12 tethering to the tumor microenvironment. Therefore, we generated *in vitro* transcribed mRNAs encoding murine single-chain IL-12 fused to diabodies targeting CSF1R and/or PD-L1, molecules expressed in the tumor microenvironment, particularly on myeloid cells. The binding capacity of chimeric constructs and the bioactivity of IL-12 were demonstrated *in vitro*. Intra-tumoral administration of naked IL-12-encoding mRNA induced significant expression of the protein of interest that peaked at 6 h and slowly declined over time up to 72 h. Interestingly, even doses as low as 0.5 µg of the IL-12-encoding mRNA achieved potent anti-tumor effects in subcutaneously injected tumors derived from B16-OVA and MC38 cell lines. Treatment delivery was associated with measurable increases of IL-12p70 and IFN-γ levels systemically. Fusion of IL-12 to the diabodies targeting PD-L1 and/or CSF1R exerted comparable efficacy against bilateral tumor models, when compared to untethered IL-12. However, tethering IL-12 to the tumor infiltrating myeloid cells resulted in nearly undetectable IL-12 and IFN-γ levels systemically. Moreover, the anti-tumor efficacy was associated with favorable modulation of the tumor immune microenvironment. In conclusion, tethering IL-12 to myeloid cell populations in the mRNA-transfected tumors attains similar anti-tumor efficacy whilst reducing potentially dangerous systemic bioavailability of IL-12.

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A STING signaling relay from tumor cells to macrophage cells contributed to combinational chemotherapy efficacy on pancreatic ductal adenocarcinoma

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The efficacy of traditional chemotherapy on pancreatic ductal adenocarcinoma (PDAC) is limited. Cisplatin in combination with gemcitabine and albumin paclitaxel (AGP) is a new combinational chemotherapy regimen that is undergoing clinical trial with promising preliminary result. Similarly, about 67% of PDAC patients who received AGP chemotherapy in our center had an overall survival time over 12 months.To understand the mechanism underlying such combinational therapy, we tested such AGP protocol on subcutaneous and orthotopic tumor models using two mouse pancreatic cancer cell lines (PDAC and KPC1199), and observed significantly improved efficacy over gemcitabine only treatment. Mass cytometry analysis of immune cell populations in tumor tissues found increased memory CD8+T cells and decreased PD1+CD8+T, Tim3+CD8+T and CD39+CD8+T cells and Treg cells. Notably, the infiltration of M2 type tumor-associated macrophages (TAMs) was also significantly reduced. Consistent with previous reports, cisplatin induced DNA damage and up-regulated the expression of type I interferon and MHC-I molecules in tumor cells by activating cGAS/STING signal pathway. Interestingly, co-culture of bone marrow macrophages with cisplatin-treated tumor cells induced phagocytosis of tumor fragments and dsDNA from tumor cells, followed by cGAS/STING signaling activation in macrophages and a skewed polarization towards M1 type. Importantly, knockout of STING in tumor cells significantly impaired AGP-induced efficacy, confirming a significant contribution of tumor-intrinsic STING signaling to such treatment. Furthermore, the efficacy of AGP regimen was also significantly reduced on PDAC tumors implanted on Stingf/f Lyzs-CRE mice, which lack of STING expression in myeloid cells. Together, our results indicates that cisplatin remodeled the immune microenvironment of PDAC by activating a relaying cGAS-STING signaling in tumor cells and subsequently macrophages to improve chemotherapy efficacy, and provided a mechanistic insight into the AGP regimen-associated molecular changes and potential biomarkers for stratifying patients for such regimen.

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Intravaginal Ty21a immunotherapy induces NK-mediated reduction of tumor growth and increases mice survival when combined with E7-vaccination or cisplatin/paclitaxel chemotherapy in the HPV16 TC-1 orthotopic cervical cancer model

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Cervical cancer is the 4th leading cause of cancer deaths in women worldwide, despite great advances in preventing this human papillomavirus (HPV)-associated cancer by prophylactic vaccination.

In non-muscle-invasive bladder cancer, standard intravesical (ives) immunotherapy with Bacillus Calmette-Guérin (BCG) successfully decreased tumor recurrence/progression, while we recently reported that another commercial vaccine, Ty21a/Vivotif, may be a more effective and safer alternative.
In the context of cervical cancer, here we investigated the intravaginal (ivag) use of these two bacterial immunotherapies. BCG, but significantly more Ty21a, increased infiltration of immune cells in the cervix-vagina (CV) of non-tumor-bearing mice including neutrophils, monocytes, dendritic cells, T cells and NK cells, similarly to data obtained previously in the bladder.
In the HPV16 TC-1 orthotopic model, repeated ivag treatments with BCG or Ty21a significantly prolonged mice survival, but long-term mice survival was not achieved. Interestingly, two ivag doses (1 and 8 days after genital TC-1 tumor challenge) of Ty21a, but not BCG, significantly decreased tumor growth until day 15. Antibody-mediated depletion of T or NK cells, show that this initial therapeutic effect of Ty21a was mediated by NK cells and was independent of CD4 or CD8 T cells, in contrast to the results obtained after ives Ty21a on the bladder tumors. Kinetic of NK cell infiltration in the genital tumors upon Ty21a suggests that activated/mature NK cells are significantly increased 24h after each instillation, but only transiently.
In contrast to bladder tumors, single therapy with Ty21a was not sufficient to increase survival of genital tumor-bearing mice. Nevertheless, we took advantage of the reduction of initial tumor growth for investigating combinatory treatments with HPV E7-vaccination or with chemotherapy. A dual therapy with ivag Ty21a (day 2 and 9) followed by E7 vaccination (day 13) resulted in improved mice survival (25 %) as compared to E7-vaccination alone. More interestingly, a tri-therapy where ivag Ty21a was also applied after E7-vaccination to increase locally E7-specific CD8 T cells, further improved mice survival (70%). Thus, the use of a single agent (ivag Ty21a) in combination with E7-vaccination is a promising treatment that may deserve investigation in patients with HSIL and cervical cancer. However, in the absence of commercially available therapeutic E7-vaccines, our data also show that combination of the ivag Ty21a (day 2 and 9) followed by the standard cisplatin/paclitaxel chemotherapy (day 10/11) , resulted in 40% mice survival as compare to chemotherapy alone (10 %), a treatment that may be more readily available to the patients.

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Metabolic underpinnings of macrophage phenotypes to modulate anti-cancer immune response

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Macrophages are critical for maintenance of tissue homeostasis, regulation of immune defense and have also been described to regulate tumor growth. One important feature of macrophages is their ability to phenotypically and functionally adapt to alterations and demands within their microenvironment, which is referred to as macrophage plasticity. Thereby, anti-inflammatory

macrophages are described with pro-tumor activities, whereas inflammatory macrophages suppress cancer cell growth.
It has been proposed that phenotypic and functional plasticity of macrophages is tightly linked to their metabolic plasticity.
Identification of metabolic switches that are associated with distinct metabolic adaptations that regulate important macrophage phenotypes and functions is an important prerequisite for the identification and development of macrophage-directed therapies, including anti-cancer immunotherapies.
Therefore, we aimed to link inflammatory macrophage phenotypes with metabolic adaptations that modulate redox homeostasis. We discovered that LPS activated inflammatory macrophages significantly suppressed reactive oxygen species (ROS) production as the principal mechanism to induce a state of total resistance to redox stress associated cell death (ferroptosis). ROS was suppressed secondary to Warburg metabolism and not as previously thought by nitric oxide (NO·), ROS scavengers downstream of tryptophan or increased glutathione (GSH). Interestingly, LPS|IFNγ polarized macrophages were sensitive to GSH depletion, yet this cell death was not due to ferroptosis but rather due to TNF-α in an autocrine loop.
It was previously shown that inflammatory macrophage phenotypes, specifically induced with IFNγ or LPS|IFNγ, exhibited anti-tumor activities that could inhibit tumor growth through the release of inflammatory cytokines or the secretion of NO·, suggesting a potential mechanism for therapeutic intervention. Since cancer cells are under oxidative stress, investigating the behavior of inflammatory macrophages in redox stress is critical to better understand the relationship between inflammatory macrophages and cancer cells for the development of novel anti-cancer immunotherapies.

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Achieving dendritic cell subset-specific targeting *in vivo* by site-directed conjugation of targeting antibodies to nanocarriers

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The major challenge of nanocarrier-based anti-cancer vaccination approaches is their targeted delivery of antigens and immunostimulatory agents to cells of interest, such as specific subtypes of dendritic cells (DCs), in order to induce robust antigen-specific anti-tumor responses. An undirected cell and body distribution of nanocarriers can lead to unwanted delivery to other immune cell types like macrophages reducing the vaccine efficacy. An often-used approach to overcome this issue is the surface functionalization of nanocarriers with targeting moieties, such as antibodies, mediating cell type-specific interaction. Numerous studies could successfully prove the targeting efficiency of antibody-conjugated carrier systems *in vitro*, however, most of them failed *in vivo* when targeting DCs that is partly due to cells of the reticuloendothelial system unspecifically clearing nanocarriers from the blood stream via Fc receptor ligation.
Therefore, this study shows a surface functionalization strategy to site-specifically attach antibodies in an orientated direction onto the nanocarrier surface. Different DC-targeting antibodies, such as

anti-CD11c, anti-CLEC9A, anti-DEC205 and anti-XCR1, were conjugated to the nanocarrier surface at their Fc domains. Anti-mouse CD11c antibody-conjugated nanocarriers specifically accumulated in the targeted organ (spleen) over time. Additionally, antibodies against CD11c and CLEC9A proved to specifically direct nanocarriers to the targeted DC subtype, conventional DCs type 1.
In conclusion, site-directed antibody conjugation to nanocarriers is essential in order to avoid unspecific uptake by non-target cells while achieving antibody-specific targeting of DC subsets. This novel conjugation technique paves the way for the development of antibody-functionalized nanocarriers for DC-based vaccination approaches in the field of cancer immunotherapy.

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Restoring immune fitness with oral *Salmonella* Typhi ZH9 to increase efficacy of immunotherapies

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Tumor-infiltrating myeloid cells supress anti-tumor immunity within the tumor microenvironment (TME) through direct and indirect inhibitory mechanisms. However, inherent myeloid plasticity offers the opportunity for systemic treatments to reprogramme these cells. One class of agents with potential to reprogramme myeloid cells are bacteria and their products, which can induce long-lasting systemic reprogramming of myeloid cells, a process termed trained immunity. Prokarium is developing a live attenuated *Salmonella enterica* serovar Typhi strain (ZH9) to be the next cancer immunotherapy platform and sought to establish whether *Salmonella* can restore immune fitness through myeloid cell training and long-term reprogramming to support T cell targeting immunotherapies.
The effects of *Salmonella* on unpolarised myeloid cells was investigated after oral *Salmonella* administration to healthy mice by phenotyping splenic myeloid cells using flow cytometry and assessing their cytokine production after *ex vivo* re-stimulation. Impact of oral *Salmonella* treatment on tissue immunosurveillance was measured in syngeneic subcutaneous colon (MC38) and experimental metastasis (4T1) models. Potential synergy with established therapies was assessed utilising an *in vitro* macrophage-T cell co-culture system with cancer patient samples and by demonstrating combination treatment *in vivo* efficacy in syngeneic murine models.
Oral *Salmonella* treatment of mice induced long-term phenotypic and functional myeloid changes, including upregulation of co-stimulatory and MHC molecules on systemic dendritic cells, monocytes and macrophages, and increased responsiveness of CD11c+ splenocytes to secondary stimuli, suggesting oral *Salmonella* reprogrammes systemic myeloid cells. Oral treatment with *Salmonella* as a monotherapy was able to change tissue immunosurveillance, resulting in delay in tumour growth in subcutaneous and experimental metastasis models, indicating that the *Salmonella*-induced trained myeloid phenotype may translate to changes in the myeloid compartment of the TME. Finally, *Salmonella* complemented other cancer therapies both *in vitro* and *in vivo*. *In vitro, Salmonella*-trained human monocytes from both heathy donors and cancer patients overcame the suppressive M2 phenotype to synergize with checkpoint inhibitors in driving T-cell proliferation. *In vivo*, oral *Salmonella* treatment synergized with anti-PD-L1 in supressing growth of subcutaneously implanted, checkpoint refractory MC38 tumors and with chemotherapy in reducing primary tumor growth and metastasis.
Oral *Salmonella* treatment can reprogram myeloid cells to induce trained immunity for re-

sensitization of solid tumors to different therapies. This opens up a new opportunity for combination therapy, leveraging trained immunity to increase immune fitness in cancer patients and thus broadening the reach of current cancer therapies.

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A novel chimeric oncolytic virus mediates a multifaceted cellular immune response in a syngeneic B16 melanoma model

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Oncolytic viruses (OVs) have recently emerged as an exciting modality in cancer immunotherapy. Our lab has engineered a novel OV vector (rVSV-NDV), which has been shown to stimulate a systemic anti-tumor immune response via immunogenic cell death. We have demonstrated that rVSV-NDV treatment leads to delayed tumor growth and prolonged survival in multiple immune-competent tumor models. A higher percentage of tumor antigen-specific $CD8^+$ T-cells circulating in the peripheral blood and evidence of abscopal effects in distant tumors were evident in a B16-OVA model after rVSV-NDV treatment; however, the bigger picture of the immune-mediated mechanism of action remained unclear. Therefore, we aimed to investigate the immune cell responses to rVSV-NDV therapy in a more detailed approach. We utilized a bilateral B16-OVA tumor model and injected rVSV-NDV or PBS directly into one of the two tumors. At defined time points, lineage and activation markers of natural killer (NK) cells, T cells, and dendritic cells (DCs) were characterized by flow cytometry from primary material extracted from the injected and distant tumors, the respective draining lymph nodes, spleen and blood. Despite the local administration of virus, global immune responses were observed. As an early immune response, on the second day after treatment, we observed a significant increase in natural killer T (NKT) cells, NK cells, T-cells, DCs and OVA-specific $CD8^+$ T cells in the draining lymph nodes in response to virus therapy, which went hand in hand with upregulation of their respective activation markers. Furthermore, on day 10 after virus treatment, an increase in $CD8^+$ T-cells in the distant tumor and a concomitant upregulation of PD-1 and CD69 activation markers was found in both tumors, as well as in the spleen and blood in a timepoint-dependent manner. Additionally, an increase in percentage of mature NK cells and activation via NKG2D and CD25 was observed in the spleen. In summary, these results demonstrate a dynamic and systemic immune-stimulatory effect in response to oncolytic rVSV-NDV therapy, which likely contributes to a multifaceted mechanism of action. These data support the further development of this novel treatment approach as a potentially valuable immunotherapy modality for solid cancers.

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Targeting the immunosuppressive tumor microenvironment with arginase-1 specific T cells

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The formation of an immunosuppressive tumor microenvironment (TME) can limit the efficacy of cancer immunotherapy by impeding anti-tumoral T-cell reactivity. Amongst the numerous mechanisms affecting anti-tumoral T-cell reactivity in the TME, expression of the metabolic enzyme arginase-1 can both impair the activation and inhibit the proliferation of T cells. Arginase-1 (Arg1) expression is described in immune regulatory cells, such as myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs). Further, several cancer cell types are also associated with Arg1 expression. Thus, T cells specifically targeting Arg1-expressing cells hold a great potential to modulate the immunosuppressive TME.
As we have previously demonstrated the presence of Arg1-specific T-cell responses in healthy individuals and cancer patients, we assessed Arg1-specific immunogenicity in Arg1 peptide vaccinated myeloproliferative neoplasm (MPN) patients. We stimulated MPN patient PBMCs with HLA class I and II Arg1 derived epitopes *in vitro* and subsequently evaluated Arg1-specific T-cell reactivity by intracellular staining for IFNγ and TNFα. We observed both CD4+ and CD8+ Arg1-specific T-cell responses towards the HLA class I and II Arg1 derived epitopes in the Arg1 peptide vaccinated MPN patients. Further, we have isolated and expanded Arg1- specific T cells and generated CD8+ Arg1-specific T cell clones, which we have used in IFNγ ELISPOT and functional target recognition assays to demonstrate Arg1 dependent recognition and cytolysis of Arg1-expressing cells, such as tumor polarized autologous monocytes and the acute monocytic leukemia cell line, MonoMac-1. Our data demonstrate the presence of Arg1-specific T-cell responses in Arg1 peptide vaccinated MPN patients and the capability of CD8+ Arg1-specific T cells to recognize and lyse Arg1-expressing cells. These results further support the potential of arginase-1 specific T cells as potent modulators of the immunosuppressive TME.

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Use of bispecific antibodies to improve transendothelial migration of T cells towards tumor cells

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The migration of effector lymphocytes from the blood stream into the tumor microenvironment is a crucial step for the anti-tumor immune response. Infiltration of $CD8^+$ T cells in the tumor bed has been correlated with a better prognosis in a variety of malignancies and poorly infiltrated tumors often show an abnormal and dysfunctional vasculature with reduced expression of adhesion molecules. As a new strategy to redirect T cells to tumor endothelial cells (EC) and increase transendothelial migration of effector T cells into the tumor bed, tetravalent bispecific monoclonal antibodies (BiMAbs) in the $(scFv1\text{-}Fc\text{-}scFv2)_2$ format were used. These BiMAbs bind to EC growth factor receptors VEGFR2 or TIE2 with N-terminal single-chain variable fragment (scFv1) antibodies and to the stimulatory/co-stimulatory T cell molecules CD3ε or CD28 with the C-terminal scFv2. Local CD3ε-mediated T cell activation is expected to result in the release of cytokines that in turn induce the expression of adhesion molecules in EC. Antibody-mediated blocking of VEGF binding to VEGFR2 could concomitantly exert anti-angiogenic effects. To assess the activation capacity of bispecific

antibodies, $CD3^+$ T cells were co-cultured with HUVEC in the presence of BiMAbs for 24 h. The treatment with αVEGFR2–αCD3 BiMAb resulted in a strong T cell activation as well as up-regulation of adhesion molecules on EC, as detected by cell surface staining and cytokine ELISA. This effect was slightly enhanced by the addition of an αTIE2–αCD28 BiMAb. The migration capacity of T cells through an EC monolayer in the presence or absence of BiMAbs was evaluated using a transwell assay. The migration of T cells was significantly increased in the presence of αVEGFR2–αCD3 BiMAb. Although additional treatment with the co-stimulatory αTIE2–αCD28 BiMAb augmented T cell activation, the migration rate of T cells was not improved. To study the killing capacity of transmigrated T cells against tumor cells, transmigrated T cells were transferred onto MCF-7 breast cancer cells spheroids in the presence or absence of BiMAbs mediating tumor cell targeting for 48 h. Tumor cell killing was quantified using an LDH release assay. We observed that T cells that had been pre-activated with αVEGFR2–αCD3 and subsequently traversed an EC monolayer, still required the subsequent addition of tumor-reactive αEpCAM1–αCD3 BiMAb in order to become cytotoxic. Killing was more prominent if migrated cells were pre-treated with a combination of stimulatory and co-stimulatory BiMAbs, αVEGFR2–αCD3 and αTIE2–αCD28. In conclusion, T cells activated by αVEGFR2–αCD3 can indirectly activate EC *in situ* resulting in a better T cell migration. This treatment can be combined with other BiMAbs that target T cells towards tumor cells and induce tumor cell killing.

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An Fc-optimized fusion protein targeting NKG2DL for induction of NK cell reactivity against ovarian cancer

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Ovarian cancer is the most lethal gynecologic malignancy. Surgery and chemotherapy still remain the standard treatments, but often fail as patients experience disease relapse and become resistant to chemotherapy. In recent decades, the introduction of monoclonal antibodies (mAbs) has revolutionized the treatment of various types of cancer, but patients with ovarian cancer have yet not benefitted. NK cells largely contribute to the efficacy of mAb treatment due to their ability to mediate antibody-dependent cellular cytotoxicity (ADCC), a feature widely considered critical for therapeutic success. Many efforts presently aim to improve this important antibody function by modifying the Fc-part to improve NK ADCC.
Ligands of the activating immune receptor NKG2D (NKG2DL) are widely expressed on malignant cells, but generally absent on healthy tissue. We aimed to take advantage of the tumor-restricted expression of NKG2DL by using them as targets for Fc-optimized NKG2D-Ig fusion proteins (NKG2D-ADCC) to target ovarian cancer cells for NK cell ADCC. NKG2D-ADCC contains the amino acid modification S239D/I332E in its Fc-part to increase affinity for the Fc-receptor CD16. Flow cytometric analyses documented varying expression patterns of NKG2DL in all tested ovarian cancer cell lines, with at least two NKG2DL expressed in each specimen. Functional analysis revealed that NKG2D-ADCC induced profound NK cell activation, degranulation and IFN-γ release. No effects were

observed upon application of a control fusion protein with unrelated specificity, confirming strictly target antigen-restricted activity of our construct. NKG2D-ADCC further potently induced NK ADCC against ovarian cancer cells in both short- and long-term cytotoxicity assays. In summary, our immunotherapeutic compound NKG2D-ADCC potently stimulates NK reactivity against ovarian cancer cells and constitutes a promising compound for immunotherapy.

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Optimized dithranol-imiquimod-based transcutaneous immunization enables tumor control

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Transcutaneous immunization (TCI) is a non-invasive vaccination method that, rather than needle-based systems, promotes strong cellular immune responses, crucial for the control of tumor growth. Previously, we presented the combined application of the TLR7 agonist imiquimod (IMQ) together with dithranol as the new TCI platform DIVA for tumor vaccination therapy. In this study, we further optimized DIVA in terms of drug dose, application pattern and number and established a new IMQ cream formulation.
Methods: For DIVA, C57BL/6 mice were treated on the ear skin with dithranol vaseline and 5% IMQ containing ointments together with ovalbumin peptides. For DIVA^2, the procedure was performed twice (day 0/1 + 7/8 or day 0/1 + 14/15). The T cell response was determined by flow cytometry and IFN-ϒ ELISpot assay, local skin inflammation was characterized by ear swelling. To evaluate tumor protection, mice received 5x10^4 MC38mOVA tumor cells subcutaneously after prophylactic DIVA or DIVA^2.
Results: Applying the adjuvants on distinct skin sites, a decrease of antigen specific CD8$^+$ T cells with a significantly reduced effector function was detectable, indicating that the local concurrence within the same tissue is required for optimal vaccination. Likewise, changing the order of dithranol and IMQ resulted in an increased skin inflammatory reaction and lower antigen specific CD8$^+$ T cells indicating that dithranol is essential for the initiation of the TCI process. For single DIVA treatment, dithranol in a concentration of 1/16%, followed by 2,5 mg IMQ per ear was sufficient to induce a potent immune response. There was no significant difference when the peptide doses were reduced up to ten-fold. In this study, we emulgated the freeze-dried nanoemulsion “IMI-Sol” to the new formulation “IMI cream” facilitating storage and application and showed that both induced comparable immune responses. DIVA^2 applied one or two weeks after the first immunization resulted in a massive increase in antigen- specific T cells and up to a ten-fold increased memory response. In a prophylactic setting, DIVA^2, but not single DIVA enabled complete control of tumor growth, resulting in an optimized survival rate.
Taken together, the described optimized transcutaneous vaccination method DIVA^2 using dithranol and IMQ as adjuvants leads to the generation of a strong cellular immune response enabling the effective control of tumor growth and has the potential for clinical development as a novel non-invasive vaccination method for peptide-based cancer vaccines in humans.

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HexaBody-CD27 enhances T-cell activation, proliferation, cytokine secretion and cytotoxic activity independently of Fc gamma receptor-mediated crosslinking

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Clustering of the costimulatory receptor CD27 on T cells induces T-cell activation, proliferation, and memory differentiation. Therefore, CD27 represents a target for cancer immunotherapy. Multiple monoclonal antibodies (mAbs) targeting CD27 that are being explored in the clinic require Fc gamma receptor (FcγR)-mediated crosslinking to induce CD27 agonism. HexaBody-CD27 (GEN1053/BNT313) is a novel CD27 mAb with an IgG1 Fc domain engineered to induce CD27 agonist activity independently of FcγR-bearing cells. The Fc domain was further modified to silence Fc-mediated antibody effector functions, with the aim to prevent T-cell depletion. Here we present preclinical characterization of the mechanism of action of HexaBody-CD27.

Target binding characteristics and functional activity of HexaBody-CD27 were analyzed using flow cytometry, cell-based reporter assays and primary human lymphocyte assays. The capacity of HexaBody-CD27 to induce tumor-infiltrating lymphocyte (TIL) proliferation was assessed using non-small cell lung cancer (NSCLC) tissue resected from patients. HexaBody-CD27 activity was investigated in human CD27 knock-in mice that were immunized with ovalbumin and treated with HexaBody-CD27 by characterizing peripheral blood and splenic T cells using flow cytometry.

HexaBody-CD27 exhibited dose-dependent CD27 agonist activity independent of crosslinking via FcγR-expressing cells. In contrast, agonist activity of benchmark anti-CD27 antibody analogs was dependent on FcγR-mediated crosslinking. HexaBody-CD27 did not functionally engage with FcγRs, and membrane-bound HexaBody-CD27 was unable to bind C1q, confirming functional silencing of the IgG Fc domain. *In vitro*, HexaBody-CD27 enhanced activation, proliferation, and proinflammatory cytokine secretion of TCR-stimulated human $CD4^+$ and $CD8^+$ T cells as well as $CD8^+$ T-cell mediated cytotoxic activity towards cognate antigen-expressing tumor cells. In TIL assays with human NSCLC tumor tissue, HexaBody-CD27 promoted expansion of $CD8^+$ T cells. In human CD27 knock-in mice, HexaBody-CD27 enhanced expansion and IFN-γ secretion of antigen-specific $CD8^+$ T cells. No decrease in percentages of circulating or splenic T cells was detected after treatment with HexaBody-CD27, whereas treatment with a benchmark anti-CD27 mAb analog resulted in a marked reduction of T cells.

HexaBody-CD27 has a functionally inert Fc domain and exhibits FcγR-crosslinking-independent CD27 agonist activity, a unique mechanism of action that distinguishes HexaBody-CD27 from benchmark mAbs targeting CD27. In preclinical studies *in vitro* and *in vivo*, HexaBody-CD27 increased T-cell activation, proliferation, cytokine secretion, and cytotoxic activity. A first-in-human clinical trial has been initiated to evaluate HexaBody-CD27 in patients with advanced solid tumors (NCT05435339).

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Immunological characterization of expanded tumor-infiltrating lymphocytes in renal cell carcinoma patients

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The utilization of expanded tumor-infiltrating lymphocytes (TILs) in adoptive TIL therapies has resulted in notable success. However, a comprehensive understanding of the effects of the TIL expansion on the immune phenotype, function, and T-cell receptor (TCR) repertoire of the infused products relative to the tumor microenvironment (TME) remains limited. In this study, we analyzed the tumor samples (n=58) from treatment-naïve renal cell carcinoma (RCC) patients, minimally cultured “pre-rapidly expanded” TILs (pre-REP TILs, n=15) and “rapidly expanded” TILs (REP TILs, n=25) according to a clinical-grade TIL production protocol, with single-cell RNA and TCRαβ-sequencing (scRNA+TCRαβ-seq), bulk TCRβ-sequencing (TCRβ-seq), and flow cytometry. We observed that the REP TILs encompassed a greater abundance of CD4+ than CD8+ T-cells, with increased LAG-3 and low PD-1 expressions in both T-cell compartments compared to the pre-REP TIL and tumor T-cells. The REP protocol preferentially expanded small clones of the CD4+ phenotype (*CD4*, *IL7R*, *KLRB1*) in the TME, indicating that the largest exhausted T-cell clones in the tumor do not expand during the rapid expansion protocol. Furthermore, by generating a catalog of RCC-associated T-cell receptor (TCR) motifs from >1000 scRNA+TCRαβ-seq and TCRβ-seq RCC, healthy and other cancer sample cohorts, we quantified the RCC-associated TCRs from the expansion protocol. The quantity of the RCC-associated TCRs were elevated in the tumors and pre-REP TILs but decreased in the REP TILs, whereas the amount of anti-viral TCRs remained low throughout the expansion. In conclusion, our findings provide an in-depth understanding of the origin, phenotype, and TCR specificity of RCC TIL products for a more rationalized production of TILs in the future.

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IL-15 immunocytokines directed to CD135 and CD133 with target cell-restricted IL-15 activity for treatment of acute myeloid leukemia

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Recently we introduced Fc-optimized antibodies targeting CD135/FLT3 and CD133 to induce antitumor immunity of NK cells against acute myeloid leukemia (AML) cells with promising results in preclinical studies; evaluation of the first compound in a clinical trial (FLYSYN, NCT02789254) was recently completed. Besides by reinforcing the capability of antibodies to induce antibody-dependent cellular cytotoxicity, NK cell immunity can be further increased using cytokines like IL-15, and multiple efforts presently aim to exploit the latter for cancer treatment. However, application of clinically effective doses of IL-15 is prevented by substantial side effects due to unspecific immune activation. We here report on modified immunocytokines (MIC) consisting of Fc-optimized CD133 and CD135 antibodies fused to mutated IL-15 with abolished binding to IL-15 receptor alpha (MIC133/MIC135). This abrogated binding allows to substitute the trans-presentation of IL-15, which physiologically is required to stimulate the IL-15 receptor beta/gamma on NK cells, by binding of the antibody part to CD133 and CD135 on leukemic cells. Comparative analysis revealed that CD135 is expressed on primary AML cells to a significantly higher extent and is less susceptible to antigen shift than CD133. Functional analyses using primary AML cells as targets revealed that MIC135 induced target-restricted NK cell anti-leukemia reactivity in a profoundly greater extent than the Fc-optimized FLYSYN antibody. Notably, in stark contrast to FLYSYN, MIC135 induced prominent NK cell proliferation, and target cell killing upon treatment with MIC135 was likewise clearly superior. Analyses regarding off-target toxicity confirmed the target-antigen restricted efficacy of MIC135 compared to anti-CD135 immunocytokines with wildtype IL-15 (IC135). Furthermore, MIC135 did not induce unwanted effects against healthy FLT3 expressing cells. Taken together, MIC135 induces NK cell reactivity against leukemia cells in a highly target cell-restricted manner and displays higher efficacy than Fc-optimized antibodies, thus constituting a promising treatment option for AML.

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Interleukin-1 receptor-associated kinase 3 acts as an immune checkpoint in myeloid cells to limit cancer immunotherapy

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Inflammatory mediators released by cancer cells promote the induction of immune suppression and tolerance in myeloid cells. Interleukin-1 receptor associated kinase 3 (IRAK3) is a pseudokinase that inhibits IL-1/TLR signaling but its role in patients treated with immune checkpoint blockade (ICB) therapy remains unclear. Using RNAseq data from the IMvigor210 trial, we found that tumors with high *IRAK3* expressions showed enriched anti-inflammatory pathways and worse clinical response to ICB therapy. Upon IRAK3 protein deletion with CRISPR/Cas9, primary human monocytes displayed altered global protein expression and phosphorylation in quantitative proteomics and released more pro-inflammatory cytokines in response to stimulation. Bone-marrow derived macrophages from an

IRAK3 CRISPR knockout (KO) mouse model demonstrated a pro-inflammatory phenotype and enhanced sensitivity to TLR agonists, compared to wild type cells. *IRAK3* deficiency delayed the growth of carcinogen-induced and oncogene-driven murine cancer cells and induced enhanced activation in myeloid cells and T cells. Upon ICB treatment, *IRAK3 KO* mice showed enrichment of TCF1+PD-1+ stem-like memory CD8+ T cells and resulted in superior growth inhibition of immunologically cold tumors in vivo. Altogether, our study demonstrated a novel cancer-driven immune tolerance program controlled by IRAK3 in humans and mice and proposed its suitability as an immunotherapy target.

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Patient-derived head and neck tumor slice cultures – a versatile tool to study oncolytic virus action

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Translating preclinical data from cell-based in vitro systems and syngeneic mouse tumor models to the clinically heterogeneous ecosystem of human tumors is a challenging task. Head and neck squamous cell carcinomas (HNSCC) display a complex architecture which makes the prediction of a treatment outcome quite difficult. To bridge this gap, we have established and employed a patient-derived HNSCC slice culturing system to assess immunomodulatory effects as well as permissivity and oncolytic virus (OV) action. In a first set of experiments, we demonstrated that the heterogenous morphology of a human tumor could be retained in these slice cultures including the preservation of different cell lineages like tumor cells, immune cells and cancer-associated fibroblasts. Upon stimulation with α-CD3/α-CD28 antibodies, cytotoxic T-cells within the slice culture were still functional and could be activated following cultivation. In a parallel approach, we uncovered that a high proportion of the patient-derived HNSCC slice cultures were susceptible to the OV VSV-GP. More specifically, VSV-GP infects a broad spectrum of tumor associated lineages including epithelial and stromal cells in permissive tumors. In sum, this human tumor ex vivo platform might complement pre-clinical studies to eventually propel cancer immune-related drug discovery and ease the translation to the clinic.

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Phosphatidylserine exposure on exhausted CD8 T cells operates as a non-classical inhibitory molecule

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In cancer and chronic infection, CD8 T cell exhaustion restricts the quality of an immune response, partly through expression of inhibitory receptors such as PD1, TIM3, and LAG3. However, the full landscape of molecules that impair CD8 T cell responses is not known and more so, inhibitory receptor focus has been strictly limited to surface proteins. Whether surface metabolites perform similar inhibitory actions in the context of exhaustion has not been explored. Here we demonstrate that phosphatidylserine (PS), a lipid primarily associated with cell death, functions as a metabolic inhibitory checkpoint molecule during live CD8 T cell exhaustion. Although live cells predominantly localize PS to the inner plasma membrane, we find that antigen specific PD1+ CD8 T cells externalize significant levels of PS to the outer plasma membrane *in vivo* during chronic Lymphocytic Choriomeningitis Virus (LCMV) infection. PS exposing CD8 T cells were not dead, stained negative for cleaved caspase3 and expanded upon serial transfer into newly infected mice. Antigen induced initial PS externalization but was not necessary for PS exposure overtime. Additionally, transcriptomics and metabolomics identified a unique PS metabolic signature within exhausted cells. In the context of cell death, externalized PS has potent immunosuppressive properties as a signaling ligand, however the roles of live cell exposed PS is incompletely understood. To determine PS contributions to an inhibitory axis on exhausted CD8 T cells, we treated LCMV chronically infected mice with PS blocking antibodies (mch1N11). Remarkably, aPS monotherapy promoted a threefold expansion of antigen specific CD8 T cells. To investigate mechanisms of PS inhibited CD8 T cells, we ran RNAseq on PD1+ Tcf1+ stem-like, PD1+ Tim3+ transitory, and PD1+ Tim3+ CD101+ terminally differentiated cells. aPS treatment drove transcriptional changes in activation associated genes and the interferon response across all populations. Pathway and functional analyses underscored that PS blockade most significantly altered the stem-like population by downregulating inhibitory signaling and increasing their proliferative state. Furthermore, we found that aPS and aPDL1 combination boosted the immune response, increased effector CD8 T cells and improved viral control in a synergistic manner, emphasizing the inhibitory potential of extracellular PS. Translationally, we probed PS dynamics in human clear cell renal cell carcinoma and human non-small cell lung cancer and found conserved PS externalization and metabolic phenotypes in live PD1+ CD8 T cells within cancer. Overall, these data demonstrate that PS externalization is not apoptosis-exclusive, but also occurs on live CD8 T cells *in vivo*. We further examine PS as a potential 'non-classical' inhibitory molecule with targetable actions and detail an unexplored aspect of CD8 T cell biology.

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CLN-617 is a first-in-class fusion protein that retains IL-2 and IL-12 in injected tumors and potently triggers systemic anti-tumor immunity

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Despite clinical evidence of anti-tumor activity, cytokine therapies have been hampered by a narrow therapeutic window and limited efficacy. Two cytokines of high interest for cancer therapy are IL-2 and IL-12, which synergize to proliferate and activate T cells and natural killer cells. However, the only approved IL-2 product, Proleukin, is rarely used in the clinic due to systemic toxicities, and no IL-12 product has been approved to date mostly due to severe dose-limiting toxicities. Here, we describe CLN-617, a first-in-class fusion protein designed for intra-tumoral (IT) injection and retention to co-deliver human IL-2 and human IL-12 in a safe and effective manner. CLN-617 is a single polypeptide chain comprised of IL-2, leukocyte-associated immunoglobulin-like receptor 2 (LAIR2), human serum albumin (HSA), and IL-12. LAIR2 and HSA functionally retain CLN-617 in the injected tumor by binding collagen and increasing molecular weight, respectively. To demonstrate retention, a murine surrogate of CLN-617 (mCLN-617) was delivered IT in MC38 tumor-bearing mice, and drug concentrations in the tumor were >10-fold greater than in serum across multiple studies. Following mCLN-617 treatment, durable and complete responses were observed with no significant body weight loss in established B16F10, CT26, and MC38 tumor models, all of which are relatively refractory to checkpoint inhibition. The majority of complete responders were protected from re-challenge, demonstrating the generation of long-term immune memory. mCLN-617 triggered a robust systemic anti-tumor immune response based on three lines of evidence. First, in mice implanted with two MC38 tumors, only one of which was treated IT, 70% of injected tumors and 40% of non-injected tumors were eradicated. When combined with IV-administered anti-PD1 antibody, >90% of both tumors showed a complete response, while anti-PD1 alone exhibited no anti-tumor activity. Second, in mice implanted with an MC38 tumor in the flank and with MC38-luciferase cells delivered to the liver via intra-splenic injection, treatment of the flank tumor mediated ~90% growth inhibition of the injected tumor either with mCLN-617 monotherapy or in combination with anti-PD1. Growth of non-injected metastatic tumors was also inhibited by mCLN-617 monotherapy, and metastatic tumor burden significantly regressed in combination with anti-PD1, while anti-PD1 alone exhibited no efficacy. Third, in mice bearing two MC38 flank tumors, the CD8:Treg ratio was enhanced in both the injected and non-injected tumors, and tumor-specific T cells expanded in peripheral blood. In conclusion, while IT-administered mCLN-617 was retained in the injected tumor, it mediated protective and systemic immunosurveillance for clearance of distal lesions. A phase I trial of CLN-617 in patients with advanced solid tumors is expected to commence in 2023.

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MHC-E, the ligand for checkpoint NKG2A, is also engaged by LILRB1 on myeloid cells

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NKG2A is a novel checkpoint in cancer immunotherapy, which is currently investigated in clinical trials using blocking antibodies. This inhibitory receptor is only expressed on killer lymphocytes, including NK cells, γδ T cells and CD8 T cells and therefore thought to unleash cytotoxic activity. The ligand of NKG2A is monomorphic MHC-E molecule (HLA-E in humans and Qa-1 in mice) in

combination with the conserved peptide called VML and Qdm, respectively. This peptide-specific ligand for NKG2A is thought to be constitutively present under steady state, although an alternative peptide repertoire for MHC-E has been described. We aimed to unravel when and where Qa-1/Qdm complexes are displayed *in vivo* to the immune system. The newly isolated nanobody 'EXX1' specifically targets this Qa-1/Qdm combination. Structural analysis revealed the docking site of this EXX1 antibody to be similar as NKG2A and the docking site of the widely used '6A8' antibody to the heavy chain, independent of peptide. Genome-wide CRISPR/Cas9 screens confirmed the requirement of Qa-1 and β2m for 6A8 binding to cells, but interestingly all members of the peptide loading complex were only essential for EXX1 binding. Knockdown of the aminopeptidase ERAAP indeed led to complete loss of Qdm presentation and sensitization for NKG2A-expressing NK cells. Using EXX1 we could also measure the stability of the Qa-1/Qdm complexes on cells. Inhibitors of protein synthesis or Golgi transport were applied to investigate the decay of surface Qa-1/Qdm. While the conventional MHC molecule H2-D^b and the Qa-1 heavy chain were rather stable, the half-life of Qa-1/Qdm complexes was as short as 30 minutes, indicating that the NKG2A ligand acts as a real time sensor for functional antigen presentation. Surprisingly, EXX1 binding was not detected on *ex vivo* isolated cells from mice, with the exception of plasmacytoid dendritic cells, even though Qa-1 heavy chains were present on all cells. Stimulation through interferon or TLRs was needed to induce display of Qa-1/Qdm complexes *in vitro* and *in vivo*, indicating that NKG2A functions as a secondary checkpoint under inflammatory conditions. The finding that the NKG2A ligand is not commonly present under homeostatic conditions suggests the presence of other Qa-1^b conformations and interacting receptors. In search for such receptors, we performed a human CRISPR activation screen with HLA-E tetramers and found LILRB1 and LILRB2 as top hits. These molecules are inhibiting receptors on macrophages, which lack NKG2A, and indeed appeared to bind to HLA-E. Importantly, phagocytosis assays with macrophages demonstrated an inhibiting role for Qa-1 and HLA-E, independent of the Qdm peptide. Altogether, our data demonstrate a converging role of two different receptors to detect defects in antigen processing and presentation by cytotoxic lymphocytes and phagocytic myeloid cells.

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Genetic ablation of macrophage glucocorticoid receptor signalling restricts primary tumour growth and overcomes resistance to immune checkpoint inhibitor treatment

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Effectiveness of checkpoint immunotherapy in cancer can be undermined by immunosuppressive tumour-associated macrophages (TAMs). Here we demonstrate that tumour-derived glucocorticoids (GC) drive immune evasion and resistance to checkpoint immunotherapy by inducing an immunosuppressive phenotype in TAM.
Transgenic melanoma models (LysMcreGRfl/fl; CD163creGRfl/fl) were generated by s.c. inoculation

of melanoma cells and tumour-bearing mice were i.p. injected with aPD-1 antibody / isotype control. Effects of TAM-expressed GR were analysed by IHC, FACS and qPCR. Analysis of skin cutaneous melanoma (SKCM) TCGA data was performed using GEPIA.
First, we tested the effect of conditional knockout of GR in TAM on tumour growth in the B16-F10luc2 and YUMM1.7 melanoma model. We demonstrate that genetic ablation of GR signalling in TAMs resulted in 50% tumour growth inhibition, along with a decreased infiltration of CD163+ and increase in MHCII+ TAMs. This was further confirmed by the equally reduced growth of YUMM1.7 tumours carrying specific GR deletion in CD163+ TAM subsets. Next, we analysed the effect of TAM-expressed GR on the response to checkpoint immunotherapy in the YUMM1.7 model, which is inherently resistant to aPD-1 checkpoint immunotherapy. As expected, aPD-1 treatment of YUMM1.7 tumour-bearing wt mice had no effect on tumour growth. However, in the absence of TAM-expressed GR, aPD-1 treatment significantly restricted tumour growth rates and reduced final volumes by more than 70%. FACS analysis revealed reprogramming of TAM towards an immunostimulatory phenotype, accompanied by a reduction of T cell exhaustion markers PD-1, TIM-3 and LAG-3.
To investigate if intratumoral de novo steroidogenesis was the source of GCs, we analysed the expression of steroidogenesis pathway enzymes in tumour tissue. The expression of HSD11B1, an enzyme that catalyses cortisol regeneration, thus amplifying its cellular action, was induced upon aPD-1 treatment of YUMM1.7 tumour-bearing wt mice. However, this effect was abrogated in the absence of TAM-expressed GR. In line with this, the expression of the rate-limiting enzyme of steroid biosynthesis Cyp11A1 was reduced in the absence of TAM-expressed GR, suggesting a reduced total de novo steroidogenesis in tumours from transgenic mice.
To investigate the translational significance of our preclinical findings, we analysed human SKCM TCGA datasets using GEPIA. Survival analysis showed that high HSD11B1 expression results in poor clinical outcomes. Additionally, HSD11B1 expression positively correlated with CD163, CD274 and PDCD1 expression.
Collectively, this study represents to first approach to reprogram immunosuppressive TAM by GR inhibition, suggesting that therapeutic targeting of GR is a promising way to sensitize melanoma to checkpoint immunotherapy.

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Continuous treatment with dexamethasone in combination with a CD19xCD3-bispecific antibody boosts CD8$^+$T cell expansion and protects against T cell exhaustion in a long-term in vitro model.

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T-cell recruiting bispecific antibodies (BsAbs) describe a novel off-the-shelf platform of T-cell directed immunotherapies. The CD3xCD19 targeting BsAb blinatumomab was the first in class to be approved in the B-cell precursor acute lymphoblastic leukemia setting resulting in unprecedented overall response rates. With recent FDA approvals, this clinical success could be expanded to follicular

lymphoma and multiple myeloma patients, respectively, and more BsAb approvals are expected in the upcoming years. BsAb therapy is associated with dose-limiting immune-related adverse events (IrAEs) as cytokine release syndrome (CRS), possibly jeopardizing treatment efficacy. Lower grade IrAE management mostly relies on treatment with the glucocorticoid dexamethasone (dexa), including a prophylactic pretreatment regime. However, conflicting data have been published addressing the effect of glucocorticoids on T-cell efficacy. Here, we aimed to provide insights into possible long-term effects of simultaneous administration of immunosuppressive dexa and BsAb treatment on T cells.
We used our previously established in vitro model system to mimic the clinical situation of continuous BsAb exposure. Healthy donor (HD) T cells were co-cultured with a CD19-expressing lymphoma cell line (OCI-Ly1) for up to 28 days and continuously treated with a CD3xCD19 BsAb (5 ng/ml) alone or in combination with dexa (300 nM). T cells were harvested every 7 days and T-cell fitness was assessed by: immunophenotyping, secretion of effector cytokines, proliferative capacity and BsAb-mediated cytotoxicity.
Interestingly, co-treatment with dexa lead to a decrease in Lag-3 expression after 14 days (Mean fluorescent intensity (MFI) ratio day 21 BsAb only=10.5±1.1, +dexa=1.7±0.4, *p=0.02), while a comparable or higher upregulation of Tim-3 and PD-1 expression was observed for both conditions (MFI ratio PD-1 day 10 BsAb only=4.5±0.1, +dexa=7.4±0.9). Overall effector cytokine (IL-2, TNF-α, IFN-γ) levels in the culture supernatant were decreased upon dexa co-treatment. However, in contrast to the BsAb only control, low levels of IFN-γ could be maintained in the dexa co-treated condition over 24 days (mean level IFN-γ day 24 BsAb only=53±26 pg/ml, +dexa=1756±393 pg/ml). Furthermore, we observed an increased $CD8^+$ T-cell expansion in the dexa co-treated condition (abs. cell count day 21 BsAb only=1613±429, +dexa=34583±19653). Finally, T cells stimulated with the combination of BsAb and dexa could maintain a significantly higher cytotoxic activity when compared to BsAb only control (mean % specific lysis day 21 BsAb only=34.2±4.4, +dexa=90.3±1.3,**p=0.004).
Based on our data we hypothesize that transient dexa treatment in BsAb patients could serve as a dual approach, managing IrAEs while simultaneously serving as protective, resting periods for T cells. Future studies are required to evaluate the overall BsAb-mediated antitumor effect of T cells co-treated with dexa.

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Manipulating the balance of pro- and anti-inflammatory signals in the tumour microenvironment to enhance cancer control

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Type I interferons (IFNs) are a family of potent cytokines with diverse functional effects on immunity. We explored this diversity in cancer and found expression of individual IFN subtypes within the tumour microenvironment (TME) resulted in a spectrum of outcomes: (i) 80% of mice inoculated

with IFNα9-expressing B16 melanoma cells had complete tumour control or (ii) mice bearing IFNα4-expressing tumours had delayed progression with all eventually succumbing to disease. We utilised bulk and single-cell RNA sequencing (RNAseq) to understand the mechanisms driving these distinct phenotypes. Bulk RNAseq profiling showed that all tumours expressing IFN had a general proinflammatory phenotype with high immune infiltration. Interestingly, IFNα4-expressing tumours displayed an 'overheated' phenotype, with single-cell RNAseq revealing a strongly Th1-polarised TME with increased proportions of type I ILCs, NK cells and inflammatory monocytes. In addition, IFNα4-expressing tumours displayed decreases in macrophages, fibroblasts and conventional type I dendritic cells (DCs) as compared to their IFNα9 counterparts. Whilst IFNα9- and IFNα4-expressing tumours had similar numbers of $CD8^+$ T cells, functionally impaired $CD8^+$ T cells were observed in the latter, characterised by impaired transition from a naïve to an effector phenotype. In addition, DCs in 'overheated' IFNα4-expressing tumours displayed both a change in subset composition and an altered phenotypic profile relative to control and IFNα9-expressing tumours. Ultimately, this imbalanced immune response was inadequate to eliminate the tumour. This led us to propose that successful anti-tumour immunity requires a finely tuned balance of pro- and anti-inflammatory signals to promote effective tumour clearance. We have validated this hypothesis by manipulating the balance of pro- and anti-inflammatory signals in the TME to 'cool down' overheated tumours and maximise the longevity of the anti-tumour immune response. We anticipate this knowledge will uncover novel immunotherapeutic targets and synergise with current immunotherapy to overcome treatment-resistant disease.

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IL-2/αIL-2 complexes massively expand systemic tumor-specific T cells and enhance abscopal responses to radiation and αPD1

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Background. Early clinical trials have provided evidence for RT-induced systemic effects in conjunction with αPD1 or IL-2 in metastatic patients, but strong abscopal responses are clinically rare. Dual combinations of αPD-1 with more effective and less toxic IL-2 derivatives are also currently under investigation. Whether a combination of RT, αPD-1, and CD122-directed IL-2/αIL-2 complexes (IL-2c) can increase abscopal effects against established non-irradiated tumors is unknown. We investigated how adding IL-2c to hRT/αPD1 affects tumor-specific $CD8^+$ T cell differentiation and the potential of this triple combination to enhance the abscopal effect compared to the respective dual treatments.
Methods. Mice bearing bilateral tumors were treated with two fractions of 8 Gy (C51 colon carcinoma model) or 12 Gy (B16 melanoma model); αPD1 was given weekly; IL-2c was given for five consecutive days. CD8 T cell-depleting and CXCR3-blocking antibodies were used to determine if the

therapeutic effects depend on $CD8^+$ and $CXCR3^+$ T cells. Differentiation stages of tumor-specific $CD8^+$ T cells in tumor-draining lymph nodes, spleen, blood, and tumors were determined flow cytometrically using MHC-I tetramers and various antibodies. Anti-tumoral effects of blood-derived and tumor-derived T cells were assessed in adoptive T cell transfer experiments.
Results. The abscopal effect was significantly stronger in triple-treated mice compared to mice treated with RT/αPD1 (C51 model: $p < 0.01$; B16 model: $p < 0.05$), RT/IL-2c (C51 model: $p < 0.01$; B16 model: $p < 0.001$) or αPD1/IL-2c (C51 model: $p < 0.0001$, B16 model: $p < 0.01$). Triple therapy improved survival and resulted in complete cures of 3/12 mice in the C51 model and 2/12 mice in the B16 model. These anti-tumor effects were associated with dramatic expansion of tumor-specific $CD8^+$ T cells. Undifferentiated stem-like ($TCF1^+ TIM3^- PD1^+$) and effector-like ($CD101^- TIM3^+ TCF1^- PD1^+$) but not terminally differentiated ($CD101^+ TIM3^+ TCF1^- PD1^+$) exhausted cells particularly strongly increased. Moreover, IL-2c induced CXCR3 mainly on non-terminally differentiated $CD8^+$ T cells. Both $CD8^+$ (C51 model: $p < 0.0001$; B16 model: $p < 0.01$) and $CXCR3^+$ (C51 model: $p < 0.0001$) T cells were crucial for the RT-induced abscopal effect upon RT/αPD1/IL-2c treatment. Finally, we found that peripheral blood from triple-treated mice is an effective source of T cells for adoptive T cell transfer.
Conclusions**.** RT/αPD-1/IL-2c triple treatment resulted in superior local and systemic expansion of tumor-specific $CD8^+$ T cells with stem- and effector-like phenotypes. Also, IL-2c strongly increased $CXCR3^+$ $CD8^+$ T cells that were associated with pronounced abscopal responses in models with an established metastasis resistant to αPD1/IL-2c and only transiently responding to RT/αPD1 or RT/IL-2c. Therefore, such triple combinations appear promising for clinical evaluation in metastatic patients.

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Polymer-based antibody mimetics (iBodies) target human PD-L1 and serve as a potent immune checkpoint blocker *in vitro*

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Cancer immunotherapy employs patient's own immune system to treat various malignancies. The interaction between programmed cell death protein 1 (PD-1) expressed on activated T cells and programmed death-ligand 1 (PD-L1) on tumor cells negatively affects signaling pathways that would otherwise allow T cells to mount an immune response against tumor cells. Using monoclonal antibodies (mAbs) to block this interaction has been shown to provide clinical benefits to treat a broad range of cancer types. However, checkpoint blockade therapy (CBT) still suffers from limitations including low efficacy, inherent or acquired resistance, immune-related adverse effects, and high cost. Thus, new and innovative approaches are needed. One possibility is to use small molecules instead of mAbs to disrupt PD-1/PD-L1 interaction. Nevertheless, small-molecule blockers often show lower solubility, stability and efficacy as compared to antibodies. We developed synthetic

antibody mimetics called iBodies based on the *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymers. The copolymers allow attachment of various functional moieties, e.g., a targeting ligand (giving it a specificity), a fluorophore (for visualization) and an affinity anchor. In addition to being biocompatible, non-immunogenic and non-toxic, the HPMA copolymers improve solubility, stability, and functional affinity (avidity) of small molecules. To generate anti-PD-L1 iBodies, we attached human PD-L1-specific small-molecule ligand to the HPMA copolymer. iBodies bind to soluble as well as membrane-bound PD-L1 with improved EC_{50} as compared to the ligand alone. A fluorescent probe enabled visualization of PD-L1-expressing cells similarly as mAb. Moreover, in a cellular CBT model, iBodies were able to rescue activation of T cells comparably to therapeutic antibodies like avelumab, atezolizumab and durvalumab. To summarize, iBodies can be used as experimental tools for detection and visualization of human PD-L1 and show therapeutic potential in cancer treatment.

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ICOS immunotherapy – Towards understanding the mechanism of action

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Checkpoint blockade therapies were suggested to improve CD8 T cells cytotoxic activity against the tumor and have shown great potential in cancer therapeutic research. Nevertheless, in the clinic, patients' response to the therapy is not optimal as a significant part of the patients does not benefit from it. T regulatory cells (Tregs), which under normal circumstances are responsible for maintaining immune homeostasis by repressing the activity of the immune system, are also found in the tumor and contribute to the immune suppressive microenvironment. In addition, it seems that in most cancers, CD8 T cells function poorly while the Tregs suppressive activity is reserved and sometimes elevated. Thus, to improve patients' response to immunotherapy, targeting Tregs and impairing their suppressive activity would potentially lead to improving overall anti-tumor immunity. It was previously shown that inducible T cell co-stimulatory (ICOS) is differentially expressed on tumor-infiltering compared to peripheral Tregs and therefore, might serve as a good target. Indeed, ICOS immunotherapy is already in clinical trials, but the mechanism of action is still unclear. To this end, we performed a single-cell RNA-seq of tumor-infiltrating immune cells enriched with Tregs from the tumor microenvironment (TME) 24 hours following a single injection of αICOS agonist to MC38 tumor-bearing mice. Our preliminary results show that αICOS treatment leads to an elevation in the CD8 T cell population. As expected, we see a trend of decrease in the Tregs population, but to our surprise, an overall less suppressive Treg phenotype accompanied by increased response to interferon signaling. This was further validated in an ex-vivo suppression assay using tumor-infiltrating Tregs. αICOS agonist treated mice showed an inferior suppression capability compared to isotype control treated mice. Moreover, our results were also recapitulated using human Tregs. Thus, although ICOS is considered a co-stimulatory receptor, under certain conditions, it can suppress Treg activity. Mechanistically, our single-cell RNA-seq data shows a reduction in CCAAT Enhancer Binding Protein Beta (Cebpb) following αICOS administration. Cebpb is known to dampen the inhibitory effect of IFNg on Tregs. We thus hypothesize that αICOS agonist causes a reduction in Cebpb levels

which synthesizes Tregs to IFNg in the TME, resulting in enhanced Treg fragility and reduced suppressive function.

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Density and ratio of stimulatory antibodies on immunofilaments influence ex vivo T cell expansion and phenotype

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The development of immunotherapy has revolutionized the treatment of cancer by influencing the anti-tumour immune response. Numerous immunotherapies focus on expanding and activating tumour-reactive T cells, for instance by re-invigorating existing T cell responses by immune checkpoint blockade or by expanding the tumour-reactive T cell pool *ex vivo* through adoptive T cell therapies (ACT). With ACT high numbers of tumour-reactive T cells are infused into a patient, consisting of autologous tumour-infiltrating lymphocytes, T cell receptor engineered T cells, or chimeric antigen receptor T cells. ACT has shown promising effects in the treatments of various cancers, however, for ACT to succeed high numbers of T cells are required.
In recent years, biomaterial-based artificial antigen presenting cells (aAPCs) have been developed for the activation and expansion of T cells. aAPC can mimic T cell activation signals by using agonistic anti-CD3 (αCD3) and anti-CD28 (αCD28) antibodies, respectively. Rigid synthetic beads presenting these agonistic antibodies are widely used in expansion protocols. However, with these standard beads the signals given to the T cells cannot be tuned. Here, we used polyisocyanopeptide based immunofilaments as nanosized aAPCs to study the effects of the ratio and density of αCD3 and αCD28 on human T cell expansion and phenotype.
We cultured human total $CD3^+$ T cells for up to 14 days with the immunofilaments and investigated the effects on T cell expansion, cytokine production, and phenotype (memory, effector, and exhaustion). We observed differences in T cell expansion depending on the density and ratio of the stimulatory signals. Additionally, the ratio and density of the stimulatory antibodies also influenced the production of the cytokines IFNγ, TNF-α, and IL-2 in the first 3 days of culture. Moreover, differences in memory phenotype and to a lesser extent in effector phenotype could be observed. Taken together these results suggest that careful finetuning of the density and ratio of stimulatory antibodies can significantly impact T cell expansion and phenotype. As such our findings can be used to optimize T cell expansion protocols for ACT.

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Comprehensive characterization of bladder microenvironment in murine MB49 bladder tumor model to identify new therapeutic targets

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In bladder cancer there is an important need for the development of therapies with fewer side effects and better efficacy than the gold-standard bacillus Calmette-Guerin (BCG) immunotherapy. A better understanding of the immunoregulation involved in bladder cancer may thus help to identify new targets for alternative or complementary immunotherapy.

To study immune regulation occurring in bladder cancer, we first assessed by flow cytometry the kinetics of immune infiltration in the orthotopic murine MB49 bladder tumor model. At steady state, the bladder seems to be a specific homing site for Type 2 innate lymphoid cells (ILC2), since a frequency of ILC2 similar to the lung was observed. Although ILC2 decrease upon tumor growth, they may be an interesting target at the onset of bladder tumor development, since ILC2 pro-tumor functions have been reported in some cancers including bladder cancer. Yet, targeting ILC2 by using ILC2-deficient, IL-33-deficient mice, or anti-IL-33 receptor treatment, did not improve mice survival upon bladder tumor implantation. In addition, no change in ILC2 infiltration between non-tumor and tumor specimen was observed in bladder tissue samples from muscle-invasive bladder cancer patients. These results suggest that ILC2 do not have a key role in the bladder tumor development. Furthermore, in absence of tumor, the bladder is also particularly infiltrated by eosinophils and M2 macrophages. During tumor growth, the level of $CD8^+$ T cells and M1 macrophages increase, while NK cells and M2 macrophages frequencies decrease but the latter still outnumbering M1 macrophages. Further characterization of tumor-infiltrating $CD8^+$ T cells showed a progressive appearance of a terminal exhausted phenotype ($PD\text{-}1^{high}TCF\text{-}1^{neg}$) upon tumor growth. In addition, we determined the Th1 vs Th2 cytokine ratio (IL-2, IFNγ, TNFα vs IL-4, IL-5, IL-13) from bladder tissues of mice with or without tumor. We found that whereas Th2 and Th1 cytokines were in the same range in the bladder microenvironment at steady state, Th1 cytokines significantly increased during tumor growth, in parallel to an increase of tumor-infiltrating $CD4^+$ $T\text{-}bet^+$ T-cells.
Overall, the establishment of a Th1 anti-tumor response might occur in the bladder upon tumor growth yet it seems that the $CD8^+$ T cells are exhausted. The balance between regulatory and effector subsets is tight and could dampen the efficiency of the anti-tumor response. Treatments targeting M2 macrophages or Th2-related pathways are ongoing in order to decrease intratumoral immunosuppression and improve the establishment of an anti-tumor response.

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Arming the oncolytic adenovirus ORCA-010 with constitutively active GSK3β to overcome melanoma induced myeloid suppression

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Melanoma incidence is on the rise on a global level and although immune therapy based on immune checkpoint blockade (ICB), has resulted in profoundly improved overall survival rates, a major part of patients is still non-responsive to the current treatment regimens or acquires resistance. Multiple factors can debilitate ICB efficacy, with a crippled myeloid compartment as a an important

contributor, since these immune cells are both responsible for proper priming and recruitment of T cells to the tumor microenvironment (TME). Spranger *et al.* showed an absence of a T cell infiltrate in melanoma to be related to a lack of the T–cell-recruiting chemokines CXCL9 and CXCL10, released by by $CD103^+$ dendritic cells (DCs) in the TME.[1] This was molecularly linked to active β-catenin signaling, which hampered the recruitment of $CD103^+$ DCs due to low levels of CCL4 in the TME. In a functional screen on arrays consisting of >1000 human kinase peptide substrates, we identified glycogen-synthase kinase-3β (GSK3β) as a pivotal kinase in both DC development and suppression, which was known to mediate β-catenin degradation. Adenovirally reinforcing expression of constitutively active GSK3β (CA.GSK3β) proved an effective strategy to overcome melanoma-induced DC suppression.[2]
The use of oncolytic viruses could potentially further enhance the anti-tumor effects of GSK3β expression by their selective oncolytic effect in tumor cells which in turn leads to local release of tumor antigens that subsequently can increasing the breadth of activated T-cells. Therefore reinforcing GSK3β expression with oncolytic viral therapy could propose a new effective strategy exerting its effect not only directly on tumor cells, but also on DCs and T cells, thereby providing a multi factorial immune activating approach.
We successfully cloned the CA.GSK3β gene into ORCA-010, a potent oncolytic virus carrying a E1AΔ24 deletion and a T1 mutation, potentiating viral release and spread, combined with a infectivity enhancing fiber RGD modification. We show efficient expression of the transgene in multiple melanoma cell lines, combined with equal to enhanced oncolytic potency of ORCA-010-CA.GSK3β vs ORCA-010. Moreover, we validated the functional effects of ORCA-010-CA.GSK3β and showed immune modulatory effects in melanoma/DC co-cultures, with a reduction in melanoma-induced M2-like macrophage differentiation and increased expression of co-stimulatory markers like CD80 and CD86. We additionally show that this coincides with a reduction in VEGF levels, secreted by melanoma cells. Overall this data reveals that arming ORCA-010 with CA.GSK3β is an effective strategy to overcome melanoma-induced suppression of myeloid cells.

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Immunomodulation induced by the drug conjugates 5-Fluorodeoxyuridine-alendronate and 5-Fluorodeoxyuridine-C-ethynylcytidine in human epithelial ovarian carcinoma

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New therapies are needed for epithelial ovarian cancer (EOC) patients faced with chemotherapy resistance. An optimal therapy should be effective in chemotherapy-naïve and/or pre-treated patients, support the anti-tumor immune response and be well tolerated without heavy side effects. We have tested the effects of two innovative duplex drugs, in which the antimetabolite 5-Fluorodeoxyuridine (5-FdU) is linked to alendronate (5-FdU-ale) or C-ethynylcytidine (5-FdU-ECyd or 5-FdU-lipid-ECyd). For the assessment of the immunomodulatory effects of each 5-FdU conjugate,

the clinically predictive model of patient-derived explant tissue culture was used. The effects on the immune landscape were analyzed by Luminex-based multiplex measurement of 50 cytokines, chemokines and growth factors in the tumor lysates. The density of immune cells was studied by automated immunostaining and virtual pathology.
In tissues originating from pre-treated patients, 5-FdU-ale effectively reshapes the tumor microenvironment, increasing the concentration of a series of factors supportive of the anti-tumor immune response and of T cell survival. Moreover, the 5-FdU-ale treatment combined with nivolumab triggers immune cell proliferation in tissues where neither treatment alone mediates any effect, suggesting synergy between 5-FdU-ale and immune checkpoint blockade. In contrast, the treatment with 5-FdU-ECyd leads to the expansion of CD8+ cytotoxic T cells in most treated tissues. Interestingly, the amplification in cytotoxic T cell numbers is significantly stronger in tissues with pre-existing tertiary lymphoid structures (TLS) at the time of treatment. 5-FdU-ECyd additionally mediates a decrease in the concentration of immunosuppressive factors in the tissue.
We have identified 5-FdU-ale and 5-FdU-ECyd as excellent candidates for the treatment of EOC because they both mediate specific immunomodulation and convert the tumor microenvironment from a “cold” into a “hot” tumor microenvironment. Our results show for the first time that duplex drugs could be used to break resistance to immunotherapy and to tackle the issue of resistance to chemotherapy. From our dataset in tissue explants, both candidates are promising and definitely warrant further translational and clinical studies.

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Cell death-optimized 5’-triphosphate RNAs for improved tumor immunotherapy

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Currently, most of the immunomodulatory approaches in antitumor therapy target and potentiate the adaptive immune system. However, only a minority of cancer patients can benefit from this therapeutic approach since adequate treatment efficacy often requires preexisting T lymphocyte infiltration. Therefore, therapeutic manipulation of the innate immune pathways holds great potential to enhance an effective adaptive immune response.
Activation of the intracellular pattern recognition receptor retinoic acid-induced gene I (RIG-I) by 5’-triphosphate double-stranded RNA (3p-RNA) was shown to induce an antiviral response characterized by the induction of type I interferons (IFN), proinflammatory cytokines and apoptosis. We have previously shown that 3p-RNA-induced apoptosis is mediated by two dsRNA-receptor families with RIG-I being essential for IFN-I-mediated priming and oligoadenylate synthase (OAS)/RNase L for cell death induction. We hypothesized that modification of the 3p-RNA characteristics influences differential activation of both receptors system leading to an optimal balance between RIG-I mediated cytokine axis and RNase-L mediated cell death axis to enhance priming and antigen availability for an effective antitumoral immune response.
A set of defined in vitro-transcribed 3p-RNA ligands were analyzed in different knockout tumor cell lines activating either only RIG-I, RIG-I and OAS or PKR (RNA-activated protein kinase) to understand the functional role of each axis and to separate cytokine response from translational inhibition and

cell death. We observed significant length-dependent effects in cytokine and cell death induction upon transfection into human and murine tumor cells. 3p-RNA-treatment induced IFN increasingly with length until a maximum was reached after which IFN levels rapidly decreased. On the other hand, cell viability steadily decreased with increasing 3p-RNA length, which was concomitant with a more effective OAS/RNase L- and PKR-mediated translation arrest. Hence, a more efficient activation of OAS/RNase L and PKR by long 3p-RNA negatively regulates IFN levels due to an early translational shutdown but increases the amount of tumor cell death and therefore antigen availability.
In conclusion, we were able to generate cell death-optimized 3p-RNA which holds great promise for the development of effective RNA-based treatments by balancing cytokine induction and cell death induction through restriction factor activation to promote immunosurveillance in the tumor microenvironment.

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Use of TCR antibody fusion proteins as bispecific agents for NK and T cell-mediated immunotherapy

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This study aimed to develop novel soluble bispecific TCR-based agents as an approach for NK and T cell-mediated immunotherapy. Bispecific agents have the unique capacity to redirect cytotoxic lymphocytes towards tumor cells by targeting different structures on the tumor cell surface. Targeted tumor antigens can be roughly divided into peptide antigens presented by major histocompatibility complex (MHC) class I molecules to antigen-specific T cell receptors (TCRs) and cell surface antigens recognized by antibodies. Peptides presented in the context of an MHC-I molecule mostly result from intracellular proteins, which make up the majority of the proteome, and thus serve as an highly attractive target for immunotherapy. However, since TCRs are characterized by a rather low affinity and are difficult to produce as soluble molecule, there are only few studies focusing on soluble TCR-based therapies to date. To achieve potent bispecific mediators a bivalent IgG-like TCR-Fc fusion format was used. The ectodomain of the TCR Vα/Cα chain was fused to the hinge/Fc part of human IgG1 and the ectodomain of the TCR Vβ/Cβ chain was expressed as a second soluble protein in tandem. To enable NK cell redirection, specific mutations known to enhance FcγRIIIa binding were introduced in the Fc fragment. Other formats made use of single chain variable fragments (scFv) recognizing CD16 or NKp46 for NK cell redirection or CD3ε for T cell redirection inserted C-terminal of TCR Cα or Cβ, respectively. A cytomegalovirus (CMV) pp65 peptide/HLA-A*02:01-specific TCR sequence was used as a model system. To analyze the influence of TCR affinity, the wildtype TCR was compared to an affinity-maturated variant. The soluble IgG-like TCRs showed a target-specific, affinity- and concentration-dependent binding leading to NK and T cell activation and cytotoxicity upon co-culture with peptide-pulsed cells. These results were further confirmed using a gp100 peptide/HLA-A*02:01-specific TCR. Using gp100-expressing melanoma cells, however, the TCR-Fc constructs failed to facilitate cytotoxicity unless synthetic gp100 peptide was added. Thus, the

NK cell-engaging TCR-Fc constructs apparently were not sensitive enough to redirect NK cells against melanoma cells presenting low quantities of naturally processed peptide. In sum, a panel of novel recombinant bispecific TCR-Fc and TCR-scFv-Fc fusion proteins were genetically engineered, produced and demonstrated to facilitate the activation and cytotoxicity of NK and T cells towards tumor cells expressing a model peptide-MHC-I complex in sufficient quantities. By multimerization approaches we presently attempt to increase binding avidities of TCR antibody complexes in order to overcome the sensitivity issues resulting from low abundance of peptide-MHC-I complexes.

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The macrophage-T cell axis in the success and failure of immunotherapy

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While most immunotherapies are focused on the induction of a potent anti-tumor T-cell response, accumulating evidence suggests the need for a fully coordinated immune response, rather than a strong anti-tumor T-cell response alone, in order to reach total tumor control. Due to the highly plastic nature of myeloid cells and their opposing roles in the tumor microenvironment (TME), the knowledge on the exact contribution of these individual immune cells to tumor regressions is very limited and their mechanism of action is still heavily debated. In this study, a unique mouse model with both primary and secondary resistance against therapeutic vaccine-induced tumor regression, was exploited to study the requirements for an optimal response to immunotherapy. Exploration of the TME by high dimensional flow cytometry, single cell RNA sequencing and Nanostring in combination with multiple immunotherapy approaches and the use of several *in vivo* models allowed for the identification of immunologic factors that are crucial for therapy response and ones that contribute to immunotherapy failure. We identified a αCsf1r-resistant ($CD115^-$) macrophage population with high CD163 expression to be responsible for both primary and secondary immunotherapy resistance by hindering $CD8^+$ T cell infiltration and effector functions. Remarkably, within the same tumors, another $CD115^+$ macrophage population of an inflammatory phenotype is the main driver of tumor regression following T-cell based immunotherapy. The presence of these inflammatory macrophages is dependent on a tumor-specific $CD8^+$ T cell response and the chemokines produced by these cells. Furthermore, they are highly important for tumor control, amongst others by depriving tumor cells of L-arginine in an iNOS-dependent manner. These findings illustrate that within the same tumor at least two distinct macrophage populations are present: a $CD115^+$ subset, of pivotal importance for therapy-induced tumor control, and a $CD115^-$ subset that dictates immunotherapy resistance. These outcomes will fuel the debate on macrophage targeting during the immunotherapy of cancer.

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Profiling immune checkpoints to identify synergistic targets for RANK inhibition in cervical cancer and exploring its potential

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Background: Conventional treatments for cervical cancer (CC) have reached a plateau, resulting in five-year survival rates of only 17% for the advanced stages. Immunotherapy presents a promising avenue to improve long-term benefits for oncology patients. Despite CC being immunogenic, only a minority of patients respond to immunotherapy. Recently, the RANK-axis has emerged as an immune suppressive pathway in cancer that can be blocked to fuel the effect of immunotherapy, but convincing evidence is still lacking. To address this gap, we conducted a study to assess the effect of RANK on tumor-immune cell interaction in CC. We then profiled a selection of immune checkpoints on CC cell lines to further explore the immune landscape in CC patient samples. The most interesting immunotherapy co-targets are being exploited in combination with RANK-blocking therapy in high-throughput *in vitro* co-cultures.

Methods: 2D co-cultures with NK-92 and various CC cell lines were utilized to assess the effect of RANK on tumor cell killing and NK activation using flow cytometry. Lentiviral transduction of LacZ (control) and RANK was performed on all CC cells, while NK-92 cells underwent Mock or RANKL mRNA nucleofection. To identify interesting co-targets, flow cytometry was used to screen immune checkpoints on the CC cell lines before and after treatment with exogenous rhRANKL. Our selection of markers was based on TCGA analysis and literature research. Immunohistochemistry for the most abundant and RANK-correlated markers was then performed on 49 formalin-fixed and paraffin-embedded CC patient samples. Finally, we are conducting high-throughput NK-tumor cell co-culture experiments to target the identified immune checkpoint axes in combination with RANK-blocking therapy using live cell imaging.

Results: Our study found that RANK suppresses tumor cell killing by NK-92 cells. We identified seven interesting co-targets for RANK-inhibition in CC, including PD-L1. IHC analysis on 49 patients showed that all the other six targets were more abundantly present than PD-L1. The combination immunotherapy co-culture experiments are ongoing.

Conclusion: Interim analysis of our study surfaces interesting (co-)targets for immunotherapy in CC. In ongoing work, we are identifying the best-in-class RANK-targeted combination immunotherapy.< div id="eid-safari-extension-is-installed" >

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Immune checkpoint blockade activates hematopoietic stem cells and reprograms early hematopoiesis.

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The antitumor function of tumor-specific T cells is curtailed by immunosuppressive mechanisms such as co-expression of immune checkpoints. Immune checkpoint blockade Immunotherapies (ICB) provide substantial clinical benefits in responding patients. The mechanisms underpinning the response to immunotherapies are multiple: ICB triggers both local (within the TME) and distal (systemic effects) immune responses. By investigating the systemic impact of immunotherapy, we found that the bone marrow (BM) swiftly respond to ICB. Using a murine model of melanoma responding to aPD1/aCTLA4 immunotherapy, we show that early hematopoietic progenitors (Lin-Sca1+Kit+, LSK) expand as early as d3 post treatment. ICB induce the proliferation of both long-term HSCs and lineage-poised Multipotent Progenitors (MPPs). Single cell RNAseq profiling of LSK identifies the expansion of IRF8+ myeloid poised subset and a FLT3+IRF8+ subset enriched in IFN-stimulated genes. The signalling pathways controlling LSK activation and its impact on lineage commitment and hematopoietic outputs are being investigated. Concomitantly to LSK expansion, we identify an early increase in BM infiltration by parenchymal, activated, cycling PD1+ T cells distinct from BM resident CD4/8+CD49a+HOBIT+ T lymphocytes. Altogether, our results show that the BM microenvironment is a highly responsive site during ICB and highlight a previously unappreciated systemic aspect of immune response to ICB, *id est* the regulation of haematopoiesis.

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Development of bispecific T-cell engager-sialidase fusion proteins for cancer immunotherapy

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Bispecific T-cell engager (BiTE)-based cancer therapies, which activate the cytotoxic T cells of a patient's own immune system, have gained momentum with the recent FDA approval of blinatumomab for the treatment of B-cell malignancies. However, this approach has had limited success against solid tumors. In addition to the problem of limited tumor accessibility, BiTE-based therapies must overcome the immunosuppressive tumor microenvironment.
Aberrant glycosylation is a hallmark of cancer. Tumor cells often upregulate characteristic terminal sialic acid-bearing glycoforms as glyco-immune checkpoints to suppress immune activation. The highly sialylated glycocalyx also serves as a physical barrier preventing effective infiltration of T and NK cells into the tumor bed. We hypothesize that by incorporating sialidase into BiTE molecules to

create sialidase fusion proteins that can selectively remove sialoglycans at the T cell-tumor cell interface, T cells may have a better chance of approaching target tumor cells. Thus, T cell-dependent tumor cell cytolysis could be enhanced and solid tumors could be treated.
Here, we describe the development of BiTE-sialidase fusion proteins that enhance tumor cell susceptibility to BiTE-mediated cytolysis by T cells via targeted desialylation at the BiTE-induced T cell-tumor cell interface. Targeted desialylation results in improved immunological synapse formation, T-cell activation and effector function. As a result, BiTE-sialidase fusion proteins show remarkably enhanced efficacy in inducing T cell-dependent tumor cell cytolysis in response to target antigens compared to the parent BiTE molecules alone. This improved function is observed in both in vitro and in vivo xenograft and syngeneic solid tumor mouse models. Our findings highlight BiTE-sialidase fusion proteins as promising candidates for developing next-generation bispecific T-cell engaging molecules for cancer immunotherapy.

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Development of a novel standardized and fully automated functional assay to assess and monitor global T cell immune function in 4 hours

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Background**:** Primary immunodeficiency is associated with human cancer. It is also well recognized that Immunosuppressive therapy affecting the individual cellular immune response increase the risk of carcinogenesis. However, in routine practice, simple, rapid and standardized immune assays to functionally assess the individual level of immunosuppression are clearly missing. For instance, in solid-organ transplant patients who are more prone to cancer development, monitoring of immunosuppressive magnitude mainly relies on drug concentration measurements not known to be sufficiently accurate. Consequently, these approaches are often not precisely tailored to each individual patient's needs. Assessing immunity is therefore paramount to implement and monitor personalized treatment. This study aims to describe the potential and clinical relevance of a novel functional assay on fully automated VIDAS® instrument to answer the unmet needs in assessing cellular immunity for routine care.
Methods: Freshly blood was collected in lithium heparin tubes from different cohort of immunosuppressed patients and healthy volunteers. Cellular immune response was then assessed in 4 hours by our automated and standardized *VIDAS® STIMM™-T*, an assay in which cytokine released by T cells from whole blood was measured after stimulation. Peripheral blood count was also performed to assess T lymphocyte subsets (CD3, CD4, CD8) by flow cytometry.
Results: Cellular immune response was assessed by the *VIDAS® STIMM™-T* in 20 healthy volunteers, 20 patients after liver transplantation (sequential sampling after transplantation) and 21 septic shock patients (sequential samples after ICU admission). We observed that *VIDAS® STIMM™-T* allowed to stratify patients into low, medium and high T cell immune response subgroups, we assessed the correlation with the patients' clinical course, independently of the total T cell number. We demonstrated, that the transplant group under immunosuppressive treatment present homogeneous IFN-G secreted concentration at W3 and W4, while at W1, W2 and the septic shock patients at all time points were more heterogeneous.
Conclusion**:** *VIDAS® STIMM™-T* embodies the next generation of assays capable of assessing and monitoring global T cell functionality in a rapid, simple, fully automated and standardized way. It may help clinicians for routine care and clinical decision-making. Further investigations will determine how the assay can be used to tailor treatment in patients with various immunosuppressive conditions: transplant, sepsis, cancer, immunotherapy…

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Very high cytomegalovirus-specific antibody titers predict clinical outcome in melanoma under PD-1 treatment and associate with a late differentiated T cell profile

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The introduction of cancer treatments by immune checkpoint inhibition (ICI) has revolutionized the research field and introduced a novel pillar of cancer therapy. While the main targets of these therapies are assumed to be T cells, little is known concerning the role of latent viral infections, such as with the highly prevalent human herpes virus cytomegalovirus (CMV), which strongly impacts the distribution of peripheral T cell populations and their differentiation profile. Therefore, we investigated whether a history of frequently recurring CMV infections represented by elevated CMV-specific IgG serum levels may inform about clinical outcomes and peripheral T cell differentiation. For this we investigated a total of 230 stage IV melanoma patients from two independent cohorts prior to anti-PD1 ICI. We quantified CMV-specific IgG serum levels and IgG-antigen and -avidity profiles of patient serum in parallel with peripheral T cell differentiation signatures. While the respective IgG-antigen and -avidity profiles were similar to healthy controls, the presence of very high CMV-specific IgG serum levels prior to starting treatment with ICI correlated with validated impaired overall and progression-free survival and with a late-stage differentiated T cell signature. Mass cytometry (CyTOF) in-depth immunophenotyping of selected patient samples identified a predominance of a late-differentiated, putatively senescent-like CD8 T cell phenotype (e.g. CD57+) in peripheral blood of patients with very high CMV-specific IgG serum levels. Preliminarily paired analyses of both blood and tumor tissue revealed a tendency towards higher CD57+ T cell frequencies in both compartments in CMV-seropositive relative to CMV-seronegative patients. The predictive capacity of CMV-specific IgG serum levels was independent of confounding factors and established markers such

as S100 serum levels. These findings suggest that T cell exhaustion mediated by a history of frequent CMV reactivations may reduce the desired treatment responses to ICI, underscoring the role of CMV and indicating a potential need for antiviral treatment already at an early stage of disease.

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Establishing a workflow to compare T cell receptor sequences in blood and tumor

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In recent years, T cell receptor (TCR) sequencing has emerged as a powerful tool in immuno-monitoring of novel immunotherapies. A diverse TCR repertoire has been shown to serve as prognostic biomarker for several cancer types, and changes in clonality and diversity of TCR show prognostic and predictive biomarker potential for immunotherapies. So far, TCR sequencing in clinical trials is most commonly applied as bulk sequencing of tumor tissue (bulk TCRseq). However, state-of-the-art single-cell RNA and TCR sequencing (sc TCRseq) offers a much deeper immune repertoire analysis with a higher level of complexity, as it allows exact pairing of the TCR α and β chains and provides additional information such as protein and gene expression of single cells. The aim of this study is to find if TCR sequences detected in bulk TCRseq from a small tumor biopsy is representative of the TCR repertoire of a surgical resected tumor. Additionally, we checked for the overlap with TCR sequences in the periphery to test whether performing sc TCRseq in blood could capture the changes in the tumor. For a comparable target commonly shared among different patients we opted to monitor antiviral bystander T cell populations. Therefore, we collected surgically resected tumors and blood samples from Cytomegalovirus (CMV) and Epstein-Barr virus (EBV) positive head and neck squamous cell carcinoma (HNCC) patients and analyzed the overlap of antiviral TCR sequences among those samples. First data indicate that we have an overlap of TCR sequences comparing single-cell tumor (sc TCRseq) and bulk tumor (bulk TCRseq) as well as blood (sc TCRseq) and bulk tumor (bulk TCRseq). Taken together, we show that sc TCR sequencing in tumor and blood yields TCR sequences that overlap in part with bulk sequencing while providing a wealth of additional information.

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Identification of novel biomarkers for long-term response on immunotherapy in non-small cell lung cancer patients

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Although immune checkpoint inhibitors (ICI) became standard of care in stage IV non-small cell lung cancer (NSCLC), responses are heterogeneous. Currently, the PD-L1 tumor proportional score is the only approved predictive biomarker for ICI yet both long-term responders (LTRs) and non-responders (NRs) with a PD-L1^{high} score have been reported. Moreover, it remains unclear how long ICI treatment should be continued after the observation of a complete metabolic response.

In the present retrospective single-center study, we compared clinicopathologic and transcriptomic data in the following two PD-L1^{high} (>60%) NSCLC cohorts: NRs with complete lack of response (6pts) and LTRs with durable metabolic remission after immunotherapy cessation (6pts). Whole exome sequencing using a large capture-based panel was performed on both primary lung tumor and metastatic biopsies to correlate our findings to specific mutations. Bulk RNAseq zoomed in on the transcriptional differences in lung tumor and lymph node biopsies between both cohorts to decipher signatures that can aid in predicting patients' responsiveness to ICI.
Comparison of baseline clinicopathologic characteristics of NR versus LTR NSCLC patients showed no significant differences. In contrast, RNAseq analysis showed that 518 genes were significantly differentially expressed between both groups (|log2 fold change| > 2, adj.p-val < 0.05). To characterize how this profile impacted the use of specific metabolic processes, we performed gene ontology analysis. Within the LTRs, we found a specific downregulation of multiple *UGT*, *AKR* and *CYP* gene family members, involved in resp. glucuronidation, carbonyl-group conjugation and oxidation of xenobiotics. We confirmed their upregulation in lung tumor tissue using a TCGA-derived lung adenocarcinoma cohort. Finally, Kaplan-Meier analyses showed a prognostic value for low *UGT1A1* and *AKR1B10* gene expression, suggestive for their biomarker potential.
To further elucidate the impact of downregulated *UGT*, *AKR and CYP* gene expression in lung tumors on response to immunotherapy, we plan to recruit more patients to consolidate our findings. To elucidate the role of tumor microenvironment, cellular deconvolution will be performed using publicly available lung tumor scRNAseq data as prior information. As such we aspire to provide relevant predictive biomarkers for ICI that can aid clinicians during patient-tailored treatment regimen optimization.

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Characterizing immunity after SARS-CoV-2 mRNA-vaccination and infection in patients with Multiple Myeloma

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Multiple Myeloma (MM) is a hematological cancer affecting plasma cells, suppressing the immune system and increasing the risk of COVID-19. mRNA-based vaccines have been used to reduce COVID-19 mortality in MM patients but results regarding serological and cellular responses have been inconsistent. Currently, it is unclear why MM patients show different functional immune responses to mRNA-based vaccines. To study these aspects, we implemented a comprehensive immune phenotyping framework monitoring humoral and cellular immune responses against the relevant virus variants after different timepoints of mRNA-based vaccination and after breakthrough infection (BT) in 100 patients with MM and 23 healthy controls. After two doses of mRNA vaccination, the humoral immune response of patients with MM was reduced by concomitant anti-MM therapy or low CD19+ lymphocyte counts. Despite an increase in the anti-SARS-CoV-2 serum antibody levels and neutralization titers after the third dose, these responses remained impaired compared to healthy controls (HC). Patients exhibiting a BT displayed higher serological responses in comparison to non-infected patients, regardless of the timepoint of BT. Furthermore, patients with MM showed an inferior T-cell response compared to healthy controls after two doses but mostly achieved a sufficient response against the wild-type (WT) strain, demonstrating adequate immunogenicity despite the disease-related immune impairment. The responses against Omicron were similarly reduced in patients with MM and HC after three vaccinations. To unravel further molecular insight, we performed single cell RNA sequencing combined with surface proteome analysis of peripheral mononuclear blood cell-derived B-, T- and NK-cells characterizing relevant subpopulations in the context of vaccination response after the second and third vaccination dose as well as after BT. Vaccination responders showed higher abundance of CD4+ cytotoxic T-cell, CD4+ memory T-cell and CD16+ CD38+ (either FAS+ or TIM3+) NK-cell populations. These populations were characterized by strong enrichment of Interferon alpha, IL-1 and IL-12 response or signaling related transcriptional signatures. Interestingly, such patterns were also evident in the same populations after BT, regardless of the previous response status of the donor. Together, patients with MM exhibited ambiguous humoral immune responses but sufficient T-cell responses against the WT strain after three doses of mRNA-based vaccination against SARS-CoV-2. At single cell resolution, vaccination response and BT were associated with cytokine-responsive patterns in peripheral immune compartments that may be beneficial for the design of improved and variant-adapted vaccination strategies in such immunocompromised patients.

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Correlation of peripheral blood immune markers with complete pathological response in a clinical trial of neoadjuvant chemoradiotherapy combined with dual immunotherapy followed by resection in locally advanced NSCLC patients.

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Non-small cell lung cancer (NSCLC) comprises approximately 80-85% of all lung cancer patients. Patients with locally advanced NSCLC have a 5-year overall survival of approximately 30%. The aim of the INCREASE trial was to explore the addition of dual immunotherapy for a subgroup of patients with locally-advanced NSCLC who were eligible for induction chemo-radiotherapy, followed by surgical resection. A total of 29 enrolled patients underwent standard neoadjuvant chemoradiotherapy (nCRT) combined with concurrent Nivolumab-ipilimumab, of which 25 underwent surgical resection. Immunomonitoring was performed in order to determine if peripheral immune activation correlated with pathological response.
Peripheral Blood Mononuclear Cell samples from three time points, (i.e. at baseline, after neoadjuvant treatment prior to surgery, and 12 weeks after surgery) were processed and extensive multicolor flowcytometry analyses were performed to monitor immune activation and correlate findings to pathological response at surgery and clinical outcome.
The neoadjuvant treatment enhanced frequencies in peripheral blood of proliferative $CD8^+$ and Thelper cells, but not of proliferative regulatory T cells (Tregs). Treatment increased on-treatment frequencies of potentially tumor-reactive CD39+ CD8+ T cells and T helper cells. Patients with a pathological complete response (pCR) had a higher ratio of activated CD8+ T cells over activated Tregs, as determined by Ki67+, CD25, and HLA-DR expression on these cell subsets. At baseline, patients with a pCR displayed lower expression levels of the PD1, CTLA-4, TIGIT, and Lag3 immune checkpoints, indicative of a lower T cell exhaustion state. Furthermore, non-pCR patients expressed lower levels of PD-L1 on B cells and an increased expression of CD39+ on activated Tregs.
Overall, we found that the addition of dual immunotherapy to standard neo-adjuvant CRT, increased immune cell activation in blood when compared to baseline, and that patients with a pCR showed a more favorable ratio of effector to Treg, and a less exhausted phenotype at baseline. INCREASE patients are undergoing follow-up and immunomonitoring results will be correlated with patterns of disease recurrence.

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High frequencies of peripheral Vδ1 T cells are associated with poor overall survival of melanoma patients undergoing PD-1 blockade and a late-differentiated phenotype

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The global incidence of melanoma, the deadliest form of skin cancer, has risen rapidly since the mid-1950s. Although immune checkpoint therapy using the Programmed Cell Death Protein (PD)-1 antibodies nivolumab and pembrolizumab significantly improved the survival of melanoma patients with distant metastasis, prognosis remains poor. About 20% of γδ T cells express PD-1, making this numerically minor population of unconventional T cells a target of checkpoint blockade. Interest in γδ T cells is growing rapidly due to their diverse functions and involvement in tumor immunity, immunological disorders and infectious diseases. Unlike αβ T cells, γδ T cells are not HLA-restricted, and their ligands and mode of antigen-recognition are not yet well characterized. The γδ T cell population encompasses heterogeneous subsets with the Vδ2 subset dominating in the peripheral blood, while Vδ1 and Vδ3 T cells are more prevalent in tissues. In previous studies, we have shown that higher frequencies of Vδ1 T cells in the peripheral blood were associated with poor overall survival of metastatic melanoma patients. Our current investigations within the framework of the DFG FOR2799 consortium confirm this correlation in three cohorts encompassing 186 patients. A high Vδ1 frequency is associated with a differentiation profile dominated by late-differentiated cells and higher frequencies of putatively replicative senescent Vδ1 T cells. Under immune checkpoint blockade we observed a decline in the frequency of this Vδ1 T cell subset and an increase in TRDV1 repertoire diversity, suggesting an expansion of other Vδ1 T cell subsets. TCR sequencing of peripheral blood mononuclear cells and corresponding tumor-infiltrating lymphocytes showed a clonal overlap between these compartments and significantly decreased frequencies of Vδ1 cells with low proliferative capacity within the tumor compartment. In order to study the tumor infiltration of γδ T cells in more detail, we performed immunohistochemical staining of formalin-fixed paraffin-embedded tissue from metastases of late stage melanoma patients before the start of checkpoint blockade. The majority of studied metastases showed γδ T cell infiltration, albeit at low numbers per area. Taken together, our data indicate a phenotypic and functional alteration of γδ T cells in late stage melanoma patients and a possible contribution of γδ T cells to melanoma immunosurveillance. Currently we are establishing a high dimensional in situ proteomics imaging

approach in order to gain a deeper insight into the subset distribution, marker expression and spatial distribution of γδ T cells in the tumor microenvironment.

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Image-based deep-learning models enable accurate ELISpot experiment classification and evaluation

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Development and evaluation of enzyme-linked immunosorbent spot (ELISpot) assays to classify immune responses is sophisticated and time-consuming. A typical experiment consists of three to four *well groups*: Replicates of experiment wells, replicates of negative control wells, a positive control well and optionally a second set of experiment wells that needs to confirm each positive response. To evaluate an experiment, appropriate settings must be chosen to take the images and perform automatic spot counting with the ELISpot reader. Then, statistical methods are available to classify the response according to the observed spot counts in each of the well groups (Distribution free resampling (DFR)). Finally, a confirmation of the agreement between the statistical test and the images is required by an expert to make sure artifacts are appropriately accounted for. This results in a typical workload of 4-8 hours for the proper classification of an ELISpot experiment with 16 96-well plates and still contains a subjective component.
Deep learning models are well known for their efficiency on image classification tasks. Here, we developed such models together with elaborate data augmentation techniques to overcome both aforementioned limitations. First, well-borders are masked and data is augmented by applying perturbations. We developed two modeling approaches. In the first, we bundle all images belonging to an experiment and feed them into a Convolutional Neural Network (CNN). In the second, we built a custom architecture combining a CNN as feature extractor for individual well images and added one of three architectures: 1. A Fully Connected Network (FCN), 2. A Long Short Term Memory Network (LSTM), 3. A Vision Transformer (ViT) to fuse information across well groups, imitating an expert evaluating all experiment wells. The output of the models is a categorical distribution of size three, providing the probability for the classification as “no response”, “positive response”, or “not evaluable”.
The best accuracy was achieved by the combination of the CNN and LSTM with 98.12% and the best F1 score with 96.60% on the held-out test data set. When compared to implemented statistical tests, this results in a substantial gain of 13% in accuracy and 30% in F1-score and eliminates the need for manual review for the vast majority of cases. To explicitly identify the outlier cases that still require manual review, we apply Monte Carlo Dropout to compute the model’s predictive uncertainty at a threshold of 0.1. To streamline the analysis, we have implemented the models, data handling and evaluation into a Web application enabling seamless visual exploration of all relevant cases. This enables much faster evaluation of our reference experiment in only 1 hour instead of 4-8 hours.

Taken together, our models deliver highly accurate results much more rapidly than before and minimize the subjective component of the evaluation.

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Detection of HER2-positive extracellular vesicles in plasma from breast cancer patients using spectral flow-cytometry

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Breast cancer is classified based on the expression of estrogen / progesterone receptors and the human epidermal growth factor receptor HER2, which is the basis for the decision on personalized therapy e.g. endocrine and anti-HER2 therapy, respectively. This classification requires tissue obtained through biopsies for immunohistochemistry and molecular analysis to determine the hormone receptor and HER2 expression status. HER2 targeting therapies are important instruments in treating breast cancer patients and have demonstrated to significantly improve survival. However, only 20-25 % of tumors do express HER2. In the course from cancer diagnosis to metastatic disease, the tumor biology and the mutational profile might change. Therefore, to verify that the correct treatment is indicated in the metastatic disease, tissue biopsy of metastatic lesion is required but in case of brain metastasis not feasible. Analysis of the tumor biology and current phenotype through exosome diagnostics from blood („liquid biopsy“) could solve the problem of this high unmet medical need to determine the HER2 status. Here, we demonstrate that spectral flow cytometric analysis of individual extracellular vesicles from peripheral blood of patients can be used to detect HER2 expression by breast cancer and allowed us to sensitively determine HER2 expression on single EVs from breast cancer cells under defined conditions *in vitro* and to determine their absolute numbers. In a proof-of-concept clinical study in breast cancer patients, spectral flow cytometric analysis of patient plasma samples at the time of cancer diagnosis detected and quantified the frequencies of circulating $HER2^{+}$EVs with high sensitivity.
This new method bears the promise to provide important information on breast cancer expression of hormone receptor and HER2, especially in situations where tissue is not readily available, and could emerge as a novel approach, to monitor breast cancer evolution over time and to tailor personalized molecular therapies.

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Single cell multiomic profiling of the antigen-specific immune response using antigen specific dCODE Dextramer® (10x) reagents and 10x Chromium Single-Cell Analysis System

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Background:Understanding the specific T- and B- cellular immunity during an induced cellular immune response is important for development of anti-tumor immunity in personalized immunotherapy. Advanced single-cell genomics technologies have enabled researchers to do single cells immune profiling, by assessing cell surface proteins, the transcriptome and TCR and/or BCR gene clonotypes. However, understanding antigen-specific recognition at the immune synapse is key to understand the specific immune response in cancer. The dCODE MHC Dextramer® technology combine single-cell genomic profiling with antigen-recognition allowing deep analysis of antigen-specific T-, and B-cells at the single cell level pairing TCR/BCR recognition adding to unveil the antigen specific immune response, in cancer and infectious diseases.
Methods: We have combined two powerful technologies, Immudex® dCODE Dextramer® (10X) reagents and the 10x Chromium Single-Cell Analysis System, to detect and characterize low-frequency antigen-specific T- and B cells, including the full sequences of the V(D)J gene segments of the antigen-specific T- and B cell receptors, as well as profile transcriptome and cell phenotyping by surface protein expression. We used a panel of dCODE Dextramer® (10) reagents directed against virus-specific antigens and negative control reagents to profile two HPBMC samples.
Results: We identified virus-specific B-cell responses over the two samples and revealed major clonotypes of all responses in the two samples, alongside with gene expression of the antigen-specific cells identified by the dCODE® (10x) Dextramer reagents. Data on antigen-specific T-cell responses is pending.
Conclusions: Here we show an assay with the ability to analyze antigen-specific immune cells, by applying combined antigen-specific detection, with surface phenotyping, and gene expression, resulting in a deep phenotypic characterization of the immune cells in the donor. The ability to do high-resolution B and T cell profiling has broader implications and utility in immuno-oncology, infectious diseases, and autoimmunity.

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An ultra-high-throughput screen for the evaluation of peptide HLA-Binder interactions

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Characterizing the binding of molecules to pHLA complexes is of great importance for the identification and safety of new T cell- based therapeutics. We have developed an ultra high throughput pHLA binding screen (named ValidaTe) which can analyze more than 5.000 binding interactions in a single measurement. One of the core components of this screen is a next generation microarray consisting of pHLA complexes. With help of the highSCORE (single color reflectometry) device, the binding properties of a therapeutic T cell receptor (TCR) was characterized in a fast and

efficient way. Moreover, we were able to demonstrate a good correlation to a dataset obtained by conventional bio-layer interferometry (BLI) measurements.

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Abstract has been withdrawn

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Epitope-specific expansion cultures with subsequent TCR identification (ESPEC-SUIT) – a platform for efficient discovery of (tumor) antigen-reactive TCRs from both CD4 and CD8 T cells

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The antigen specificity of a T cell is determined by the sequence of its T cell receptor (TCR), which is inherited by daughter cells after T cell activation and subsequent proliferation. Determination of the TCR sequence is therefore the key to tracing individual T cell clones through time and space and to gaining insight into their reactivity, affinity and history of expansion.
Epitope-specific expansion cultures (ESPEC) make use of short-term peptide-driven *in vitro* re-stimulation of *in vivo* primed T cells, to enrich for T cells with relevant antigen specificity. Post-ESPEC functional readouts such as ELISpot are used to confirm expansion of cells reactive to the antigen-of-interest but not to control antigens.
Subsequent TCR identification (SUIT) selects TCRs for functional validation based on TCR repertoire sequencing of peptide stimulated, unstimulated and pre-expansion samples. To facilitate analysis, T cell subpopulations can be enriched based either on their expression of surface markers such as CD4/CD8, or their cytokine production upon re-stimulation.
We performed ESPEC for >20 patients with various tumor types and see a clear narrowing of the repertoire in peptide stimulated cultures, accompanied by significant clonal expansion of a subgroup of TCRs compared to unstimulated and baseline samples.
For n=11 patients we also performed SUIT and of >500 candidates we selected 243 top-expanded TCRs for functional validation, revealing on-target reactivity for 40-100% of TCRs, depending on the antigen and the number of TCRs included in the screen. Most TCRs included in this study are specific for recurrent or private neoepitopes and were retrieved from patients receiving neoepitope vaccination, mostly as short or long peptide vaccine formulations. However, we have discovered CD4 and CD8 T cell-derived TCRs from unvaccinated patients and found the ESPEC protocol to also enrich for relevant specificities when tumor-infiltrating lymphocytes were used as starting material. When traced to tumor tissue, post-ESPEC expanded cells show a distinct gene expression signature shared with validated tumor-reactive clones, confirming the suitability of the assay for discovery of tumor-specific TCRs.

Ongoing scale-up of both TCR cloning and screening capacity will increase the amount of TCRs that can be tested per patient and will thus allow an even deeper insight into the *in vivo* behavior of clonotypes with known specificity for individual antigens or targeting multiple epitopes with distinct qualities.

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Using the whole spectrum - five colour EliSpot for more detailed immunomonitoring

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Laboratory diagnostics evolved in the last decades and become an indispensable part in personalised medical treatment. Even new diagnostic fields like companion diagnostics arose. From a primarily visual evaluation of assay results, computer-assisted digitalisation and automatic evaluation become normal in high-throughput analyses in routine laboratories in these days. Furthermore, technological progress is only possible as a combination of laboratory tests, reader devices and software tools for evaluation to guarantee diagnostically conclusive results.
In order to meet all these challenges a new five colour EliSpot assay system has been develop for the simultaneous detection of Interferon gamma (IFN-γ), Interleukin-2 (IL-2), IL-6, IL-17A and Tumor necrosis factor alpha (TNF-α) on single cell level. The secretion of the pro-inflammatory cytokine IFN-γ, produced by activated type 1 T helper cells and also NK or cytotoxic T cells, is indicating an active ongoing infection. IFN-γ activates macrophages and induces the expression of MHC-II-complexes on professional antigen presenting cells. IL-2 activates the proliferation of lymphocytes and promotes their differentiation to effector and memory T cells. It also prevents differentiation into IL-17 secreting Th 17 cells. The cytokine IL-6 is connected with autoimmunity or chronic inflammation and is responsible for the acute phase response. IL-6 is produced by a variety of cell types, even those not associated with immunological function like muscle cells. IL-17A is also a pro-inflammatory cytokine linked to localised chronic inflammation and autoimmunity. The secretion of the classical pro-inflammatory cytokine TNF-α is associated with the induction of fever and the alerting of other cells of the immune system.
Especially for the detection of rare events like IL-17A secreting t cells in general or even more in combination with other cytokines, the EliSpot clearly outperform other assay systems with its superior sensitivity of 1 cell out of 200000 PBMCs. Additionally, to gain the maximal scientific output of this multi cytokine assays a new software version for the evaluation of results has been develop. The new count combination function of the AID reader software 8.1 allows the individual user specific combination of different cytokines via logical operators for a more precise characterisation of different cell populations.

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Positive Control Peptide Pools for T cell Immune Monitoring

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Immune monitoring of T-cell responses has a central role in the development of immunotherapeutics and vaccines. The platforms used for this purpose mainly include ELISpot, Interferon-gamma release assays (IGRAs) and Intracellular Cytokine Staining combined with Flow Cytometry (ICS-FC). All of these are activation-dependent and as such require positive stimulation controls indicating correct assay performance. Compared with conventional positive assay controls based on unspecific polyclonal stimulation (*e.g.* PHA/Ionomycin), peptide pools have the advantage of providing a natural TCR-mediated signal. A range of frequently used positive control pools, containing a limited number of epitopes from selected pathogens, are described in the literature, named e. g. EF (EBV, Flu), CEF (CMV, EBV, Flu) and CEFT (CMV, EBV, Flu, Tetanus) peptide pools.
We recently introduced an extended positive control pool called 'CEFX' (CMV, EBV, Flu + many others). CEFX contains 176 peptides, including numerous CD4 T-cell-stimulating peptides, all carefully selected from 17 different organisms to provide broad HLA-coverage. Cross-comparison revealed that this positive control pool provides much stronger and more universal stimulation than previous ones and was successfully used on all main assay platforms. Here we show results from CEFX stimulation in 648 donors, revealing strong and age-independent activation of T cells. 644/648 (99.4 %) of the donors showed a detectable response in ICS with a mean response of 627/648 (96.8 %) and 620/648 (95.7 %) of CD4 and CD8 T-cells, respectively.
Because the SARS-CoV-2 pandemic, combined with a global vaccination campaign, has induced strong T cell immunity against this virus in a majority of the world's population, we have now further extended the CEFX pool by a number of strongly T-cell stimulating SARS-CoV-2-derived peptides based on published sequences. The new positive control peptide pool called CEFSX (containing selected peptides from CMV, EBV, Flu, SARS-CoV-2, + many others) shows even better stimulation than CEFX and probably represents the most effective and up-to-date peptide-based control pool for T cell immune monitoring to date.

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In vitro expansion of Wilms' tumor protein 1 epitope-specific primary T cells from healthy human peripheral blood mononuclear cells

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The nearly universal tumor-associated antigen (TAA) Wilms' tumor protein 1 (WT1) is overexpressed in a variety of solid and hematological cancers. However, as a self-antigen also expressed in healthy tissues, T-cell clones of high affinity against self-antigens are usually eliminated after negative selection in the thymus. Therefore, frequency of high-affinity T-cell receptors (TCRs) towards TAAs in circulating T cells is low.
Here, we provide an *in vitro* T-cell expansion protocol to generate $WT1_{37-45}$-specific TCRs using HLA-A2 healthy donor peripheral blood. Purified $CD8^+$ T cells are expanded in two rounds in vitro stimulation (IVS). For the first IVS of 8 days, autologous dendritic cells (DCs) are used, differentiated from freshly isolated $CD14^+$ monocytes and after maturation pulsed with immunodominant HLA-A2-restricted $WT1_{37-45}$ epitope. After priming of the naïve $CD8^+$ T cells, irradiated autologous peptide-pulsed $CD14^-$ and $CD8^-$ depleted peripheral blood lymphocytes (PBLs) are used for the second IVS of 8 days. Expanded WT1-specific T cells are detected with fluorochrome-labeled WT1-specific tetramers. An increase of $WT1_{37-45}$ tetramer positive T cells can be expected after the 2nd IVS. Subsequently, expanded T-cell clones can serve as starting material for downstream assays such as (i) assessment of epitope-specific T cell reactivity, (ii) TCR repertoire sequencing or (iii) (single-cell) sorting to generate T-cell clones for discovery of epitope-specific TCR sequences for e.g., development of TCR-engineered cell therapy.

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High dimensional analysis of peripheral blood mononuclear cells in AML patients shows a beneficial tumor reactive T-cell environment at start of treatment in patients responding to an allogenic leukemia-derived cancer vaccine (vididencel)

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Priming the immune system to eradicate or control residual disease could be an effective strategy in AML maintenance therapy. In this phase 2 study (ADVANCE-II, Clintrials.gov: NCT03697707) AML patients in first complete remission (CR1), with measurable residual disease (MRD), and ineligible for allo-transplant, were treated with an allogenic leukemia-derived dendritic cell vaccine (vididencel). AML patients who are in CR but with MRD are at high risk to relapse. Here we analyzed circulating immune cells at start of treatment to investigate intrinsic differences in the immune system, which could have an impact on clinical outcome.
A total of 20 evaluable AML-patients received 4 biweekly intradermal injections with vididencel, followed by 2 booster injections at week 14 and 18. MRD was assessed at baseline, week 14, 20 and 32. Peripheral blood was drawn on day 1, 3, 42, 44, week 11, 18, 20 and 32 and peripheral blood mononuclear cells (PBMCs) cryopreserved. Multiparametric flow cytometry (40 marker panel; Cytek 5 laser Aurora) was performed, followed by high dimensional reduction using FlowSom.
Seven out of 20 patients showed a MRD response with five patients showing MRD conversion (to

MRD negative), and two with a reduction of at least 1Log10 in MRD level. Seven patients remained in CR with MRD positive status and only six patients relapsed.
Specific T-cell responses to tumor antigens like WT1, PRAME and RHAMM, also endogenously expressed in vididencel, were measured by IFNy ELISPOT and observed in almost all patients, showing that DCP-001 is able to induce specific T-cell responses (ASH2022 #713). Patients who remained in CR or even achieved a MRD negative status, had higher levels of circulating cDC1, cDC2, B-cells as well as relative higher number of $CD4^+$ $CD161^+$ effector memory (EM) , $CD4^+$ $KLRG1^+$ and $CD4^+$ $PD\text{-}1^+$ $ICOS^+$ T-cells, while having lower levels of $CD8^+$ central memory (CM) T cells, $CD8^+$ $LAG\text{-}3^+$, $CD8^+$ $CD161^+$ EM and $CD4^+$ $LAG\text{-}3^+$ T-cells compared to the patients who relapsed in the first 32 weeks of the study.
In conclusion, analysis of baseline immune cell composition in these intensively treated AML patients indicates that, patients with a lower frequency of T-cells with inhibitory markers (such as $CD8^+$ LAG-3) and a higher proportion of both DCs and tumor reactive T-cells, are more likely to have a better clinical response after vididencel treatment.

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Intradermal tilsotolimod versus placebo as adjuvant treatment in patients with stage II pT3-4/cN0 melanoma: interim efficacy and safety results of the randomized phase II INTRIM study.

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Background: Adjuvant immunotherapy is currently moving into earlier stages of melanoma. CPG7909, a Toll-like receptor-9 (TLR9) agonist, injected at the primary tumour excision site in patients with clinical stage I/II melanoma, was found to boost loco-regional and systemic anti-melanoma immunity. (Koster *et al.* Clin Cancer Res 2017) In an exploratory analysis this was accompanied by a significant decrease in tumour-positive sentinel lymph node (SLN) rates and improved recurrence-free survival (RFS). To confirm these results we performed a randomized,

placebo-controlled and double-blind phase II study of TLR9 agonist tilsotolimod (IMO-2125) in patients with stage II pT3-4/cN0 melanoma.
Methods: Patients with pT3-4/cN0 melanoma, scheduled to undergo wide local excision and sentinel node biopsy (SNB), were randomized 1:1 to a single intradermal injection of 8mg of tilsotolimod vs. placebo at the primary tumour excision site, 7-10 days prior to surgery. In twenty patients, blood and SLN sampling was carried out for immune monitoring.
The primary endpoint was SLN tumour status; secondary endpoints were SLN and peripheral blood immune status, RFS and overall survival (OS) after 2, 5 and 10 years.
Results: From January 2020 to February 2022, 108 patients received IMO-2125 (n=54) or placebo (n=54). At interim analysis, the primary endpoint had been reached[SC1] and enrollment was stopped, i.e. tilsotolimod-administered patients had a significantly reduced tumour positive SLN rate compared to placebo-treated patients (13% vs. 45%, P<0.0001), exceeding the pre-specified P-value of significance of 0.008. Notably, more patients in the placebo group had ulcerated melanoma, but stratified analysis for ulceration showed a similar trend. Local injection of tilsotolimod was mainly associated with grade-1 and -2 injection site reactions (54%), accompanied by transient grade-3 confusion in one elderly patient. Preliminary immune monitoring data show the expected type-I IFN response in peripheral blood and activation of plasmacytoid dendritic cells in the SLN upon tilsotolimod administration. Further analyses are ongoing: Conclusions: Single-dose tilsotolimod is reasonably safe and significantly reduces tumour positive SLN rates in pT3-T4 melanoma patients. Provided that SLN status accurately reflects future RFS, tilsotolimod has the potential to improve outcomes of early-stage melanoma patients at relatively low cost and toxicity.

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High-dimensional *in situ* proteomics imaging to assess γδ T cells in human tissues

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The role, function and orchestration of γδ T cells and their heterogenous subsets in human tissues is largely unknown. This involves immunity per se, but also their role in physiological and particularly malignant processes. These unconventional T cells that lack MHC-restriction and whose ligands remain undefined for the most part, bridge adaptive and innate immunity. This fact, as well as their involvement in the immediate early immune response and their powerful cytotoxic potential, make them highly promising particularly for exploitation in new immune therapy strategies. However, it remains unclear which cell subsets are ultimately responsible for the desired effects, as for example in cancer also pro-tumor functionalities are described for certain phenotypes.
As a first step towards closing this gap, we here describe a high-dimensional *in situ* proteomics

analysis approach to explore the spatial orchestration of human tissue-resident or -invasive γδ T cell subsets in the local microenvironment at single cell resolution. To this end, we use the MICS (MACSima imaging cyclic staining) technology, which allows examining up to 150 markers on a single tissue section. The basis of the MICS technology is immunohistochemistry visualizing structures of interest using fluorescence-labeled antibodies. For the here presented approach, we have identified and established commercially available antibodies specific for the Vδ1, Vδ2 and Vγ9 chains of the γδ T cell receptor (TCR) that allow the assessment of the vast majority of tissue resident γδ T cell subsets, when combined with a pan-γδ TCR specific antibody. Further, status and function-related conclusions can be drawn through the complementary determination of defined T cell differentiation, proliferation or activation markers. A selection of these markers, paired with additional lineage-specific immune cell and tissue-specific markers, enable a classification by means of spatial orchestration of the studied tissues. Using unbiased bioinformatic analysis pipelines, we are not only able to assign the individual γδ T cell to a precise phenotype but may also draw conclusions concerning complex biological processes, such as cancer rejection, in the investigated tissues through the cellular neighborhoods that can also be assigned to detailed cellular phenotypes. The pipeline presented here will serve as a key technology in our DFG research unit 2799 “Receiving and Translating Signals via the γδ T Cell Receptor” to study the role of γδ T cells under immune checkpoint blockade in cancer patients.

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The CAR forms individual synapses independently of the endogenous TCR, however, the TCR has crucial relevance in maintaining CAR T cell functionality.

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CAR and TCRs use the same downstream signaling machinery in the engineered T cell for activation. It is unresolved whether CAR and TCR mutually cross-activate upon engaging their respective antigen. Here we demonstrate that upon activation, the CAR did not induce phosphorylation of TCR associated CD3ç and, vice versa, indicating that the CAR and TCR did not cross-signal to trigger T cell effector functions. On the membrane level, TCR and CAR formed separate synapses upon antigen engagement as revealed by total internal reflection fluorescence (TIRF) and fast AiryScan microscopy. Upon engaging their respective antigen, however, CAR and TCR could co-operate in triggering effector functions through combinatorial signaling allowing logic “AND” gating in target recognition. Data have major implication as tonic signaling through the physiologic TCR/CD3 complex can support CAR-mediated T cell activation. The endogenous TCR likely has crucial relevance for maintaining CAR T cell functional capacities in the long-term.

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Colon cancer cells treated with mastic essential oil release damage-associated molecular patterns (DAMPs) characteristic of immunogenic cell death

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The identification of immunogenic cell death has challenged the classical idea that programmed cell death is unable to elicit adaptive immune responses. Cancer cells dying in this manner prove immunogenic, due to a combination of tumor cell antigens and DAMPs released from dying cancer cells that act as potent immunostimulators. Herein, we evaluated the ability of mastic essential oil to induce immunogenic cell death in colon cancer cells of human and murine origin. To this end, HT29 and CT26 cells were treated with 0.02 % (v/v) of essential oil for up to 24 hours and various DAMPs, associated with immunogenic cell death were evaluated. Intriguingly, in both cell lines exposure of calreticulin, an endoplasmatic reticulum chaperon, on the outer layer of the cytoplasmic membrane was detected using flow cytometry, with a variation in the time of maximum exposure. Similarly, exposure of heat shock proteins was also detected in treated cancer cells. In addition, the translocation of high mobility group box 1 (HMGB1) protein, outside the nucleus was assessed with Western blot and was found to be increased when cancer cells were incubated in the presence of the essential oil. Interestingly, this effect was found to be time-dependent, for up to 24 hours of

treatment. Finally, the gene expression of type I interferons was estimated using real-time PCR. It was found that expression of interferon alpha 2 and interferon beta was elevated in CT26 and HT29 cells, also in a time-dependent manner and beginning as early as 12 hours post treatment with mastic essential oil. All of the above observations have been linked with the emergence of immunogenic cell death. Our findings suggest that treatment with mastic essential oil induced the release of DAMPs, capable of eliciting adaptive immune responses against dying cancer cells. Therefore, this plant-derived extract may hold promise as an alternative, adjuvant anticancer agent. Further studies are underway in order to confirm these results and also if mastic oil-treated cancer cells can induce tumor-specific adaptive immune responses and that the effect is transferable in in vivo models. This project has received funding from the Hellenic Foundation for Research and Innovation (HFRI) and the General Sekretariat for Research and Technology (GSRT), under grant agreement HFRI-FM17C3-2007.

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Deciphering the metabolic capabilities of precursor exhausted T cells (T_{pex}) to identify novel immunotherapeutic approaches

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Tumor-specific CD8 T cells have the potential to control tumor growth but often acquire a state of exhaustion due to prolong stimulation within the tumor microenvironment (TME). The limited capacity for rejuvenation of T cells exhaustion in tumors hampers current immunotherapies and fails to achieve long-term responses. In line with this, it became clear that exhausted T cells comprise a series of T cell states with multiple phenotypic trajectories, functional traits, and transcriptional programs, ranging from precursors of exhausted T cells (Tpex) to terminally differentiated exhausted T cells. With more understanding of the pivotal role of Tpex in the maintenance of tumor-reactive CD8 T cells and their superior response to immune checkpoint blockade (ICB)therapy, Tpex has garnered tremendous attention as a major therapeutic target for immune interventions. Nonetheless, it needs to be underlined that we are still far from fully understanding Tpex distinct cellular dynamics, developmental pathways, and molecular mechanisms. While more research is needed to understand Tpex biology, we developed a human antigen-specific ex vivo model for CD8 T cell exhaustion, representing the heterogeneity of exhausted T cells in TME. By taking advantage of the single-cell immunometabolism and transcriptome profiling technique, we delineated that Tpex from our ex vivo platform mirroring the Tpex features in tumors resected from cancer patients. Our platform allows us to investigate the specific pathways in the development and maintenance of Tpex in patients and may lead to the discovery of novel Tpex-based therapeutic strategies in the fight against cancer.

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CRISPR/Cas9 gene editing of immune checkpoint receptor NKG2A improves the anti-leukemic efficacy of primary CD33-targeting CAR-NK cells

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Recently, we reported on the successful generation of primary CD33-targeting chimeric antigen receptor (CAR; CAR33)-NK cells, which are highly effective against acute myeloid leukemia (AML) *in vitro* as well as in AML-xenograft mouse models (Albinger et al., *Blood Cancer J* 2022). Yet, NK cell function can be impaired by high levels of the inhibitory immune checkpoint receptors such as NKG2A (natural killer group 2A) expressed on NK cells (Bexte et al., *Oncoimmunology* 2022). By applying CRISPR/Cas9 gene editing to knockout (KO) the *killer cell lectin like receptor C1* (*KLRC1)* gene, we could significantly reduce NKG2A cell surface expression and thereby improved CAR33-NK cell functionality *in vitro* and *in vivo*.
CD33-targeting CAR-NK cells were generated by lentiviral transduction of peripheral blood-derived (PB-)NK cells. Nucleofection with CRISPR-Cas9 technology was used to KO the NKG2A-encoding *KLRC1* locus in CAR33-NK cells. The CAR33- and NKG2A-expression as well as cytotoxicity were analysed using flow cytometry and IncuCyte® after feeder cell-free, IL-15/IL-2-based expansion. The *in vivo*-efficacy was evaluated in OCI-AML2 (GFP^+, Luc^+) xenografted NSG-SGM3 mouse models. Lentiviral transduction of PB-NK cells resulted in up to 60% CAR33-positive cells, while *KLRC1* gene disruption resulted in 50% reduction of NKG2A cell surface expression. CITE-Seq and qPCR analysis revealed a distinct gene regulation pattern in CAR33- and CAR33-*KLRC1*^{ko}-NK cells. Upregulation of CXCR4, CD16 and CD70 in CAR- and CAR-KO-NK cells indicated a mature and activated NK cell state. CAR33-*KLRC1*^{ko}-NK cells showed significantly higher elimination of $CD33^+$/$HLA\text{-}E^+$ OCI-AML2 cells as well as primary patient material in *in vitro* cytotoxicity assays compared to *KLRC1*^{ko}-NK or CAR33-NK cells. Furthermore, a reduction of leukemic burden was observed *in vivo* following a single injection of a low dose ($3x10^6$ cells) of CAR33-*KLRC1*^{ko}-NK cells compared to CAR33-NK cell treatment in an NSG-SGM3 AML-xenograft mouse model. Two injections of $3x10^6$ CAR33-*KLRC1*^{ko}-NK cells each showed superior efficacy compared to *KLRC1*^{ko}-NK or CAR33-NK cell treatment and led to a complete elimination of AML and leukemia-initiating cells in the bone marrow, which was confirmed by bone marrow re-engraftment analysis. Application of CAR33-*KLRC1*^{ko}-NK cells *in vivo* appeared to be safe and did not induce any side effects in line with histologic analysis of lung, liver and colon.
Removing an inhibitory receptor in CAR-NK cells induced an anti-leukemic NK cell phenotype which showed a highly beneficial effect for the treatment of AML. This double genetic modification has the potential to enable NK cells to bypass inhibition following contact with malignant cells not only in context of AML, but also in a broad range of other malignant diseases.

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T cell therapy drives inter- and intraclonal loss of mutant p53 heterozygosity in cancer

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In this study, we use a murine fibrosarcoma (Ag104A) that arose spontaneously in an aging C3H mouse to analyze neoantigen-specific T cell therapy using T cell receptor-engineered T cells (TCR-Ts). The tumor cell line generated from the autochthonous Ag104A tumor was never cloned, engineered, or cultured for an extended period. Accordingly, all neoantigens in Ag104A are naturally expressed and established tumors reflect the heterogeneity of the autochthonous cancer. We employed next generation sequencing to decipher the genomic landscape of Ag104A on single cell level. Function of neoantigen-specific TCR-Ts was analyzed in cell culture experiments and *in vivo* using the original Ag104A tumor cell line and derivating clones. A tumor-specific TCR (M2/3) was isolated from mice immunized with Ag104A cancer cells. The target of M2/3 TCR-Ts was determined using tandem minigenes and was identified as an H-2K^k-presented peptide derived from mutant *Trp53* (mp53). T cell therapy with M2/3 TCR-Ts resulted in growth arrest of established Ag104A tumors that otherwise progressed rapidly. However, all tumors relapsed 4-5 weeks after treatment and escaping tumors lost mp53 on genomic DNA, while wild-type p53 was retained. Single-cell sequencing of the relapsed variants and the original Ag104A tumor cell population revealed that M2/3 TCR-Ts eliminated all mp53-expressing cancer cells and selected a population of tumor cells that expressed only wild-type p53, consistent with as little as 0.2% of the original tumor cell population. Interestingly, M2/3 TCR-Ts also induced escape variants of a clonal Ag104A tumor that, like the majority of all Ag104A cancer cells, showed a heterozygous expression pattern for p53/mp53. Sequencing of such recurrent variants revealed loss of heterozygosity due to a deletion in a copy of chromosome 11 that encoded for mp53. Here, we show that while mp53-specific TCR-Ts efficiently eliminate all tumor cells carrying the mutation, the success of therapy is significantly compromised by inter- and intraclonal loss of heterozygosity of the targeted p53 mutation. Our results suggest that only neoantigens that already exhibit loss of heterozygosity can serve as rejection antigens.

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Activation of tumor reactive lymphocytes by shingosine-1-phosphate

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T-cells can mediate long-term and clinically relevant anti-tumor directed immune responses. They are activated through their nominal T-cell receptor (TCR), associated with the CD3 complex followed by phosphorylation of the TCR zeta protein. This triggers a cascade of signaling events resulting in reprogrammed gene expression, proliferation and immune effector functions, categorized by Th1/Th2/Th17 responses. Within the T-cell population, there is a more specialized group - Tumor Infiltrating Lymphocytes (TILs) – can not only recognize autologous tumor cells but have an increased potential to enter and persist in tumor tissue.
Sphingosine-1-phosphate (S1P) is a signaling sphingolipid, produced by sphingosine kinases, and reversibly degraded by S1P phosphatases, or irreversibly by S1P lyases. S1P is recognized by a G-protein coupled receptor family (S1PR1-5), that signals in an extracellular or intracellular manner, in a duality known as “inside-out signaling”. S1P is a potent modulator of cellular signaling and differentiation – and has been used as prognostic marker for survival in patients with cancer. S1P function and S1P receptor expression were analyzed in Peripheral Blood Mononuclear Cells (PBMCs) and TIL obtained from patients with pancreatic ductal adenocarcinoma (PDAC).
TILs from PDAC (n=11) were stimulated with 250nM S1P and maximal TCR stimulation using a human α-CD3 monoclonal antibody (4 groups: S1P+ α-CD3/ S1P/ α-CD3/ nil). S1P resulted in a significantly higher (p=0.0007) number of CD3 molecules per T-cell and a significantly higher (p=0.0013) internalization of the sphingosine-1-phosphate receptor 1 (S1PR1). In contrast, stimulation of TILs with α-CD3 was associated with a significantly higher (p=0.0080) internalization of S1PR1. The co-stimulation of TILs with both S1P and α-CD3 resulted in a significant higher (p=0.0007) internalization of the S1PR1.
PBMCs were primed with 250nM of S1P for 30 minutes - which resulted in upregulation of TCR molecules per T-cell – and were then by incubated with 60 different tumor associated antigens for 7 days (in the absence of S1P). This resulted in a different quality of T-cell recognition of commonly shared tumor-associated antigens, defined by IFN-γ, IL-17A and IL-4 production.
These results suggested a close association between CD3 and S1PR1 expression, and a S1PR1 mediated signaling in T-cell activation and antigen recognition. Since a higher expression of CD3 molecules on the cell surface has been associated with a lower activation threshold, S1P may represent a useful modulator to increase T-cell activation against transformed cells. This was supported by an increased quality and quantity of T-cell recognition of commonly shared tumor targets in PBMCs after S1P priming.
In summary, S1P seems a viable immune modulator to increase T-cell precursor reactivity directed against cancer–associated target molecules in active cellular therapies.

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Bcl-2-shRNA carrying exosomes isolated from engineered NK cells cause tumor regression in a triple negative breast cancer mouse model

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Evasion of apoptosis is one of the hallmarks of cancer, and it can also promote therapy resistance. Silencing anti-apoptotic genes, such as *Bcl-2*, in tumors can be an effective gene therapy strategy. Exosomes are lipid bilayer enclosed extracellular vesicles that have been recently gaining popularity as biocompatible and efficient gene delivery vectors. The contents of the exosomes is representative of its cellular origin, and it is known that Natural Killer (NK)-derived exosomes contain pro-apoptotic molecules such as FasL and TRAIL.Thus, the apoptosis inducing effect of NK-derived exosomes can be combined with their gene-carrying capacity to induce an effective apoptotic response. We have previously isolated and characterized the exosomes derived from engineered NK92MI cells that have been lentivirally transduced with Bcl-2 shRNA. We have also shown that these exosomes can successfully carry the shRNA into human breast cancer cell lines in vitro, leading to induction of apoptosis. In this study, we tested the anti-tumoral effects of these exosomes in in vivo settings. We generated a xenograft triple negative breast cancer model in athymic mice and administered intratumorally Bcl-2 shRNA-containing NK exosomes biweekly for a total of 5 times. At the end of the study, the tumor diameter increased by 50% in the group treated by exosomes derived from scrambled-shRNA transduced NK cells, whereas tumor diameter decreased by 65% in the group treated with Bcl-2 shRNA containing NK exosomes. The silencing of Bcl-2 has been demonstrated by immunohistochemical staining of the tumor tissues and apoptosis induction (both intrinsic and extrinsic pathway) has been shown by TUNNEL staining and qRT-PCR. With this study, it has been shown for the first time that shRNAs in transduced NK cells can be successfully loaded into exosomes and that these exosomes are an efficient shRNA carriers in in vivo settings. Since Bcl-2 expression is increased in many cancer types, exosomes from engineered NK cells can be used in other cancers (e.g. colon and lung cancers). Thus, with this study, preliminary in vivo results have been generated for boosting the anti-tumoral effect of NK cell exosomes by a facile gene-loading approach. This research has been supported by The Scientific and Technological Research Council of Turkey, TÜBİTAK-SBAG-1001 (217S455)

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BAFF-R-specific chimeric antigen receptor T cells are efficient against murine model of mantle-cell lymphoma

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Chimeric antigen receptor (CAR) T cells targeting CD19 have proved to be an efficient immunotherapy against B-cell leukemia and lymphomas. However, some patients relapse with CD19-negative cancer cells and not all lymphoma types express CD19 to a high degree. There is therefore interest in identifying other targets for CAR therapy.
BAFF receptor (BAFF-R) is a TNF receptor superfamily receptor which plays an important role in B-cell

survival through its binding to the soluble ligand B-cell activating factor (BAFF). Lymphoma cell lines lacking BAFF-R have reduced growth potential *in vitro* and *in vivo*. Recently, BAFF-R-specific CARs have proved efficient in animal models. We thus find BAFF-R an attractive target for CAR therapy. We first examined BAFF-R expression in leukocytes and hematological cancers by single-cell RNAseq and spectral flow cytometry and found high expression in B-cell cancers, including mantle-cell lymphoma and follicular lymphoma. We generated monoclonal antibodies against BAFF-R and examined their reactivity against the human proteome and by using human tissue arrays. One BAFF-R antibody had little poly-reactivity towards other proteins and cells, and we generated a BAFF-R mono-specific CAR based on heavy and light chains of this antibody. By carefully examining immediate T cell activation, we found that calcium flux, CD69 expression, and NFkB, AP-1, and NFAT signaling was comparable between our BAFF-R specific CAR and an identical CAR specific for CD19. T cells transduced with BAFF-R-specific CARs performed similarly to CD19-specific CAR in terms of proliferation, cytokine production, and cytotoxic killing against multiple lymphoma cell lines *in vitro*. Importantly, BAFF-R CARs could kill cells that lack CD19 to the same degree as wildtype cancer cells. Using a mantle-cell lymphoma murine model we show that both BAFF-R- and CD19-specific CARs control tumor-growth.
Taken together, we have developed an efficient BAFF-R-specific CAR with the potential to work on its own or in combination with CD19-specific therapies.

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Key chemokine network modulation enhances CAR T cell therapy effects in human solid tumor explants

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Chimeric antigen receptor T cells (CAR) are a promising cellular therapy strategy, which has been shown successfully in hematological malignancies. In solid tumors, however, CAR infiltration remains a major obstacle. T cell infiltration and positioning is highly influenced by key chemokines like MIG, IP10 and fractalkine (FTK). In colorectal cancer liver metastases (CRC-LM), however, these chemokines are mainly concentrated in the invasive margin and not in the tumor epithelium (TE). Likewise, T cells are mainly observed in this region and not in the TE. We recently established a fully human tissue-based ex vivo cell migration analysis model, which successfully reproduces exogenous T cell infiltration and distribution in human tumor explants showing associations of infiltration with key chemokine concentrations. Here we show how CAR infiltration into the TE is enhanced by key chemokine network modulation. In brief, autologous CEA-CAR and CA125-CAR were manufactured from peripheral blood of a CRC-LM and an ovarian cancer (Ov-Ca) patient, labeled with a fluorescent dye and added to the appropriate patient explant model in the presence of combined neutralization of MIG, IP10 and FTK. After tissue processing, staining and imaging, CAR were quantified.

Surprisingly, key chemokine inhibition significantly enhanced CAR infiltration into the TE in both cancer entities despite decrease of MIG, IP10 and FTK concentrations. In comparison to control T cells (Mock), significantly more CAR infiltrated the TE under chemokine blockade underscoring that CAR could supersede simple T cells. Moreover, multiplex cytokine analysis still revealed much higher concentrations of the chemokines MIG, IP10, CCL5 and MIP1β accompanied by higher IL2, IL12, TNFα and IFNγ compared to Mock allowing CAR infiltration, activation and most likely engagement with the tumor. Interestingly, we observed almost similar cytokine and chemokine changes in CRC-LM and Ov-Ca. Indeed, the inhibited chemokines in this study have very important physiological functions and patient treatment would have crucial effects. Thus, we considered an alternative way of chemokine modulation. The oncolytic virus ParvOryx has shown efficacy, tolerability and safety in a phase 2 clinical trial and modulation of the tumor microenvironment including T cell infiltration was observed. In fact, combined treatment of CRC-LM and Ov-Ca explants with CAR and ParvOryx again showed enhanced CAR infiltration and higher MIG, IP10, MIP1β, IL2, IL12, TNFα and IFNγ concentrations compared to Mock treatments. In summary, our results show that key chemokine networks can be modulated by combined chemokine blockade or ParvOryx treatment leading to enhanced CAR effects in solid tumors.

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Gut microbial taxa are linked to beneficial or adverse CAR T-cell immunophenotypes and outcomes in lymphoma patients treated with CD19-targeted CAR T-cells

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Introduction: The gut microbiome modulates T-cell immunity and thereby potentially impacts efficacy of cancer immunotherapies e.g. through the secretion of distinct metabolites. Especially, we and others demonstrated that antibiotic driven gut microbial taxa shifts mitigated efficacy of CD19-targeted CAR T-cells. Here, we hypothesize that an antibiotic driven dysbiosis of the gut microbiome prior to CAR T-cell infusion is linked to dysfunctional CAR T-cell immunophenotypes as well as an immunosuppressive protein milieu *in vivo*.
Methods**:** Patients with relapsed / refractory B-NHL were treated with CD19-targeted CAR T-cells (n=55). Peripheral blood and fecal biospecimens were collected. CAR T-cell expansion and co-expression of PD-1, TIM-3, LAG-3 and CD244 were studied by flow cytometry at day 7 after infusion. Protein signatures were assessed through a multiplexed proteomics assay at day of infusion. Metagenomic shotgun sequencing was performed on 108 stool samples prior and after infusion. Patients being exposed to antibiotics up to three weeks prior to CAR T (pAbx) as a surrogate for a disrupted microbiome community structure were retrospectively compared to patients, who did not

receive antibiotic treatment prior to CAR T (cAbx).
Results**:** PAbx showed inferior CAR T-cell expansion at day 7 after infusion compared to cAbx. UMAP analysis revealed an upregulation of PD-1, TIM-3, LAG-3 and CD244 on CAR T-cells in pAbx but not cAbx. Accordingly, pAbx exhibited higher frequencies of several dysfunctional CAR T-cell phenotypes compared to cAbx. In addition, we detected higher levels of LDH, CRP, Ferritin and PCT suggestive for higher tumor burden and inflammation at the day of infusion not only in pAbx but also in patients, who exhibited inferior CAR T-cell expansion. A further in-depth characterization of the overlapping protein milieu revealed, in particular, high levels of IL-6, PD-1 and MUC-16 in pAbx and patients with low CAR T-cell expansion. Interestingly, a set of distinct gut microbes at baseline correlated with either non-dysfunctional (e.g., Gordonibacter pamelaeae) or highly dysfunctional (e.g., Enterococcus faecium) CAR T-cell immunophenotypes at day 7. In addition, the presence of Enterococcus faecium coincided with higher mean levels of IL-6, PD-1 and MUC-16. Notably, a monodomination pattern of some of these taxa (e.g., Enterococcus faecium) was driven by prior-antibiotic treatment. Finally, the abundance of Enterococcus faecium at baseline was linked to inferior overall survival, whereas the abundance of Gordonibacter pamelaeae was associated higher tumor shrinkage from baseline to day 30.
Conclusion**:** We describe associations between antibiotic driven taxa shifts in the pre-infusion gut microbiome and dysfunctional phenotypic features of CAR T-cells as well as immunosuppressive protein signatures in patients with r/r B-NHL. Our findings might serve as a predictive biomarker for microbiome-based patient assessment and response prediction.

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Adoptive transfer of T cells transduced with an HLA-independent T-cell receptor against Tyrosinase-related protein 2

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From tumor-reactive lymphocytes of melanoma patient Ma-Mel-86 with HLA I-negative disease, we have cloned two distinct αβ T-cell receptors (TCRs) against the melanocytic differentiation antigen tyrosinase-related protein 2 (TRP2). These TCRs recognize TRP2 in an HLA-independent fashion. One of the two anti-TRP2 TCRs cross-reacts with the murine TRP2 homolog. This TCR was chosen for *in vivo* mouse experiments to explore both efficacy and safety and to establish a basis for its clinical application. The TCR chains were codon-optimized and chimerized with homologous murine constant regions (cTCR).
We performed adoptive T-cell transfer in NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl} (NSG) mice. Each mouse was

subcutaneously inoculated with $1x10^6$ cells of the HLA-deficient melanoma-cell line Ma-Mel-86b stably transduced with a bicistronic retroviral construct encoding human TRP2 and nanoluciferase (NLUC). Seven days after tumor inoculation, we treated the mice with buffy coat-derived T cells transduced with a tricistronic retroviral construct encoding the anti-TRP2 cTCR [TRBV_mTRBC_P2A_TRAV_mTRAC] and firefly luciferase (FLUC). On the day of transfer we injected $5x10^6$ anti-TRP2 cTCR/CD3 double-positive T cells per mouse via tail vein and $7{,}2x10^5$ IU IL-2 intraperitoneally. Control mice were treated accordingly with T cells from the same buffy coat transduced with FLUC only.
According to physical examination, *in vivo* bioluminescence imaging, and immunohistochemistry, T cells transduced with anti-TRP2 cTCR effectively eliminated the TRP2-overexpressing tumor cells, whereas tumors in the control group were continuously growing. While anti-TRP2 cTCR-engineered T cells mainly localized and persisted at the tumor sites, T cells transferred in the control group mostly localized and persisted in the spleen. Immunohistochemistry did not reveal any signs of off-tumor/on-target effects in tissues naturally expressing Trp2.
Based on our findings, the adoptive transfer of T cells transduced with naturally occurring HLA-independent TCR against TRP2 appears both effective and safe. This encourages further preclinical and clinical development. In a next step, we address the concern that inflammation-induced downregulation of TRP2 might occur after transfer of HLA-independent anti-TRP2 T cells which could lead to immune evasion as reported with HLA-restricted anti-gp100 and anti-Melan-A/MART-1 T cells and currently perform an *in vivo* experiment with melanoma cells naturally expressing TRP2.

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Identification of hepatocyte-restricted antigens, epitopes, and T cell receptors to treat recurrent hepatocellular carcinoma after liver transplantation

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HCC recurrence in the context of an HLA-mismatched donor liver provides the unique setting that liver antigens from HCC versus the liver allograft are presented by different alleles of Human Leukocyte Antigen (HLA). Here, we present the development of an adoptive therapy with T cell receptor (TCR)-engineered T cells directed against hepatocyte-restricted antigens (HRAs) presented by the recipient, but not donor HLA.
We have applied an integrative approach of *in silico* antigen and epitope prediction, immunopeptidomics, and *in vitro* laboratory tools to stringently select and validate HRAs, their immunogenic epitopes, as well as corresponding TCRs.
58 presumed liver antigens retrieved from the human protein ATLAS were further evaluated for liver-restricted expression in 6 public RNA databases and 1 protein database (HIPED), shortlisting 14

candidate HRAs. 3/14 HRAs did not show RNA expression in healthy tissues, except for liver, in another five tissue datasets (n=1,709) and validated using qPCR. Two HRAs demonstrated RNA expression in >70% of HCC patients (n=421). Immunopeptidomics of HCC-derived hepatocytes (n=12), together with *in silico* predictions of immunogenicity, revealed 36 HLA-A2-restricted epitopes. These epitopes were tested and ranked according to *in vitro* HLA-A2 binding ability. Epitope-specific T cells were enriched from healthy donors for 6 of these epitopes using an *in vitro* co-culture with autologous antigen presenting cells. Eleven TCRαβs directed against 4 HRA-derived epitopes were selected following epitope-MHC-directed fluorescence-activated sorting of T cells. Five TCRs were functionally expressed upon gene transfer into T cells and recognized their cognate peptide, of which 4 TCRs harboured a stringent safety profile according to amino acid scanning, and are expected to mediate no to negligible cross-reactivity.
We have identified HRAs, epitopes and corresponding TCRs, of which the lead TCRs will be further exploited for the treatment of recurrent HCC after liver transplantation with adoptive therapy of TCR-engineered T cells.

*YSR and DK contributed equally to this research project.
*SIB, RD, and DS contributed equally to this research project.
*Target antigens, epitopes, and T cell receptors are not disclosed due to patent filing

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While both improving anti-tumor reactivity, TIGIT editing preserves proliferation abilities of melanoma specific T-cells, in the difference to PD-1 editing

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Our project is part of the development of innovative cellular therapies, based on the injection of tumor-specific T-cells with optimized functions by genomic editing of genes encoding for immune checkpoint (IC). Our team previously showed that adoptive transfer of melanoma-specific PD-1^{KO} T-cells resulted in improved control of melanoma tumor growth when transplanted into immunodeficient mice. However, the deletion of PD-1 in these cells also decreased their proliferative abilities, which represents a limitation to complete tumor control efficacy. The impact of genomic deletion of IC on essential properties of CD8^{+} T-cells is a crucial issue to consider for the development of this therapeutic strategy. Our project aims to determine if this feature is specific to PD-1 deletion or if it can also be observed upon TIGIT disruption, whose inhibition pathway acts synergistically with that of PD-1. Our results confirmed that melanoma-specific PD-1^{KO} T-cells exhibit a dysregulated cell cycle whereas TIGIT^{KO} T-cells have preserved proliferative capacities. Moreover, TIGIT^{KO} T-cells express lower levels of PD-1, which would give them an additional advantage within the tumor microenvironment. Consistently, transcriptomic analyses revealed the modulation of genes involved in the transcriptomic regulation of *PDCD1*, in TIGIT^{KO} T-cells. Furthermore, we showed *in vitro* the

superior antitumor activity of TIGIT^{KO} T-cells, compared to their WT counterpart. Finally, we set up a preclinical model with immunodeficient mice engrafted with PD-L1^{pos}/CD155^{pos} human melanoma tumors. In this model, adoptive cell transfer of TIGIT^{KO} T-cells significantly delayed tumor growth compared to WT T-cells.
These results highlight the need to carefully document the consequences of IC-editing, not only on anti-tumor properties but also on essential properties of T cells such as proliferation or metabolism. In this respect, TIGIT editing appears to meet all the requirements for an innovative therapeutic application, improving anti-tumor T-cell reactivity while preserving T-cell persistence abilities.

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TIL therapy as potential new therapeutic option for neuroblastoma patients

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Adoptive transfer of tumor infiltrating lymphocytes (TILs) is a potent therapeutic option for human solid cancers, as proven by the phase III clinical trial for metastatic melanoma. The potential of TIL therapy is also being explored for other so-called 'hot tumors' that display a high mutational burden and high immune cell infiltration, such as bladder cancer, head and neck cancer, breast cancer and recently also non-small cell lung cancer (NSCLC). However, also 'cold tumors' can be responsive to TIL therapy. This could potentially hold true also for pediatric tumors, such as neuroblastoma. High risk neuroblastoma patients have a dismal survival rate, and novel treatment modalities are in dire need. For neuroblastoma patients, a high TIL density correlates with a good patient outcome. Therefore, we hypothesized that TIL therapy may be a putative therapeutic option. We therefore studied whether tumor reactive TIL products can be generated from neuroblastoma tumor lesions.
We collected tumor material from 19 neuroblastoma patients, and phenotypically defined the composition of TILs in tumor lesions that were treated with first line chemotherapy. With the clinically approved expansion protocol used for melanoma TIL products, we found that half of the cohort reached high expansion numbers, comparable to those of adult tumors. Patients showing limited expansion numbers tended to be of younger age, and they had received more treatment regimens. Overall, low CD3 T cell infiltrates resulted in limited expansion capacity.
To determine the tumor reactivity, we co-cultured expanded TIL products with autologous tumor digest, and measured the production of pro-inflammatory cytokines (IFNγ and TNF) as well as the expression of the co-stimulation marker CD137 (4-1BB) and of CD107α, indicative for killing capacity. Overall, 5 out of 8 (62.5%) tested TIL products showed responsiveness to tumor tissue. Interestingly, the anti-tumor response did not originate from conventional CD4^{+} and CD8^{+} T cells, but rather from unconventional γδ T cells.
These findings provide new insights in the anti-tumor responses in 'cold' pediatric tumors, and suggest a window of opportunity for therapeutic approaches with TIL therapy.

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Initiation of a phase I/II trial of CRISPR-Cas9-mediated PD-1-deficient tumour-infiltrating lymphocyte-based adoptive T-cell therapy in metastatic melanoma

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Therapeutic antibodies blocking the engagement of inhibitory T cell receptors, such as programmed cell death protein 1 (PD-1) or cytotoxic T-lymphocyte-associated protein 4 (CTLA4) have been highly successful. Combination of these antibodies with other treatments often outperforms the individual therapy. Adoptive T-cell therapy with in vitro-expanded tumour-infiltrating lymphocytes (TILs, TIL therapy) has been shown to induce durable responses in patients with metastatic melanoma (MM), and recently our institution participated in a phase III trial demonstrating the superior efficacy of TIL therapy vs ipilimumab (CTLA4-blocking) for MM. Previously we hypothesized that these approaches could be coupled by disrupting PD-1 expression in TIL therapy products using CRISPR-Cas9. In addition to potentially improving TIL therapy outcomes, this approach could reduce the systemic toxicities associated with PD-1-blocking treatment by only inhibiting PD-1 on TILs. Our pre-clinical data demonstrated that this approach was feasible, had minimal impact on existing workflows, and was highly efficient. No off-target editing or negative impact of PD-1 disruption on TIL expansion potential, cytotoxicity, or tumour-reactivity was detected. Here, we report on the feasibility of scaling our pre-clinical experiments to clinical scale. PD-1 disruption efficiency of patient-ready CRISPR-Cas9 reagents in expanded TILs was measured via flow cytometry and compared to our pre-clinical study results. Degradation of patient-ready RNP complexes during the TIL expansion process was determined via western blot detection of Cas9 protein. >50 potential off-target editing sites were predicted using a combination of bioinformatic tools. These sites, as well as the on-target site, were investigated post-expansion in TIL samples from both the pre-clinical study and our patient-ready reagent studies using next-generation sequencing approaches. PD-1 disruption by patient-ready reagents was comparable to our pre-clinical studies at both the protein and gene level. Cas9 was shown to be completely degraded/cleared prior to the end of TIL expansion, suggesting complete RNP complex clearance. Off-target analyses revealed no clear signs of off-target editing in any loci. We demonstrate that we can produce a patient-ready highly PD-1-deficient TIL therapy product using CRISPR-Cas9 and a non-labour intensive, simple, non-viral workflow. Based on this, we are initiating a phase I/II TIL therapy trial in MM employing CRISPR-Cas9-mediated PD-1-deficient TILs.

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CD56 dim & bright NK cells – what happens during ex vivo culture?

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Human Natural Killer (NK) cells in peripheral blood have traditionally been classified into two major populations according to their expression levels of CD56 and CD16. During *in vitro* culture of freshly isolated NK cells, we observed an increase in the proportion of NK cells that express high levels of CD56, while NK cells with lower CD56 expression gradually disappeared over time. This phenomenon results in NK cell populations that are indistinguishable based on CD56 expression at the end of a 14-day *in vitro* culture and raises the question of the impact of *ex vivo* manufacturing process and the use of cytokines on NK cell phenotype and functionality.
In order to study the two subsets separately, we developed a sorting strategy using MACSQuant Tyto that allowed to separate CD56 bright and dim NK cells with over 98% purity and 99% viability. To assess whether differences in expansion might explain the increase in the proportion of NK cells with high CD56 expression during culture, we monitored cell numbers over the course of two weeks and measured similar expansion of the two separated subsets. During cultivation, we observed an increase in CD56 intensity in the NK cell population sorted for low expression of CD56. However, other markers remained typical of the CD56 dim population. Meanwhile, the NK cells sorted for high CD56 expression maintained expression of other markers that are typical for CD56 bright NK cells.
We also tested transducibility with CAR constructs using lentiviral vectors pseudotyped with a modified baboon envelope glycoprotein. Both sorted subsets were transducible with similar efficiencies. CAR-transduced and untransduced NK cells from both sorted subsets were then tested for natural, as well as CAR-mediated cytotoxicity. Hereby, the NK cells that had been sorted for high CD56 expression outperformed those that had been sorted for lower CD56 intensity. This is contrary to the traditional classification of CD56 dim NK cells being more cytotoxic but is consistent with a previous study demonstrating the high antitumor activity of IL-15-primed CD56 bright NK cells (Wagner et al., 2017).
Together, these observations suggest that, when kept separately during culture, the shift towards a higher CD56 expression is mostly due to upregulation without changes in the other markers that are traditionally used to define the CD56 dim and bright subsets. Intriguingly, both subsets are able to be genetically modified by lentiviral vectors with similar efficiencies and possess highly potent antitumor functionality in *in vitro* cytotoxicity assays.

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Next-Generation, Inducible IL-7–Expressing, Tumor-Infiltrating Lymphocytes by Lentiviral Vector Genetic Modification for Clinical Application

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Tumor-infiltrating lymphocyte (TIL) therapy has shown some of the most favorable responses in refractory metastatic melanoma, possibly due to the immunogenicity of this cancer. TILs are isolated from a patient's own tumor tissues, rapidly expanded in vitro, and then adoptively transferred back into the patient. These TILs can recognize and attack cancer cells in large numbers with high specificity. In multiple Phase 1/2 trials of melanoma, TIL therapy has demonstrated a robust response rate of up to 50%; however, durable complete responses were only seen in about 15% of treated patients. Interleukin 7 (IL-7) is a potent stimulator of T-cell proliferation and survival. However, T-cells are incapable of producing their own IL-7 and rely on secretion from surrounding stromal cells. We hypothesize that the introduction of an inducible IL-7 gene to TILs (next generation TIL; ADP-TILIL7) will improve their ability to engraft, proliferate, and survive, while maintaining diverse TIL specificity. This may translate into improved clinical activity and durability of response when given to patients. To investigate the ability of IL-7 to enhance T-cell functionality, we first used T-cells from healthy donor peripheral blood transduced with an affinity enhanced T-cell receptor (TCR) specific for the cancer testis antigen MAGE A4 combined with IL-7 under the control of a nuclear factor of activated T-cells (NFAT) inducible promoter as a model system (ADP-A2M4IL7). Production of inducible IL-7 by ADP-A2M4IL7 led to improved T-cell expansion and retention of functionality upon repeated stimulation in vitro, without impacting immediate T-cell activity or T-cell specificity. Next, we focused on introducing the human IL-7 gene under the control of the same NFAT inducible promoter used in the model system into patient-derived TILs to generate T-cells that secrete IL-7 upon recognition of their cognate antigen. We detail how transduction of TILs was best achieved before the rapid expansion step, and we report increased IL-7 production with the inclusion of a transduction-enhancing poloxamer. To test the functionality of the lentiviral vector, we developed an assay to determine the production of IL-7 upon non-specific TCR stimulation in vitro and found that ADP-TILIL7 reliably secreted levels of IL-7 that had been shown to increase survival in assays with ADP-A2M4IL7 cells. Finally, transduction and exposure to IL-7 did not reduce the proliferation potential or change the phenotype of the TILs, indicating that ADP-TILIL7 is as fit for purpose as conventional TILs. Based on our preclinical studies, a single-center Phase 1 clinical trial will be initiated with ADP-TILIL7 to treat patients with metastatic melanoma.

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Advancing the Development of Cell-based Immunotherapies with the Measurement of Cell Avidity

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As cellular immunotherapies become increasingly complex in design, methods to identify and advance the most promising therapeutics candidates must also evolve. Current strategies leveraging in vitro assays to predict in vivo results, such as killing assays and cytokine secretion assays, may prove insufficient, as only 1 in 8 cellular therapies receive approval. Instead of focusing on downstream metrics of immune cell function, measuring the initial interaction between an immune cell and a cancer cell may offer unique insights required to increase confidence in candidate selection.

We developed the z-Movi Cell Avidity Analyzer to directly quantify the strength of the interaction between effector and target cells at single cell resolution. Unlike affinity, cell avidity is the overall strength of interactions between all receptor-ligand pairs at the interface between a cancer cell and

an immune cell.
Here, we discuss recent studies applying measurements to inform CAR-NK, CAR-T, and TCR design and selection, demonstrating the utility as a novel biomarker for developing potent and safe immunotherapies.
Higher avidity has been significantly correlated with improved tumor control in murine models, but has also been associated with toxicities in patients, suggesting tuning of cell therapies to the desired avidity for ideal function is needed.

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A click chemistry approach to the alteration of cellular surfaces

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Cancer immunotherapy, especially the adoptive cell immunotherapy, has become an exciting new therapeutic strategy that complements traditional approaches. It takes advantage of the immune system's ability to control and eliminate cancer. Despite the apparent success of T-cells modified by chimeric antigen receptor (CAR-T), their introduction into routine clinical practice is slow. Their genetic modifications take time and cost money. The crucial part of a CAR receptor is the fragment of tumor-recognizing antibody located on the cell surface. We have developed a chemical strategy allowing easy, rapid and efficient modification of cellular surfaces. Our modification procedure enables installation of many tumor-targeting moieties such as antibodies, small molecules aptamers or peptides on the glycoproteins of an immune cell. It involves the metabolic incorporation of a new sugar analog bearing chemically reactive moiety. Our approach is fully compatible with the cell environment and orthogonal to the existing Azide-DBCO click chemistry with much faster reaction kinetics at low concentrations. The cells are cultivated in the presence of a metabolic precursor for 48 hours and subsequently reacted for 30 minutes with a 2.5 micromolar concentration of the tumor-targeting moiety in a complete cultivation media. To demonstrate the potential of the method, we modified the surface on a natural killer cell resembling cell line (NK92), with an antibody or a small molecule that recognizes the target cell line. We then co-cultured modified or unmodified NK92 cells with the target cells for 4-hours at a 2.5:1 effector-target ratio. We observed up to 45% increase in the number of necrotic chronic B-cell leukemia cells when co-incubated with modified anti-CD20 NK92 cells versus the unmodified control. Similarly, when navigated to a cervical cancer cell line expressing carbonic anhydrase 9 by a high-affinity small molecule binder, the targeted NK92 produced 40% more apoptotic cells. In summary, we can efficiently alter the surfaces of cultivated cell lines and primary cells, such as peripheral blood mononucleocytes or NK-cell and demonstrate their increased efficacy in targeting. We believe that the methodology presented could allow the production of modified immune cells equipped with more tumor-aiming molecules leading to a more effective immune therapy with lower costs.

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Automated manufacture of dNPM1 TCR-engineered T cells for therapy

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Treatment of relapsed or refractory acute myeloid leukemia (AML) still represents a challenge for physicians and is mainly associated with poor prognosis with a 5-year survival rate in Europe of only 25%. A neoantigen encoded by mutated nucleophosmin 1 (dNPM1) is expressed in approximately 30% of patients and presents an attractive target for immunotherapy with T cell receptor (TCR)-engineered T cells. Manufacturing of TCR-modified T cells, however, is still limited by a complex, time-consuming and laborious procedure. Therefore, this study specifically addressed the requirements for a scaled manufacture of dNPM1-specific T cells in an automated, closed and Good Manufacturing Practice (GMP)-compliant process using the CliniMACS Prodigy. Small scale studies demonstrated that the HLA-A*02:01 restricted recombinant TCR is dependent on CD8 co-receptor interaction to bind the peptide-HLA complex with high affinity. We therefore focused on the development of a CD8 T cell-specific process. Thus, in 23 individual runs, 2E8 CD8-positive T cells were enriched from cryopreserved leukapheresis, subsequently activated, lentivirally transduced, expanded and finally formulated. By adjusting and optimizing culture conditions, we additionally reduced the manufacturing time from twelve to eight days while still achieving a clinically relevant yield of up to 5.5E9 dNPM1 TCR-engineered T cells (4.2E9 ± 1.4E9). The cellular product mainly consisted of highly viable CD8-positive T cells (97.5% ± 0.978%) with an early memory phenotype. The cellular product was stable for up to 48h. Overall, mean vector copy number was 2.9 ± 1.1. Importantly, TCR-engineered T cells specifically lysed dNPM1-positive target cells *in vitro* as well as *in vivo*. In conclusion, we demonstrated that our CD8 process is robust and reproducibly yielded suitable numbers of potent dNPM1 TCR-engineered T cells, preparing the way for initiating a clinical trial to treat patients with relapsed or refractory AML.

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Systematic off-target screening of T-cell receptors prior to clinical application

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Transfer of patient T cells genetically engineered to express T-cell receptor sequences with a desired reactivity (TCR-Ts) is currently widely explored to treat metastatic cancer. Incomplete pre-clinical assessment of TCR off-target reactivity has led to fatal adverse effects in past patient trials. Rapid increase in clinically applied TCRs demands accumulation of standardized pre-clinical data, yet there is no consensus regarding which parameters to evaluate.
We propose a blueprint for mapping of TCR-reactivity, validated with two HLA-A*02:01-restricted TCRs: the clinically proven, affinity-enhanced NY-ESO-1-specific TCR 1G4-α95:LY(1G4), and the CD20-specific TCR A23. Potential off-target epitopes recognized by TCR-Ts were mapped in five steps: Generation of a TCR “fingerprint” from functional responses to cells loaded with a positional peptide scanning matrix of the target epitope (1) forms basis for a computational search in the human proteome (2), followed by reactivity screening against a second peptide library (3), and against naturally processed and presented off-target peptides from short mRNAs (4), and full-length proteins (5). TCR-Ts were screened for recognition of unintended HLA alleles using a cell line panel representing an extensive HLA-library. As proxy for clinical efficacy, and to some degree safety, a syngeneic, HLA-A*02:01-transgenic mouse cancer model was treated with A23 TCR-Ts.
Based on TCR fingerprints, computational analysis identified candidate off-target peptides (1G4:11, A23:30), of which few were recognized (1G4:1, A23:11). Even fewer were processed and presented from mRNAs encoding 30-mer peptides (1G4:1, A23:3), and none by cells with native expression of the corresponding full-length proteins. These results are consistent with the reported safety profile of 1G4 in patients. A23 TCR-Ts eradicated established CD20-expressing tumors without detectable toxicities in the HLA-A*02:01-transgenic mice. However, both TCRs cross-recognized three HLA alleles of the A02-supertype. Notably, 1G4-reactivity was observed in absence of NY-ESO-1 expression, suggesting that 1G4 TCR-Ts could cause toxicities in patients with these HLA alleles.
We established a broadly applicable pipeline for systematic pre-clinical characterization of TCRs. Validation demonstrated the importance of screening TCR-Ts for recognition of full-length candidate off-targets, and for recognition of unintended HLA alleles, of importance for patient selection. Use of this approach should inform clinical decision-making.

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Exploiting circulating lymphocytes and cell-free DNA as a source to develop minimally-invasive personalized T-cell therapies

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Adoptive transfer of neoantigen-specific TILs has demonstrated antitumor activity in selected patients with distinct types of epithelial cancers. However, the need of a tumor biopsy to both

identify candidate neoantigens and reactive T cells or TCRs limits the broad applicability of personalized T-cell therapies. Here we explored cell-free DNA (cfDNA) as an alternative source to identify candidate neoantigens. In addition, we aimed to define a phenotypic signature, beyond the previously reported use of PD-1 expression alone, capable of improving the detection of circulating neoantigen-specific T cells and TCRs. To this end, we sorted $CD8^+$ and $CD4^+$ circulating T cells from 6 patients with different epithelial tumors based on the expression of PD-1 alone into $PD\text{-}1^{hi}$, $PD\text{-}1^{dim}$ and $PD\text{-}1^-$ and in combination with cell-surface receptors CD27, CD39, CD38, HLA-DR and 4-1BB, obtaining up to 35 $CD8^+$ and 35 $CD4^+$ T-cell populations for some of the patients studied. The *ex vivo* expanded T-cell subsets were screened using a personalized high-throughput screening strategy, enabling the detection of T cells targeting neoantigens identified using WES of cfDNA as well as additional mutations identified exclusively in the tumor. Using this approach, we detected neoantigen-specific T cells in 5 out of 6 patients with breast, colorectal or head and neck cancer. The overlap between NSMs identified in cfDNA and tumor varied notably between patients with 2 out of 6 patients displaying no overlap between tumor and cfDNA WES. Nevertheless, cfDNA preferentially detected clonal tumor somatic mutations and enabled the identification of neoantigen-specific T-cell responses in 4 of 5 patients harboring neoantigen reactivities. More specifically, using cfDNA we identified 7 of 13 neoantigens all of which were also detected in the tumor. Both $CD8^+$ and $CD4^+$ neoantigen-reactive T cells were preferentially enriched in T-cell subsets expressing high levels of PD-1, either alone or in combination with other cell-surface markers, being $PD\text{-}1^{hi}CD39^+$ the combination of markers that more consistently identified $CD4^+$ and $CD8^+$ neoantigen reactivities. More importantly, the frequency of neoantigen-specific lymphocytes contained in the $CD8^+PD1^{hi}CD39^+$ population was consistently higher than in any other $CD8^+$ population and it outperformed unselected TIL expanded in high dose IL-2 in the detection of $CD8^+$ neoantigen reactivities. Our results underscore peripheral blood as an alternative source to identify cancer-specific neoantigens and $CD8^+$ and $CD4^+$ neoantigen-specific T lymphocytes and TCRs from patients with epithelial cancers.

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T cell receptor specific for tumor-restricted Ropporin-1 to treat triple negative breast cancer

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Triple negative breast cancer (TNBC) lacks classical targets for hormone and/or antibody therapy, and responses to immune checkpoint inhibitors are rare and generally not sustained. However, the prognostic value of infiltrating CD8 lymphocytes and proven antigenicity of TNBC argue that this disease is amenable to adoptive T cell therapy. We applied an integrative approach using *in silico* analyses and laboratory experiments to discover a tumor-restricted intracellular antigen; immunogenic, non-cross-reactive epitopes derived from it; as well as sensitive and specific T cell

receptors (TCRs) against it. We identified the target Ropporin (ROPN1), which showed neither gene nor protein expression in healthy human tissue databases (n=1,709) and over 15 major healthy organs according to qRT-PCR and immune-histochemical (IHC) staining. Notably, this target demonstrated homogenous protein expression in >80% of TNBC patients (n=756 gene expression; n=386 IHC). Epitope predictions and immunopeptidomics using cancer cell lines and tissues enabled identification of 12 HLA-A2-binding epitopes. Epitope-specific T cells were successfully enriched from naïve T cell repertoires for 9 epitopes, which yielded more than 25 clonal TCRs. TCRs directed against 5 epitopes were functionally expressed upon gene transfer into T cells, and TCRs directed against 3 epitopes recognized endogenously processed PCT-1. One TCR (FLY-A) demonstrated preferential pairing between the therapeutic TCR alpha and beta chains and harbored a stringent recognition motif according to positional amino acid scanning of the cognate epitope. From an efficacy perspective, this TCR mediated dose-dependent killing of patient-derived TNBC 3D organoids *in vitro* and a breast cancer cell line in an *in vivo* murine study. Importantly, in both studies, treatment with FLY-A T cells significantly outperformed the standard of care treatments cisplatin and sacituzumab govitecam. ROPN1 has been identified as a promising target for adoptive T cell therapy for TNBC patients and a specific and active TCR has been selected as the lead candidate for clinical development.

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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 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 protocols and product quality to make this therapy available to other tumor entities and more cancer patients.
In this project, the *ex vivo* addition of 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+ T cells exhibited a higher fraction of cells expressing the costimulatory marker CD28 and the exhaustion marker BTLA, 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. Furthermore, the specificity of T-cell populations towards tumor-associated antigens and HPV-peptides was investigated in Cervical Cancer TILs. The CD39-CD69- cells seemed to be enriched in TILs specific for HPV peptides in TILs expanded with TIL stimulators compared to IL-2 expanded TILs.
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.

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Re-Arm NK cells in Patients with acute myeloid leukemia

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Acute myeloid leukemia (AML) is the most common cause of leukemia-related deaths. In these patients, natural killer cells are frequently dysfunctional and characterized by low expression of activating receptors. We established an *in vitro* system, to study the effect of AML-Blasts on NK cells. AML blasts potently suppressed cytokine secretion and tumor cell lysis by NK cells. On a mechanistic level, AML blast-derived PGE2 leads to hyperphosphorylation of Lck at Y192 and Y505, thereby impairing downstream signal transduction in NK cells. Neutralizing PGE2 production by blocking COX2 using inhibitors or blocking PGE2 signaling via EP2/4 antagonists abolished the suppressive effect of AML blasts. The clinical significance of this mechanism was supported by the finding that stimulation of NK cells via CD16 was equally blocked by PGE2 secreted by AML blasts, implying that

clinical applications in which NK cells are to be activated are significantly attenuated without prior blockade of PGE2. This was confirmed by enabling NK cells to lyse blasts when stimulated by anti-CD123 upon blockade of PGE2.

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CD37 is a safe anti-AML CAR target

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Acute myeloid leukemia (AML) is the most common form of hematological malignancy affecting myeloid progenitors. This cancer is highly heterogeneous and remains difficult to cure despite the introduction of chimeric antigen receptor (CAR) technology in the panel of treatment options. The lack of tumour-specific antigens appears as one of the main challenges. Indeed, the current anti-AML CAR constructs target proteins also expressed in hematopoietic stem cells (HSCs) therefore carrying the risk of myeloablation. There is thus a clear unmet need for non-toxic CAR target to treat AML. Recently, the mRNA encoding the leukocyte-specific tetraspanin 37 (CD37), a marker thought to be restricted to mature B cells, was detected in AML, we herein confirmed these data. However, the presence of the protein led to conflicting results, some groups saw it, others could not detect it. When we evaluated AML recognition by the HH1 antibody, an anti-CD37 antibody developed in our institution and previously designed as a CAR, we detected a signal, whereas a commercial antibody stained only weakly or not. Thus, in AML, CD37 detection appeared antibody dependent. This prompted us to further test our anti-CD37CAR in different AML models and compare its outcome with the clinically relevant anti-CD33 CAR construct. We observed that CD37CAR T cells were comparable to CD33CAR T cells in different models: among others, they specifically kill various AML cells (lines and primary) in a CD37-dependent manner; they secreted inflammatory cytokines and controlled tumor development in xenograft models including patient-derived xenograft (PDX). Importantly, colony formation assay demonstrated that CD37CAR was not toxic against bone marrow derived HSCs, whereas CD33CAR T cells completely eradicated the myeloid progenitors. Our work provides the first non-toxic AML CAR target, broadly expressed and strongly supports a future clinical evaluation of CD37CAR T cell therapy in the clinic.

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Dissection of the tumor-reactive and bystander T-cell repertoires in a murine model for pancreatic cancer

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We investigated the nature of the tumor-infiltrating T-cell response in an orthotopic mouse model for pancreatic ductal adenocarcinoma (PDAC) at the clonal level by means of single-cell sequencing of FACS-purified CD3+ T-cells using the 10x Genomics platform. These tumors, like the human disease, are not sensitive to PD-1/PD-L1 immune checkpoint blockade. Nevertheless tumor regression can be induced - in a T-cell dependent manner - by applying MEK inhibitors in combination with agonist anti-CD40 Ab.
Accordingly, molecular cloning and in vitro functional screening of T-cell receptors (TCRs) from single-cell data sets revealed that a significant fraction of the expanded the CD8+ and CD4+ T-cell clonotypes were tumor-reactive. Notably, the majority of T-cells belonging to tumor-reactive CD8+ clonotypes displayed a gene signature bearing witness of extensive T-cell exhaustion. We also isolated tumor-reactive TCRs from CD4+ T-regulatory cells, further explaining the lack of an effective anti-tumor T-cell response, even in the context of PD-1/PD-L1 blockade.
Differential gene expression analysis between data sets representing tumor-reactive versus non-tumor-reactive T-cell clonotypes rendered a gene signature by means of which tumor-reactive TCRs could be identified in mouse data sets with a true positive rate of 100%. This gene signature also effectively distinguishes between tumor-reactive and bystander T-cells in in human cancers, highlighting the relevance of our findings for the immunotherapy of difficult-to-treat indications.
Analysis of the specificity of the tumor-reactive TCR repertoire by means of a syngeneic tumor cell line panel, synthetic peptides and the natural immunopeptidome revealed that this featured three reactivity patterns: neo-epitope-specific, pancreatic tumor-specific, and cross-reactive to other tumor types of C57BL/6 mouse origin.

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Combination of bispecific killer cell engagers and NKG2D-CAR effector cells for enhanced antitumor activity

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NKG2D is an activating receptor of natural killer (NK) cells and other lymphocytes that mediates lysis of malignant cells through recognition of stress-induced ligands such as MICA. These ligands are broadly expressed by various cancer types and serve as targets for adoptive immunotherapy. However, shedding or downregulation of NKG2D ligands (NKG2DLs) can prevent NKG2D activation, resulting in escape of cancer cells from immune surveillance. To enable tumor-specific targeting independent of membrane-anchored NKG2DLs, we previously developed a bispecific antibody (NKAB-ErbB2) that redirected lymphocytes endogenously expressing NKG2D or carrying an NKG2D-based chimeric antigen receptor (NKAR) to the tumor-associated surface antigen ErbB2 (HER2) and enhanced specific antitumor activity.
Here, we extended this approach to target solid tumors that express epidermal growth factor receptor (EGFR), and malignancies of B-cell origin characterized by CD19 and CD20 expression. We generated recombinant NKAB-EGFR, NKAB-CD19 and NKAB-CD20 antibodies, which all displayed bispecific binding to NKG2D and the respective target antigen. In combination with NKAR-engineered NK-92 cells, the NKAB molecules facilitated specific recognition and increased killing of tumor cells *in vitro*. Moreover, cancer cells with heterogeneous antigen expression were effectively eliminated by employing combinations of NKAB-ErbB2 and NKAB-EGFR, or NKAB-CD19 and NKAB-CD20 molecules, respectively. To further enhance effector cell activity, the initial NKG2D-CD3ζ NKAR receptor was co-expressed together with the IL-15 superagonist RD-IL15, or modified to include a 2B4 costimulatory protein domain. This resulted in growth and cytotoxicity of NKAR-NK-92 cells independent from exogenous IL-2, and enhanced NKG2D-CAR activity, respectively.
Our results demonstrate that combining NKAB antibodies with effector cells carrying an activating NKAR receptor represents a powerful and versatile approach to simultaneously enhance tumor-antigen-specific as well as NKG2D-CAR-mediated cytotoxicity, which may be particularly useful to target cancers with heterogeneous antigen expression and prevent immune escape.

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Pre-clinical characterization of a bispecific Vγ9Vδ2-T cell engager directed against prostate specific membrane antigen

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There is a high unmet medical need for patients with metastatic castration-resistant prostate cancer who progress on anti-androgen and taxane based therapy regimens. Exploiting HLA-independent Vγ9Vδ2-T cells that constitute a sizable proinflammatory antitumor effector cell population could represent a promising therapeutic approach. Here, we describe the development and pre-clinical characterization of an Fc-domain containing PSMA-Vδ2 bispecific T cell engager (bsTCE) aiming to

target Vγ9Vδ2-T cells to lyse PSMA$^+$ prostate tumor cells. We found that the PSMA-Vδ2 bsTCE activates healthy donor- and prostate cancer patient-derived Vγ9Vδ2-T cells and induces lysis of PSMA-expressing prostate cancer cell lines and prostate cancer patient-derived tumor cells. Activating receptors DNAM-1 and NKG2D contributed to Vγ9Vδ2-T activation and tumor cell lysis when using sub-optimal concentrations of PSMA-Vδ2 bsTCE. These activating receptors were expressed by prostate cancer patient-derived Vγ9Vδ2-T cells and their subsequent ligands were found to be significantly more expressed by cells derived from malignant prostate tissues compared to non-malignant tissues. The PSMA-Vδ2 bsTCE induced preferential lysis of PSMA$^+$ tumor cells and did not mediate lysis of non-malignant prostate tissue. *In vivo* antitumor efficacy of the PSMA-Vδ2 bsTCE was confirmed in NCG mice using human PBMC as effector cells. Based on these data PSMA-Vδ2 bsTCE represents a promising and novel anti-tumor strategy for prostate cancer patients. As such it is currently being evaluated in a phase 1/2a clinical trial in patients with metastatic castration-resistant prostate cancer, who proved refractory to standard of care therapy options (NCT05369000).

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Generation and effector function of ALECSAT cells used for adoptive cell therapy of solid tumors depends on the presence of antigen-unloaded mature dendritic cells

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Adoptive immunotherapy of solid tumors represents significant challenge. We have previously developed the adoptive immunotherapy based on employment of the CD4-enriched population of activated lymphocytes with epigenetically induced expression of a variety of cancer/testis antigens as antigen presenting cells for ex vivo immunization. The product, called ALECSAT, showed antitumor activity in patients with recurrent glioblastoma, leading to tumor regression in a subset of the patients. One of the limiting factors of ALECSAT therapy can be insufficient number of cells generated using the first version of ALECSAT technology (ALECSAT-1). Here we demonstrate that addition of antigen unloaded dendritic cells at the immunization step of the ALECSAT production process significantly (up to eight-ten-fold) increases total number of generated cells. This modification (called ALECSAT-2) does not induce significant changes in the principal characteristics of ALECSAT cells such as expression of CD62L and chemokine receptor CXCR3 that can drive lymphocytes to tumor site, and of costimulatory molecule CD226 required for the lytic activity of lymphocytes. We have also demonstrated that the addition of dendritic cells to the final ALECSAT cells induces formation of mixed lymphocyte/dendritic cell clusters and release of interferon-γ. Based on the known role of intratumoral dendritic cells in the antitumor activity of the adoptively transferred lymphocytes, we propose a model of possible mechanism of action of ALECSAT cells where the central role belongs to crosstalk between closely apposed dendritic cells and lymphocytes leading to their mutual activation and increase in the antitumor activity of the ALECSAT cells. We also discuss possibility of increasing the efficacy of ALECSAT therapy by its combining with dendritic cell therapy.

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The independent role of CD8 molecule in enhancing the reactivity of T cells redirected with chimeric antigen receptor

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The CD8 molecule is an important component of the T-cell receptor (TCR) signaling pathway. CD8 functions as a co-receptor by binding to the alpha 3 domain of the human leukocyte antigen (HLA) class I molecule, resulting in the recruitment of Lck molecule to the TCR complex and subsequently amplifies the signaling cascade, ultimately leading to T-cell activation. However, the role of CD8 in T cells redirected with chimeric antigen receptor (CAR) has still been unclear. In this study, we aimed to investigate the role of CD8 in CAR-T cells by using the endogenous TCR-defect Jurkat 76 cell line (J76) expressing a CAR with or without CD8, and evaluated the reactivity of a panel of CAR-T cells targeting an HLA-peptide complex or a non-HLA surface molecule to determine the contribution of CD8 in CAR-T-cell function. We first established CD8-expressing J76 cells (J76/CD8). Then, we prepared five different HLA-A*02:01-restricted NY-ESO-1_{157}-specific (A2/NY-ESO-1_{157}) single-chain variable fragments (scFvs) and five different CD19-specific scFvs with various avidity which were integrated into the second-generation CAR (CD28/CD3ζ) gene. These CAR genes were individually reconstituted into J76 cells and J76/CD8 cells to assess their reactivity for target cells by measuring the degrees of CD69 upregulation. An A2/NY-ESO-1_{157}-specific TCR gene was also prepared to compare the roles of CD8 between CAR-T cells and TCR-T cells. To evaluate the target-specific binding of CAR-T cells, we used A2/NY-ESO-1_{157} tetramers and in house soluble CD19 dimers which possess two extracellular domains of the CD19 molecule. As the results, the binding ability of TCR-T cells was enhanced in the presence of CD8 on the cell surface. In contrast, A2/NY-ESO-1_{157} CAR-T cells showed similar structural avidities irrespective of the CD8 expression. As expected, both CD8-positive and negative CD19 CAR-T cells were similarly stained with soluble CD19 dimer. Interestingly, A2/NY-ESO-1_{157} CAR-T cells, TCR-T cells, and CD19 CAR-T cells with CD8 displayed enhanced reactivity for target cells when compared to those cells without CD8. Moreover, expression of an HLA class I (HLA-A*02:01) on target cells had little effect on the reactivity of CD19 CAR-T cells in the presence or absence of an HLA-A*02:01-restricted endogenous TCR. Finally, the level of phosphorylation of Lck (Tyr394) appeared to increase in both CD8-expressing A2/NY-ESO-1_{157} and CD19 CAR-T cells following coculture with target cells, suggesting that CD8 can recruit Lck and subsequently enhance the CAR-T-cell signaling. In conclusion, these results suggest that CD8 is likely to augment the reactivity of CAR-T cells, irrespective of the interaction between CD8 and an HLA class I. This may be explained by the role of CD8 as a carrier protein that can associate with Lck. These findings have also important implications for the development of next-generation CAR-T cells that are able to improve antitumor immune responses.

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Blocking the CD47/SIRPα immune checkpoint enhances myeloid cell mediated killing of BCP-ALL cells by an IgA2 variant of daratumumab

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The development of immunotherapeutic approaches for the most common childhood malignancy - B-cell precursor acute lymphoblastic leukemia (BCP-ALL) - has become the focus of attention in recent years. Myeloid cells represent an interesting effector cell population for therapeutic antibodies as they are part of the tumor microenvironment and the most abundant immune cell population in peripheral blood. One promising antigen is CD38, which can be targeted by the monoclonal antibody daratumumab (DARA), already approved for multiple myeloma. Being of immunoglobulin (Ig)G1 isotype, DARA activates the complement system and recruits immune cells which express the Fcγ receptors FcγRI (CD64), FcγRIIa (CD32a) and FcγRIIIa (CD16a), such as NK cells, macrophages and neutrophils. However, the activation of neutrophils by IgG1 antibodies is poor, whereas targeting the IgA receptor FcαRI (CD89) on neutrophils triggers intracellular signaling more effectively and leads to improved tumor cell killing. Inspired by previous promising results with an IgA2 variant of DARA against T-ALL, this study compared the efficacy of both DARA-IgG1 and DARA-IgA2 against BCP-ALL and how this therapeutic approach can be enhanced by blocking the innate CD47/SIRPa immune checkpoint axis with the Fc silent CD47 antibody variant 5F9-IgG2σ. CD47 is a protein often overexpressed on cancer cells that, upon binding to the signal regulatory protein (SIRP)a on myeloid cells, induces tumor immune escape. Six BCP-ALL cell lines of diverse differentiation stages and genetic subtypes (697, SEM, REH, MHH-CALL-4, MUTZ5, and TOM-1) served as targets. CD38 and CD47 expression on all cell lines was shown by quantitative flow cytometry. Antibody-dependent cell-mediated cytotoxicity (ADCC) by GM-CSF stimulated neutrophils was measured in ^{51}Cr-release assays, and antibody-dependent cell-mediated phagocytosis (ADCP) by M-CSF stimulated macrophages was assessed by live cell imaging with automated real time microscopy. While in ADCP both DARA isotypes performed similarly well, in ADCC the BCP-ALL lysis rates of DARA-IgA2 were significantly higher compared to DARA-IgG1 (e.g. REH: IgG1 1,8 ± 1,7 %, IgA2 21,3 ± 1,1 %). Regardless of the isotype of the antibody, the anti-tumor efficacy of both neutrophils and macrophages was significantly enhanced by CD47/SIRPa checkpoint blockade (e.g. REH: IgG1 8,8 ± 6,7 %, IgA2 49 ± 7,2%). In summary, we could show that while the isotype switch of DARA from IgG1 to IgA2 is comparably efficient in macrophage activation, it could improve neutrophil mediated killing of tumor cells. The tumor cell killing capability of DARA can be significantly enhanced by CD47 blockade regardless of the isotype. Consequently, combining DARA as

IgG1 or IgA2 together with CD47/SIRPα checkpoint blockade is worth further evaluation for BCP-ALL immunotherapy.

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An intrinsic radiohapten capture system for CAR T cells that reports biodistribution and function

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Background: CAR T cell therapy is an important tool for treatment of hematologic malignancies, but development outside of this disease paradigm has stalled. As such, there is an unmet need to understand why CAR T cell therapy fails, which would be aided through the creation of tools to track the cells' biodistribution, persistence and function in vivo. To accomplish this, we engineered a class of theranostic "THOR" CAR T cells that express a membrane-bound scFv, huC825, that binds DOTA-radiohaptens with pM affinity; these hapten chelands can be conjugated with radionuclides with therapeutic potential or diagnostic imaging capabilities.
Methods: 19BBz anti-CD19 CAR T cells were engineered to express huC825 (THOR cells). We tested the potential to track THOR cells in vivo using PET/CT following intravenous DOTA-based radiohapten tracer ([86Y]Y-ABD) administration in immunodeficient NSG mice bearing subcutaneous CD19+ human Raji tumors. To observe if there was correlation of PET/CT signal with T cell numbers, we harvested tissue samples from the same mice and quantified the number of CD3+ cells using immunohistochemistry. We measured the activation and exhaustion status of THOR cells ex-vivo using quantitative flow cytometry.
Results: THOR cells were successfully transduced and demonstrated similar in vitro effector functions as parent 19BBz CAR T cells. Serial tracking experiments in vivo with [86Y]Y-ABD radiotracer showed THOR cells can be observed at day 7 post T cell administration, peak at day 14 at the tumor site, and persist until at least day 28. Using a high tumor burden model, we observed higher levels of uptake in the liver (8.8 ± 1.8 %ID/gmax), compared to the primary tumor (5.2 ± 0.97 %ID/gmax) at day 14 post T cell administration. CD3 staining by immunohistochemistry revealed weak correlation of PET/CT signal with THOR cell number, with the spleen showing the highest number of T-cells, yet low levels of uptake (3.1 ± 0.96 %ID/gmean). THOR cells in liver, harvested from mice in this high tumor burden model, had 5- and 12- fold higher levels of huC825 expression compared to the primary tumor and spleen, which indicated that the discrepancy between PET/CT uptake and T cell number was due to differences in huC825 expression. Furthermore, huC825 expression positively correlated with

presence of CD25 (R2=0.933, p=0.0007), a T cell activation marker, and negatively correlated with PD1 and LAG3 (PD1: R2=0.7143, p=0.0576; LAG3: R2=0.9524, p=0.0011), T cell exhaustion markers. Conclusions: We show the pharmacokinetics of THOR cells can be probed in vivo following systemic administration of radiotracer using PET/CT imaging. Furthermore, we demonstrate the ability to measure THOR cells by PET/CT is dependent upon their activation and exhaustion status, thus indicating that the THOR platform has the potential to demonstrate cell biodistribution, persistence, and possibly predict the anti-tumor activity and off-tumor toxicities of CAR T therapies

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Stem cell derived natural killer cells exert efficient ADCC through endogenous CD16 and facilitate monoantigen-specific dual tumor targeting via engament of chimeric antigen receptor and monoclonal antibody.

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Natural killer cells (NK) are gaining traction as cell therapy products with potent anti-tumor potential for use in combination with therapeutic agents such as monoclonal antibodies (mAb) or specific engagers for further enhancement of cytotoxic efficacy. Glycostem's ex vivo expansion and differentiation method in a fully closed automated manufacturing platform (uNiK^{TM}), generates GTA002 (oNKord®), an "off-the-shelf" allogeneic cryopreserved NK cell product from umbilical cord blood derived CD34^{+} progenitors. Safety and tolerability of a non-cryopreserved predecessor was demonstrated in an earlier Phase I trial in elderly AML patients (Dolstra et al. 2017). One of the important outcomes of this study was the notable increase in the CD16 expression of infused NK cells. Thus, we next exploited the potential of further enhancing and focusing cryopreserved NK cell anti-tumor responses in an antigen (Ag)-specific manner, either via antibody-dependent cellular cytotoxicity (ADCC) and/or through a chimeric antigen receptor (CAR) on genetically modified NK cells (viveNK^{TM}).
Similar to its predesscor non-cryopreserved NK cells, GTA002 significantly upregulated CD16 expression in vivo in immunodeficient NCG mice. This spurred the optimization of the culture process to upregulate CD16 expression to study the ADCC potential of GTA002 in vitro. ADCC was assessed against CD19^{+} and HER2^{+} targets at low effector-to-target (E:T) ratios by end-point flow cytometry assays as well as impedance- and live imaging- (2D & 3D) based real time analysis. Next, we engineered anti-CD19 (FMC63) CAR-NK cells as well as CD16-NK cells using the viveNK^{TM} platform to benchmark and to evaluate the effect of simultaneous activation of CD19-CAR mediated cytotoxicity and CD19-induced ADCC of NK cells expressing engineered or endogenous CD16.
Furthermore, expression of important activating and inhibitory receptors and intracellular levels of TNF, IFNg, perforin and granzyme B were measured by flow cytometry to investigate their role for efficient cytotoxicity of GTA002 and CD19-viveNK^{TM} cells. We detected simultaneous tumor targeting both via preserved innate NK cell responses as well as Ag-specific targeting either via ADCC or CAR-mediated killing. Interestingly, CD19-CAR exerted further enhanced Ag-specific killing of a NK cell resistant B-ALL cell line (NALM6) at low E:T ratios, depicting a superposed monoantigen targeting potential.

Overall, the enhancement of the inherent cytotoxicity of GTA002 by harnessing ADCC through combination therapy or by introduction of CAR-mediated Ag-specific cytotoxicity demonstrate the great potential of multimodal targeting against a variety of challenging cancers using a highly safe "off-the-shelf" NK cell-based cellular therapeutic.

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Identifying new features of T cell senescence unveils high anti-tumor activity despite impaired proliferation and disturbed metabolic activity: a new avenue for cancer immunotherapy

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This study aims to characterize the mechanisms of senescence-induced T cell dysfunction for adoptive T cell therapy for cancer. Despite the success of T cell-based cancer immunotherapy, a large fraction of patients still does not benefit from this treatment. Naturally occurring and tumor-induced T cell dysfunction often leads to impaired anti-tumor responses in these patients. While exhaustion is a well-described mechanism driving T cell towards a non-responsive state, senescence in T cells has some similar characteristics but remains less well understood. In this study, we characterized $CD8^+$ T cells from healthy, but aged donors extensively regarding their cellular phenotype and differentiation status by flow cytometry. We then correlated these findings with the functional features (proliferation, cytotoxicity) and metabolic activity (metabolic flux assays, microscopy). Using this multi-analysis approach, we could identify a distinct subpopulation of non-proliferative T cells within the "conventional" senescent T cell population by a combination of surface markers (e.g., CD28, CD57 and CD45RA). Furthermore, this senescent state was characterized by an impaired mitochondrial function as shown by reduced respiration and membrane potential. Altered mitochondrial function also correlated with morphological changes of the mitochondria as demonstrated by electron microscopy. Again, only the combination of multiple "senescent marker" was sufficient to identify the senescent population with disturbed mitochondrial function. When these T cells were exposed to challenging culture conditions such as glucose starvation or hypoxia, they showed a high extent of mitochondrial depolarization indicating less tolerance to this metabolic "stress". In turn, senescent inducing treatments caused mitochondrial damage and inhibition of mitochondrial respiration promoted the onset of senescent in healthy T cells. On a functional level, the senescent T cells showed a superior cytotoxic potential indicated by high production of effector molecules and degranulation. However, a direct proof of potent anti-tumor cytotoxicity of this T cell population is still missing. Therefore, we introduced a tumor antigen-specific T cell receptor (TCR) by RNA-electroporation into different T cell populations and test their cytolytic activity. Despite, being non-proliferative and prone to apoptosis, the senescent T cells elicited a strong anti-tumor response in long-term tumor cytolytic assays. These results highlight the potential of senescent effector T cell populations in cancer immunotherapy. A better understanding of senescence in T cells and strategies

to revert senescence-induced dysfunction may help improving T cell-based cancer immunotherapies.

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Elimination of primary human acute myeloid leukemia *in vivo* by T-cell receptor-mediated targeting of a shared neoantigen

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Acute myeloid leukemia (AML) is characterized by a number of recurrent mutations that serve as potential targets for directed adoptive T-cell therapy, but so far successful clinically relevant options are lacking. We identified a T-cell receptor (TCR) reactive to a peptide derived from the shared D835Y mutation in FMS-like tyrosine kinase 3 (FLT3) presented in context of HLA-A*02:01 (HLA-A2), exploiting our previously developed technology utilizing healthy donor T-cells. T-cells redirected with the FLT3^{D835Y} TCR recognized mutant FLT3 peptide at concentrations as low as 10-100pM *in vitro*. No reactivity was observed against a panel of 25 HLA-A2+ cell lines lacking the FLT3^{D835Y} mutation derived from a variety of tissues and expressing a variety of additional HLA alleles, indicating high peptide:HLA-specificity. Further, we showed that the FLT3^{D835Y} TCR efficiently eliminated leukemic cells in seven HLA-A2+ patients harboring the mutation, while healthy B- and T-cells were spared. No reactivity was observed in response to HLA-A2 patient leukemia cells carrying different amino acids in the same position (wild type, D835E or D835H) or with mutations in other positions. We next investigated the efficacy of the FLT3^{D835Y} TCR *in vivo*, using patient derived xenograft (PDX) models engrafted with FLT3^{D835Y} mutated leukemia cells from two patients, one aggressive and one more slowly growing CD34+ AML. Immunodeficient mice were stably engrafted with primary patient samples and we confirmed by whole exome sequencing and droplet digital PCR that the engrafted cells recapitulated the mutational landscape found in the primary patient, with FLT3^{D835Y} as a dominating clone. The PDX mice were then treated with either FLT3^{D835Y} TCR or control TCR T-cells. In both models, the FLT3^{D835Y} leukemic cells were efficiently eliminated by the FLT3^{D835Y} TCR T-cells, assessed by flow cytometry and droplet digital PCR, while control TCR T-cell treated mice had massive leukemia infiltration in the bone marrow. The efficient elimination of leukemic cells ultimatly lead to a rescue of the endogenous mouse hematopoiesis, which was highly supprerssed by the leukemia in the control TCR treated mice. Lastly, we showed that the FLT3^{D835Y} TCR T-cells killed the

leukemia propagating cells; no mice transplanted with AML cells pre-cultured for 48 hours with FLT3^{D835Y} TCR T-cells developed leukemia, while all mice transplanted with AML cells pre-cultured with control T-cells or without T-cells engrafted with leukemia during the 7 months follow-up. To conclude, we identified a T-cell receptor with promosing therapeutic potential against AML patients with FLT3^{D835Y} mutation in context of HLA-A2, which is supported by efficient elimination of leukemic cells *in vivo* in multiple PDX models.

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Tumor – Reactive γδ TILs from patients with epithelial cancer

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Pancreatic cancer is the 7th leading cause of cancer-related deaths worldwide. Treatment options are still limited. One of the challenges in TIL expansion from PDAC lesions is to obtain high number of *bona-fide* tumor specific T-cells for cellular therapies. Current TIL expansion methods may facilitate classical and non-classical MHC restricted T-cells with the objective to i) identify clonal TCRs that could be used for transgenic expression in recipient effector cells directed against commonly shared tumor antigens and ii) to ensure tumor recognition in the case of MHC class I / II loss variants in cancer lesions. We tailored therefore TIL expansion using a combination of cytokines and an NRLP3 activator which resulted in increased expansion of the VD1+ TIL population. Negative sorting, resulting in >90% pure TCR gamma delta TIL showed that the NRLP3 driven TIL recognize autologous and allogeneic pancreatic cancer cells in an CD1d-restricted fashion defined by IFN-gamma production. T-cell receptor sequencing revealed a limited repertoire usage of TCR gamma – delta chains with unusual Vgamma3 and Vgamma8 usage. These oligoclonal TCR gamma – delta T-cell lines recognized mitochondrial material isolated from pancreatic cancer cell lines or mitochondria isolated from autologous PBMCs pre-treated with doxorubicin, yet not mitochondria isolated from untreated (autologous) control PBMCs presented by autologous IL-4/GM-CSF generated dendritic cells. NRLP3 stimulation increased the number of mutant target epitopes recognized defined by IFN-gamma production in TCR alpha-beta+ TIL, which could be abrogated using an NRLP3 pathway inhibitor. NRLP3 activated TCR alpha-beta T-cells recognized the autologous tumor either in an MHC class I or class II-restricted fashion, substantiated by the molecular recognition analysis of mutant MHC class I or class II target epitopes, defined by DNA exome sequencing. Deep TCR sequencing, spatial transcriptomics of the tumor lesion and RNAseq of the corresponding TIL exhibited an oligoclonal TCR expansion pattern, 7/7 TIL lines showed that NRLP3 stimulation increased mRNA and protein expression of CXCL9 and CXCL10 production in TIL which facilitates tissue invasion. In summary, NRLP3 pathway stimulation increased the quality and quantity of tumor-reactive and mutant epitope specific TIL restricted by classical MHC molecules, increases expression of chemokines facilitating tissue invasion along with the expansion of a TCR gamma-delta CD1d restricted T-cell subpopulation engaged in surveillance of damaged mitochondrial products. This allows a more clinically favourable expansion of TIL for patients with PDAC and enables the identification of TCR gamma-delta TCRs that

could serve as blueprints for anti-cancer directed, CD1d restricted cellular immune responses that recognize commonly shared tumor-associated targets provided by damaged mitochondria.

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Expitope 3.0 – An Advanced *in silico* Webtool Empowered with Machine Learning for Enhanced pHLA Epitope Prediction and Safety Assessment

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The development of T cell receptor (TCR)-T cells for adoptive immunotherapy using highly sensitive TCRs faces critical safety issues of recognition of the target peptide HLA (pHLA) complex on healthy tissues (on target/off tumor recognition) or TCR cross-recognition of pHLA complexes that differ from the target sequence (off-target recognition). In both cases, unwanted T cell responses may occur that result in significant, potentially lethal, toxicity. To minimize safety risks, *in silico* assessment of pHLA epitope expression in various tissues and cell lines allows broad and deep screening for potential cross-reactivity and off-target toxicity. Expitope, was developed to identify pHLA epitopes that could be suitable targets for TCR isolation and prediction of pHLA binding affinities, as an important measure of target suitability. In addition, Expitope allows to identify related epitopes (up to 50% mismatch), and to evaluate their safety by assessing their expression patterns in healthy tissues.
Expitope 3.0 is a faster, fully revised version of this webtool including several new features:
1) New databases were added: the current Refseq database (125,000 unique sequences) was merged with Uniprot, Genecode and Ensemble, increasing the total number of unique gene sequences to 370,000. The databases ProteomicsDB, TCGA and the long read GTEx also enlarge the number of screened epitopes.
2) To improve the epitope prediction, netChops3.1 and netMHC4.0 were replaced by Pepsickle and MHCflurry2.0. Implementing the open-source Pepsickle allows the user more freedom and higher accuracy for determining either proteasomal or immunoproteasomal cleavage and presentation of epitopes. The added TAP matrix (04) allows Expitope 3.0 to choose the relevant information for its prediction model.
3) Expitope 3.0 represents the first version of Expitope to use machine learning to improve pHLA epitope prediction. The HLA Ligand Atlas (a compendium of pHLAs based on mass spectrometry data from 29 human tissues) was used to train Expitope 3.0 to predict the binding strength of the peptide for HLA allotypes, based on individual computed scores. Expitope 3.0 classifies every peptide as a “strong”, “weak” or “non-binding” epitope. Among a variety of machine learning approaches, the multilayer perceptron (MLP) performed best on classification (area under the curve 0.94) and was subsequently implemented.
Taken together, the upcoming publicly available Expitope 3.0 webtool allows quicker analysis of pHLA epitopes in antigens, with broader search of databases and higher prediction accuracy enhancing its use for safety assessments.

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T cell receptors equipped with ICOS demonstrate enhanced control of melanoma recurrence and T cell longevity upon adoptive therapy *in vivo*

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Adoptive therapy using a patient's own T cells engineered to express a new T Cell Receptor (TCR) enables specific recognition and killing of antigen-positive tumor cells and has already shown clinical successes in several solid tumor types. However, in most cases, these clinical effects are at best transient since tumor micro-environments generally do not provide co-stimulation to T cells, thereby halting their functioning and leading to non-durable anti-tumor responses. In this study, we have developed TCRs harboring different co-stimulatory molecules and tested these novel TCRs *in vivo* for their therapeutic efficacy in a melanoma mouse model.

These new TCRs contain human variable domains targetting the human gp100/HLA-A2 antigen, followed by murine TCR-C domain and intracellular domains of either CD28, ICOS, 4-1BB, CD40L or OX40 co-stimulatory molecules. This panel of co-stimulatory TCRs, as well as the wt TCR, were introduced in mouse T cells and tested for their effect on tumor recurrence as well as peripheral T cell persistence in an immune competent melanoma mouse model, expressing the target antigen. To assess the mechanism of action, we tested signaling-deficient co-stimulatory TCRs, such as the TCR:ICOS, in which tyrosine phosphorylation sites were mutated to hamper downstream activation. Anti-melanoma responses were correlated to the co-signaling and maturation phenotypes of circulating and tumor-infiltrating TCR+ T cells.

Co-stimulatory TCRs containing either CD28, ICOS or OX40 were surface expressed and significantly enhanced the anti-tumor response of adoptively transferred T cells. Delay in tumor recurrence was most pronounced by TCR:ICOS T cells, which demonstrated 140 days of relapse-free survival compared to 30-35 days for TCR:OX40 and TCR:CD28, and 22 days for wt TCR T cells. In addition, TCR:ICOS CD8 T cells showed the longest persistence in the blood where >100 cells/µL were detectable 10 weeks after transfer, compared to 30-60 cells /µL for TCR:OX40 and TCR:CD28, and 7 cells /µL for wt TCR T cells. These effects were abrogated upon introduction of a PI3K signaling-deficient mutation. Notably, TCR:ICOS CD8 T cells in regressing tumors were characterized by a young phenotype and expression of CD137.

Taken together, equipping T cells with a co-stimulatory TCR containing ICOS enhances control of melanoma recurrence and T cell longevity. These effects depended on ICOS-mediated activation of the PI3K pathway and were related to markers of T cell fitness. These findings put the TCR:ICOS forward as a candidate TCR format to enhance the efficacy and durability of adoptive T cell therapy.

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IL-12 induces transcriptional and epigenetic programming of TIL from pancreatic cancer favoring Th1 differentiation, tissue homing and polyfunctionality

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IL-12 mediates pleiotropic effects on the innate and adaptive immune system inducing Th1 differentiation with promising results in preclinical tumor models. Early clinical trials testing local or systemic administration of IL-12 in patients with cancer demonstrated that tolerated doses of IL-12 produce minimal benefit while higher doses induce toxicities that outweigh clinical responses. IL-12 has yet to live up to its clinical promise as compared to preclinical models. Cancer immunotherapy with tumor-infiltrating lymphocytes (TILs) faces the challenge of expanding highly functional T cells and tumor-reactive T-cells which show a Th1 response along with persistence in the tumor microenvironment (TME). Controlled Expression of IL-12 in adaptive cell therapy is a viable option. We tested therefore the effect of different exogenously added IL-12 doses on the quality and quantity of T-cell responses directed against molecular defined targets in pancreatic cancer. TILs were expanded from primary PDAC (pancreatic adenocarcinoma cancer) lesions and primed with high (1ng/mL) and low dose (0.01ng/mL) IL-12 for three days, followed by transcriptome and methylome sequencing. TIL priming with high dose IL-12 resulted in the differential expression of 600 genes, which included up-regulation of genes associated with Th1 polarization such as *CCR1*, *IFNG*, *GZMB* ($\log_2$ fold changes of 2.12, 2.60, 2.15) and IL-18-mediated responses (IL18R1 and IL18RAP, $\log_2$ fold change of 2.09 and 1.99, respectively). Up-regulation of *IL18R1* and *IL18RAP* ($\log_2$ fold change of 0.72 and 0.73, respectively) was observed even after priming with 0.01ng/mL of IL-12. Conversely, genes linked to T regulatory cells in the TME and impaired PDAC TIL function, such as *CCR8* and *CD160*, were down-regulated ($\log_2$ fold change of -1.58 and -3.04, respectively). Key epigenetic changes observed after priming with IL-12 a) were the hypomethylation of *BHLHE40*, which was accompanied by up-regulation of its mRNA in association with HIF1a. BHLHE40 is a transcription factor which has been associated with tissue residency, metabolic fitness, polyfunctionality and antigen-specificity of TILs; and b) hypomethylation of *CYTIP* and increased mRNA expression, which has been shown to lower the TCR activation threshold in T cells which have not yet encountered their nominal target antigen. TILs often exhibit metabolic dysfunction and exhaustion within tumors associated with different mechanisms of immune-suppression in the TME. IL-12-priming of TIL from pancreatic cancer lesions may therefore present a viable strategy to protect TIL from immune-suppressive factors and increase factors in TIL associated with tissue access, persistence, metabolic fitness and T-cell polyfunctionality in order to enhance the therapeutic potential of active cellular immunotherapy, while avoiding potential deleterious side effects associated with local or systemic IL-12 administration to patients with pancreatic cancer.

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The tumor-reactive CD8+ T-cell repertoire in human pancreatic cancer as revealed by single-cell sequencing

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Although pancreatic ductal adenocarcinoma (PDAC) is generally considered a poorly immunogenic cancer, we found clear evidence for a tumor-reactive T-cell response in samples of primary resected PDAC. Immunohistochemistry revealed prominent T-cell infiltrates in the majority (~ 70%) of tumor biopsies. These tumor-infiltrating lymphocytes (TILs) could be isolated and expanded *ex vivo* with similar efficiency as those isolated from melanoma. Furthermore, comparison of the T-cell receptor (TCR) repertoire between TIL and PBMC isolates from patients pointed at the selective expansion of T-cell subsets in the tumors.

More recently, we applied single-cell sequencing of TILs in combination with functional TCR-screening to dissect the natural tumor-infiltrating T-cell repertoire in nine surgically resected primary PDAC samples, including three genetically unstable tumors. In accordance with our previously published data, we identified multiple expanded T-cell clonotypes the TCRs of which mediated reactivity against autologous tumor cells in *in vitro* functional assays. Whereas the fraction of tumor-reactive clonotypes was highest in the genetically instable tumors, we also identified such clonotypes in all 6 regular PDAC samples.

In total, we performed functional TCR screening for 158 T-cell clonotypes, of which 93 mediated reactivity against autologous tumor cells. This data set, which also includes 65 bystander clonotypes, resulted in a gene signature that accurately predicts tumor-reactivity of T-cell clonotypes in PDAC samples. Importantly, this signature also effectively discriminates between tumor-reactive and bystander T-cell clonotypes in external single-cell sequencing TIL data sets of other tumor types, in particular primary lung cancer and metastatic melanoma, breast and colorectal cancers

Identification of the T-cell epitopes targeted by the natural tumor-reactive T-cell response will allow assessment of the potential of this T-cell repertoire for personalized therapy, as well as address the question to which extent mutanome-encoded neo-antigens play a role in this respect.

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A novel and unexpected target antigen for CAR therapy in metastatic osteosarcoma

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Osteosarcoma (OS) is an aggressive malignant tumour, mainly affecting young adults. The introduction of chemotherapy in the 1970's greatly improved survival rates, but since then no major breakthroughs have been made. Current chemotherapy remains the standard of care with a 54% and 71% event-free and overall survival for localized disease, respectively. OS frequently metastasizes, through the bloodstream and mostly into the lungs. Patients with primary metastatic disease have a very poor prognosis, as have those who relapse the first years after having completed adjuvant chemotherapy despite surgical resection of lung metastases. Thus, there is a clear need to identify specific drugs or innovative therapeutic approaches to detect and eliminate the chemotherapy resistant micrometastases. An emerging path to treat solid tumours is the use of immunotherapy, which has shown remarkable effects in some cancer types. The use of immune checkpoint inhibitors has been disappointing in OS, while the use of Chimeric Antigen Receptor (CAR) T cells still remains largely unexplored. Indeed, for CAR T-cell therapy to be efficacious and safe they should recognize an epitope selectively expressed on the cell surface of OS and OS metastases, but not on healthy tissues. In this study we validated two antibodies, TP-1 and TP-3, which were generated from hybridomas in our hospital more than three decades ago and shown to be highly reactive to biopsies and specific to OS tissues (90%), however their target remained unknown. We designed scFv based on their sequences, linked them to a second generation chimeric antigen receptor (CAR) signaling tail and called them OSCAR-1 and OSCAR-3.

By screening a library of more than 5000 membrane proteins, we first identified their antigen as an isoform of a common and ubiquitous protein whose membrane location seemed to be a hallmark of OS development, and hence a highly specific and hard-to-predict target. We next confirmed the antigen specificity by knock-in and knock-out of the target gene.

T cells transduced with OSCAR-1 and OSCAR-3 induced efficient and effective killing of OS cell lines

and OS metastases derived cells, but were not reactive to hematopoietic stem cells or any healthy tissues reported to express the target at mRNA level. The only exception was mesenchymal cell derived osteoblasts. We tested the CARs in three in vivo models and observed an efficient control of tumour growth and survival, even in very aggressive and metastatic OS models.
OSCARs are promising therapeutic molecules which are presently being submitted for clinical validation.

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Development of large-scale expansion protocol for NKG2C-positive NK cells for treatment of glioblastoma

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Natural Killer (NK) cells have proven as a promising candidate for immunotherapy of tumors. Glioblastoma, a WHO grade IV glioma and the most common primary adult brain tumor, express elevated levels of HLA-E and therefore block NK cells with expression of the inhibitory NKG2A receptor. Of note, glioblastoma cells also overexpress non-classical HLA-G, which provides a nonameric peptide that, in complex with HLA-E, has the highest affinity for NKG2A described so far. However, these HLA-E-peptide complexes are recognized by a small NK cell subset expressing the activating NKG2C receptor, preferentially found in human cytomegalovirus (HCMV)-seropositive donors. The NKG2C+ NK cell subset in peripheral blood mostly represent differentiated NK cells with high lytic capacity as well as less cytokine production and is therefore a potential candidate for immunotherapy of glioblastoma. However, low frequencies of NKG2C+ NK cells and therefore their low absolute cell numbers in peripheral blood limits further clinical use. In this project, we aim to establish a large-scale *ex vivo* expansion of functional NKG2C+ NK cells from peripheral blood employing bioreactors and using a recently developed PC-3 feeder cell line genetically engineered with interleukin 2 (IL-2), membrane-bound IL-15, as well as a first generation single chain trimeric HLA-E molecule. Isolated NK cells from healthy HCMV-seropositive donors were expanded in bottom-gas permeable membrane bioreactors for 11 days using different PC-3 feeder cell to NK cells ratios. NK cells were characterized for expression of NKG2A/C, activating/inhibitory receptors and for appearance of maturation and exhaustion markers by flow cytometry analysis. Co-cultivation of NK cells from 14 donors with feeder cells at a ratio of 10:1 resulted in the best mean expansion factor (40-fold ± 13.2 SEM) of total NK cells. Additionally, a selective expansion of NKG2C+ NK cells was achieved resulting in an NK cell product skewed to NKG2C+ cells (day 0: 7.5% ± 3.2% SEM; day 11: 57.9% ± 16.6% SEM). Expanded NK cells from 6 donors exhibited significantly increased numbers of CD16+, CD25+, CD57+ and KIR+ cells indicating maturation and a shift to memory-like NK cells. The NKG2C+ NK cells showed cytolytic activity against two primary glioblastoma cell lines which was significantly increased by KIR:HLA mismatch and when boosted by treatment with 50 IU IL-2. We have established a convenient protocol for selective and large-scale expansion of NKG2C+ NK cells,

which easily can be shifted to a GMP-compliant process. Our results furthermore demonstrate an efficient cytotoxicity of NKG2C+ NK cells primed with low doses of IL-2 towards primary glioblastoma cells. Further *in vivo* studies are warranted for future translation into the clinics.

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CAR Detection Reagents - Applications for flow cytometry, cellular enrichment, and microscopic imaging.

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Cellular immunotherapies represent without any doubt a key milestone in the treatment of various malignancies, and the development of new therapies is currently happening at an incredibly fast pace. Among those, the use of cells expressing a Chimeric Antigen Receptor (CAR) targeting hematological malignancies has shown remarkable efficacy in research and clinical applications. CAR cell therapies are based on genetically engineered immunologic effector cells, such as T cells or NK cells, expressing synthetic protein receptors tailored for precise molecular recognition of a defined cell surface antigen. Specific and reproducible detection of CAR expression is of utmost importance, not only for research and pre-clinical applications, but also for clinical manufacturing of cellular products and patient immunomonitoring. To fulfill these critical requirements, we developed CAR Detection Reagents for flow cytometry with outstanding quality and high specificity. Our antigen-based Detection Reagents mimic the CAR interaction with the tumor cells for the most relevant CAR T cell targets in the field, such as CD19, CD22, CD33, and BCMA. These biotinylated Fc-antigen homodimers allow high flexibility in panel design as well as superior brightness of CAR staining. This has been tested on model cell lines (SupT1) and on primary CAR T cells. Our Detection Reagents feature an engineered IgG1 Fc region devoid of binding to Fcγ receptors that allows background-free analysis as demonstrated by staining experiments on lysed whole blood. This is a functional prerequisite for reliable enumeration of CAR T cells even in settings with particularly sparse abundance, e.g., for patient immunomonitoring purposes. Our Detection Reagents can not only be used for precise flow cytometric CAR detection but also for the fast magnetic isolation of CAR cells from freshly drawn anticoagulated whole blood or PBMCs, as shown for CD19 CAR. Additionally, we developed anti-idiotype antibodies that can be used as specific identifiers for the CD19 FMC63 CAR, which is widely used for the most advanced CAR T cell therapies in the field, including commercial products. The CD19 FMC63 CAR anti-idiotype antibody was successfully conjugated to several fluorophores allowing fast and easy CAR T cell identification. Besides the application in flow cytometry this anti-idiotype antibody can also be used for imaging applications: Here we demonstrate the precise detection and enumeration of CD19 FMC63 CAR T cells in fresh-frozen splenic tissues isolated from tumor-harboring mice. These multifaceted applications demonstrate that CAR Detection Reagents are versatile tools for CAR T cell research and clinical therapies.

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Analysis of the bio-distribution and targeting efficacy of CAR T-cell therapy in a whole mouse body

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Chimeric antigen receptor-engineered T cells (CAR-T) therapies have been successfully implemented in clinical care for plasma cell malignancies. However, their usability is heavily restrained in solid tumors due to poor bio-distribution, low targeting efficacy, and immune suppression. Therefore, understanding the contribution of these factors is required to design and increase the effectiveness of future treatments for solid tumors.
Using our in-house clearing and imaging technique, we study the therapeutical effect of anti-EpCAM CAR T-cell therapy in a colorectal cancer mouse model. This technique allows for the visualization of thousands of micro-metastases not visible with standard imaging techniques in a whole mouse body. In combination with our mass-spectrometry-based proteomics (DISCO-MS) and deep learning-based pipeline (DeepMACT) technologies, we aim to investigate the bio-distribution of CAR T-cell therapy and the role of the immunosuppressive tumor microenvironment.
Our results show the dissemination of targeted and untargeted micro- and macro-metastasis in a whole mouse body. DeepMACT results provide quantitative information on the location, size, and shape, of all individual metastases. Additionally, immunolabelling of blood vessels and the lymphatic systems reveal possible routes for CAR T-cell penetration through tissues and organs. Finally, DISCO-MS results show differences between targeted and untargeted metastasis and their tumor microenvironment.
Our results show that whole-body analysis of treated cancer metastasis with clearing, omic, and deep-learning technologies is vital for a detailed understanding of the current roadblocks in CAR T-cell therapy.

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mRNAs encoding IL-12 and a decoy-resistant variant of IL-18 synergize to engineer T cells for efficacious intratumoral adoptive immunotherapy

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Poster Session II

New Targets & New Leads

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Interleukin-12 (IL-12) is a potent immunotherapeutic cytokine in mouse models which application as a systemic agent in the clinical setting is hampered by IFNγ-dependent toxicity. Engineering T cells with IL-12 is highly efficacious in mouse models but has resulted in serious adverse events in clinical settings. On the other hand, IL-18 is a myeloid-derived cytokine that elicits IFN-γ expression on T and NK lymphocytes. IL-12 and IL-18 are known to synergize in terms of eliciting massive IFNγ production. For cancer immunotherapy, IL-18 has the caveat of being down-regulated in its function by a decoy receptor termed IL-18BP, which is reportedly abundant in tumor tissues. Recently, a mutant sequence of mouse IL-18 termed DRIL18 which preserves its bioactivity but lacks binding to IL-18BP has been reported to exert T-cell-dependent antitumor activity upon systemic delivery. We previously reported that transient engineering of tumor-specific CD8 T cells with IL-12 mRNA enhanced their systemic therapeutic efficacy when delivered intratumorally. In this study, we sought to improve the therapeutic strategy of intratumoral delivery of T cells transiently engineered to express IL-12 with IL-18 mRNA electroporation.

We mixed $CD8^+$ T cells (TCR transgenic, TILs and CAR-T cells) engineered with mRNAs to transiently express either single-chain IL-12 (scIL-12) or an IL-18 decoy-resistant variant (DRIL18) that is not functionally hampered by IL-18BP. These electroporated $CD8^+$ T cells were mixed and injected repeatedly into mouse tumors for antitumor efficacy experiments. RNA-seq was performed to assess the functional changes induced after mRNA electroporation. Additionally, T-cell metabolic modifications and glycosylation profile functional changes were analyzed using seahorse and cell adhesion assays.

We observed that pmel-1 TCR-transgenic T cells electroporated with scIL-12 or DRIL18 mRNAs exerted powerful therapeutic effects in local and distant melanoma lesions. These effects were associated with T-cell metabolic fitness, enhanced miR-155 control of immunosuppressive target genes, enhanced expression of various cytokines and unique changes in the glycosylation profile of surface proteins, enabling enhanced adhesiveness to E-selectin. Efficacy of this intratumoral immunotherapeutic strategy was recapitulated using other clinically relevant adoptive T cell therapies as tumor-infiltrating lymphocytes (TILs) and CAR T cells upon IL-12 and DRIL18 mRNA electroporation

In conclusion, we report on a substantial improvement of adoptive T-cell therapies strategy based on mRNA transient gene-transfer and repeated intratumoral delivery. The synergistic immunobiology of IL-12 and IL-18, best represented in the form of DRIL18, holds promise for efficacious outcomes in the treatment of metastatic cancer patients.

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Robust anti-tumor activity of CAR-engineered umbilical cord blood CD34^{+} progenitor cell-derived NK against B-cell leukaemia provides new prospect for cancer immunotherapy

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Natural Killer (NK) cells present a highly promising ''off-the-shelf'' cell therapy opportunity for cancer patients considering their minimal side effects and great intrinsic cytotoxic capacity against several malignancies. Their potential can be further enhanced and focused by introducing chimeric antigen receptor (CAR)-expression to facilitate antigen-specific persistent tumor killing. Glycostem Therapeutics' *ex vivo* expansion and differentiation method in a fully closed, automated, feeder-free manufacturing platform, uNiK^{TM}, differentiates umbilical cord blood-derived CD34^{+} stem cells into functional allogeneic cryopreserved NK cells (oNKord®). Implementing a genetic engineering step to the uNiK^{TM} platform facilitates the generation of ''off-the-shelf'' antigen-specific viveNK™ cells.
In this proof-of-concept study, we provide the pre-clinical characterization of CD19-specific CAR-NK cells generated via LV transduction at the stem cell stage with various CAR cassettes consisting of different hinge, transmembrane and intracellular signaling domain combinations. Engineered cells showed high and stable surface CAR-expression (n=15 donors) equal or below vector copy number of 5/per cell, within a Multiplicity of Infection (MOI) range of 1-20. Strikingly, MOI5 is sufficient to achieve up to 91% CAR-expression. CD19-CAR-NK cells, including 1st generation CAR-cassettes, exerted significant CD19 antigen-specific degranulation, cytokine production and cytotoxicity against resistant B-cell leukemia cells at low effector:target ratios such as 1:1 and 1:3. Additionally, comparative mechanistic evaluation of CAR-NK cells in steady state and in action has been performed using several methods demonstrating antigen-specific cellular avidity, NK cell receptor phenotype and granule load, as well as single-cell RNA-sequencing-based profiling of CD19-CAR-NK cells. Intriguingly, our novel transduction step integrated to the uNiK^{TM} platform is also adaptable to a variety of different hematopoietic stem cell sources, including induced pluripotent stem cells. Finally, *in vivo* evaluation of CD19-CAR-NK cells selected cassettes against resistant B cell leukemia cells such as NALM-6 is currently ongoing.
Overall, our data demonstrate the applicability of an efficient engineering platform where highly functional, antigen-directed, ''off-the-shelf'' cryopreserved CAR-NK cells can be generated as a novel candidate for adoptive cancer immunotherapy against several hematological or solid tumors.

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Dendritic cells' role in ovarian cancer immunotherapy vaccine

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Dendritic cells plays a role in the immunotherapy of ovarian cancer. According to the current range of ovarian cancer immunotherapy approaches, it is appropriate to improve dendritic cells vaccines

(DCV) for better cancer management. We aimed to investigate allogenic DCV preparation with different ovarian cancer (OC) cell lines (A2780, SKOV3, COV362, OV7), their molecular profile, and their impact on dendritic cell maturation.
Flow cytometry analysis was used to determine the expression of CD44, CD73, CD105, CD274 stemness-related markers in cancer cell lines. Gene expression was assessed by RT-PCR in OC cell lines. DCs were matured with prepared lysates of ovarian cancer cell lines and their mixture. Flow cytometry analysis was performed after DC maturation, surface markers such as CD11c, CD80, CD83, MHCII, and CCR7 were analyzed. Evaluation of gene expression in DC was also performed by RT-PCR. Pearson correlation analysis was used to investigate the relationship of the ovarian cancer cell lines molecular profile and dendritic cells maturation level.
Studied OC cell lines have different molecular profiles. Individual OvCa cell lines and their mixture have different effects on the maturity of dendritic cells. The highest expression of genes associated with immunogenicity and the lowest with tolerogenicity – were achieved using a mixture of all OC cell lines lysates for the maturation of DC. The OV7 cell line with the mesenchymal and stem-like phenotype is able to induce the highest expression of maturation markers on DC. Genes encoding the major transcription factor for epithelial-mesenchymal transformation SNAIL1 and the multidrug resistance active transport carrier ABCG2 are strongly correlated with essential DL maturation markers CD80 and MHCII and CD86 and STAT1 transcripts ($p < 0.05$).
In early-phase clinical trials where it is not always possible to use the patient's autologous tumor material could be used the mixture of OC cell lines lysates for allogenic DCV preparation. Patient's tumor material and Single-cell transcriptional profiling will be further investigated to improve DCV immunotherapy of ovarian cancer.

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Generation of melanoma antigen-specific CD8$^+$ T cells from induced pluripotent stem cells

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Adoptive T cell therapy is a promising approach in personalized medicine based on the infusion of antigen-specific T cells, which target and specifically kill cancer cells. The basic concept involves the isolation of tumor-infiltrating lymphocytes, their modification, expansion *in vitro* and finally re-transfusion into the patient. However, the *in vitro* generation of sufficient quantities of tumor-specific T cells that do not exhibit an exhausted phenotype is difficult to achieve. Since induced pluripotent stem cells (iPSCs) have the potential to give rise to nearly unlimited amounts of any cell type of the three germ layers, including CD8$^+$ T cells, they could enable T cell-based immunotherapy on a large scale. In this context, we used fibroblasts from the *RET* transgenic mouse model of skin malignant melanoma, which is characterized by the overexpression of the human *RET* transgene in melanin-containing cells. We have successfully reprogrammed these fibroblasts into iPSCs by the Yamanaka factors via lentiviral reprogramming. We have also transfected these iPSCs with different Scaffold/Matrix Attachment Region (S/MAR) DNA vectors that carry melanoma antigen-specific T cell receptors (TCRs). For the differentiation towards the lymphoid lineage, we used a 2D co-culture system with bioengineered OP9 murine stromal cells. The co-culture of iPSCs on OP9 feeder cells was used to successfully generate hematopoietic stem cells (HSCs). Further differentiation of HSCs into

immature T cells was achieved by long-term co-culture with stromal cells expressing the Notch Delta ligand 1 (DLL1), which is crucial for T cell development and differentiation in the thymus. Stimulation of these immature T cells will generate terminally differentiated $CD8^+$ T cells, and their anti-tumor activity against melanoma antigens will be tested *in vitro*. In the same way, functional *in vivo* experiments will be carried out using the Ret transgenic mouse model. Generating almost unlimited melanoma antigen-specific $CD8^+$ T cells from iPSCs will take adoptive T cell therapy on a new level.

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A network of RNA binding proteins controls the expression of the CAR-T cells targets CD19 and CD20 in B-ALL

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CART-19 therapy has revolutionized the treatment of pediatric B-ALL patients in relapse under standard therapies. However, about 50% of the patients eventually become resistant to the treatment. 30-60% of those relapses lack the CD19 target antigen, localised on exon 2, due to mutations or aberrant splicing. In these patients, CD20 and CD22 are considered as alternative targets. Whereas CD19 is ubiquitously expressed, CD20 is expressed in circa 50% of cases, and CD22 in 80–90%.
RNA binding proteins (RBPs) modulate gene expression, for example by regulating the splicing. Here we analyzed RBPs expression using RNA seq data of 706 samples and using qRT-PCR analysis of normal B cells and B-ALL samples at initial diagnosis. CRISPR/Cas9-mediated knockout (KO) in the leukemic cell line 697 was used to assess the effect of selected RBPs on CD19 and CD20 expression. Expression of RBPs is deregulated in B-ALL patients in a subtype-dependent manner. Compared to normal B cells, *PTBP1* and *NONO* were less expressed in isolated leukemic blasts than in normal B cells. CD19 but not CD20 surface expression was significantly reduced after PTBP1 KO. CD19 isoform distribution changed upon PTBP1 KO. While the exon 2 wild type variant was less abundant, intron 2 retention (In2Ret) was significantly upregulated. A two-nucleotide deletion affecting the binding sites of PTBP1 was detected in 35% of B-ALL samples at initial diagnosis. Interestingly, the two-nucleotide deletion accumulated in B-ALL patients harboring the ETV6-RUNX1 gene fusion. Moreover, Intron 2 retention was increased in blasts compared to normal B cells at diagnosis. KO of NONO increased CD20 expression without affecting CD19 expression. High expression of *NONO* was found particularly in the hypodiploidy subtype.
Different RBPs are embedded in so-called mRNA regulons that coordinately regulate numerous cellular processes including immune response mechanisms. Deregulation of PTBP1, due either to expression changes or to alteration in binding capabilities, implies an accumulation of the CD19 epitope-negative splicing variant In2Ret that finally result in decreased levels of CD19 protein. NONO affects CD20 expression. It is conceivable that disease- as well as patient-specific features define the isoform distribution leading to the accumulation of therapy-resistant CD19 and CD20 variants.

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Nanosystems and T cell therapy for cancer: potential applications

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Some T-cell cancer immunotherapies, like chimeric antigen receptor (CAR) T-cell therapy, require gene-modified T cells to redirect their antigen specificity and overcome tumor escape mechanisms. The T-cells genetic modification has been done through viral vectors, which is associated with safety concerns, high cost and production challenges, and more recently also through electroporation, which can be extremely cytotoxic. In this context, nanosystems constitute an alternative to overcome the challenges associated with current methods, resulting in a safe and cost-effective platform. In this work, we proposed to develop polymer-based nanosystems to efficiently deliver genetic material into T cells, with reduced cytotoxicity. A panel of polymers were screened as potential nanoplatforms for T cell engineering, using Jurkat cell line and primary T cells as models. These nanosystems were prepared in different polymer/genetic material ratios, submitted to physicochemical characterization, and evaluated in terms of transfection activity and toxicity. Their cellular internalization was also analyzed. The best-performing formulation achieved high biological activity and reduced toxicity, when compared to the gold standard bPEI-based polyplexes, even in primary T cells. The developed nanosystems resulted in high cellular uptake, being observed inside almost 80% T cells. Moreover, they were able to deliver different types of genetic material to T cells, and presented a size of 150 nm, high capacity to complex the genetic material and zeta potential of +30-35 mV. The developed nanosystems not only exhibited suitable physicochemical properties for gene delivery, but also proved to be an effective strategy for T-cell engineering. The positive charge of the polymeric mixtures led to strong electrostatic interactions with the nucleic acids, promoting the formation of stable and small polyplexes that resulted in high internalization in T cells. With their continuous optimization, they may have a strong potential for future clinical applications, improving the current T-cell-based cancer treatments.

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Combining electroporation and lentiviral transduction for highly efficient gene engineering in primary CAR NK cells for cancer immunotherapy

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Although significant progress has been made in treating cancer, still various challenges remain. Natural killer (NK) cells are specialised effectors of the innate immune system and exhibit potent anti-tumour activity. Promising clinical outcomes have been achieved in adoptive immunotherapy with alloreactive NK cells. However, remissions induced by adoptively infused NK cells are only transient, as tumour cells evade the innate anti-tumour activity. To tackle these different immunoevasive mechanisms in the recent years more approaches have been developed to enhance

the anti-tumour reactivity of immune cells via genetic engineering. Providing NK cells with antigen specific cytotoxicity via chimeric antigen receptors (CARs) via lentiviral transduction showed effectiveness against haematological malignancies in preclinical studies. The CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 (CRISPR-associated) ribonucleoprotein (RNP) system allows site specific gene-editing. Herein, we introduce a protocol allowing efficient genetic engineering of peripheral blood-derived primary human NK cell combining both methods to genetically engineer NK cells. The protocol enables flexible electroporation for transfection at various different time points after cell isolation, even without prior cell activation. This methods enables introduction of various different genetic modifications in different ways thereby diversifying potential genetic modifications. As an example this protocol was used to combine CAR-directed cytotoxicity after transduction with BaEVRless lentiviral particles with a knock-out of the inhibitory receptor NKG2A, reducing immunosuppressive signalling of tumour cells via HLA-E, resulting in an enhanced tumour cell lysis both in the presence and absence of the CAR target. In the future, this protocol may pave the way for the generation of potent NK cell products with sophisticated genetic modifications for therapeutic use.

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Human leukocyte antigen (HLA) matching of cancer patient-derived xenografts (PDX) models with immune cell-humanized mice

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Matching of human leukocyte antigen (HLA) profiles of patient-derived xenografts (PDX) with human immune cell populations that are used for humanization of mouse models is a prerequisite for testing immune checkpoint inhibitors and mimics closer the clinical situation.
In a first step, we analyzed individual HLA profiles of a broad panel of established PDX models from 18 different tumor entities and compared it to the HLA frequencies and haplotypes of the German population. For that, HLA class I, II and non-class types of 291 established PDX models were determined in 4-digit resolution based on RNA-sequencing data. For comparative analyses to the German population, HLA allele and haplotype frequencies of 8862 German stem cell donors (GSCD) provided by The Allele Frequency Net Database were engaged. PDX HLA profiles were comparable to GSCD HLA profiles regarding frequencies of most of the alleles and haplotypes but also in certain variations. We observed allele homozygosity at ≥ 1 HLA class I loci in more than 50 % of the determined individual HLA profiles of PDX models which is associated with metastatic cancers or decreased survival rates. In a second step, known peripheral blood mononuclear cell (PBMC) donors were analyzed for HLA profiles and a HLA matching to the PDX models was performed according to the donor-recipient HLA matching criteria recommended by the Blood and Marrow Transplant Clinical Trials Network. HLA matching in HLA-A, -B, -C and DRB1 revealed 15 matches including PDX from 9 tumor entities and currently 10 known blood donors. Lastly, HLA-matched PDX and immune cells were engaged in an in vivo model and tested for the response to checkpoint inhibition. For that, mice were transplanted with two head and neck squamous cell cancer models and humanized with HLA-matched PBMCs after tumor engraftment. Check point inhibition was performed with

Nivolumab which showed strong tumor growth inhibition in one model and moderate tumor growth inhibition in the second model in a HLA-matched setting. Further PDX models in a HLA-matched setting are under investigation for the response to immune checkpoint inhibition.
The generated comprehensive HLA profile portfolio containing matching information on a broad panel of PDX models and PBMC donors supports the investigation of prognostic markers and enables personalized, preclinical immune-oncology studies to encourage the development of novel immune-therapeutic strategies.

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Modulation of the tumor microenvironment by genetically engineered CAR-NK cells

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Natural killer (NK) cells play a critical role in antitumor immunity due to their cytotoxic and immunomodulatory effector functions. To enhance their therapeutic potential, NK cells can be modified to express chimeric antigen receptors (CARs) that facilitate selective recognition and killing of tumor cells. We previously generated the continuously expanding HER2 (ErbB2)-specific CAR-NK cell line NK-92/5.28.z, which is currently investigated in the CAR2BRAIN phase I clinical trial (NCT03383978) in patients with recurrent HER2-positive glioblastoma (GB). In immunocompetent mouse GB models, NK-92/5.28.z cells in addition to direct tumor-specific cytotoxicity induced endogenous antitumor immunity resulting in cures and long-term protection against rechallenge.
To better overcome immunosuppressive effects of the tumor microenvironment (TME) and further enhance the immunomodulatory activity, here we developed variants of the CAR-NK cells in which we either blocked the secretion of anti-inflammatory IL-10 with an endoplasmic reticulum-retained intracellular IL-10 antibody (anti-IL10ER), or expressed a PD-L1-targeted IL-15 immunocytokine (anti-PDL1-RD-IL15). Trapping IL-10 in activated NK-92/5.28.z cells prevented polarization of cocultured macrophages towards an immunosuppressive M2-like phenotype and supported maturation of dendritic cells *in vitro*. Immunocytokine secreting CAR-NK cells stimulated growth in the absence of IL-2 in an autocrine fashion, blocked the PD-1/PD-L1 immune checkpoint and activated cytotoxicity of bystander immune cells. In an immunocompetent GB mouse model, in comparison to unmodified NK-92/5.28.z cells treatment with the functionally enhanced CAR-NK cells increased the number of tumor-infiltrating immune cells and further reduced the amount of glioma cells. Our data demonstrate induction of a more pronounced pro-inflammatory immune response by the modified NK-92/5.28.z variants, indicating an enhanced therapeutic potential.

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Identification and functional analysis of tumor-reactive CD4+ effector and regulatory T-cell subsets in human pancreatic cancer

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Pancreatic ductal adenocarcinoma (PDAC) is a highly malignant disease for which surgical resection is the only effective treatment to date. The devastating recurrence rate limits the long-term impact of this treatment and calls for effective adjuvant modalities. Unfortunately, the tumor is resistant to cytostatic and targeted regimens. Although PDAC has generally been considered a cold, poorly immunogenic cancer, we and others found clear evidence for a tumor-reactive T-cell response. Nevertheless, it is evident that this response is too weak to be effectively mobilized by means of currently available immunostimulatory regimens, such as immune checkpoint blockade. We are therefore focusing on the development of personalized T-cell therapy involving the transfer of patient-derived, tumor-reactive TCRs into autologous T-cells.
Dissection of the intratumoral T-cell repertoire in 9 primary human pancreatic cancer samples by means of single cell sequencing not only resulted in the isolation of multiple tumor-reactive T-cell receptors (TCRs) from HLA class I-restricted CD8+ T-cells, but also from HLA class II-restricted CD4+ T-cells. Based on transcriptome profiles, we can distinguish between TCRs derived from CD4+ effector T-cells and CD4+ FOXP3+ T-regulatory cells. Interestingly, both show strong anti-tumor reactivity in *in vitro* assays, as well as highly effective tumor cell killing by native CD4+ and CD8+ T-cells.
In view of the importance of CD4+ T-cell help, in addition to CD8+ effector T-cells, for achieving lasting, therapeutic anti-tumor responses, we are currently further evaluating the merit of CD4+ T-cell derived TCRs from the natural tumor-reactive T-cell repertoire for personalized T-cell therapy. Key questions in this respect are (i) whether the target epitopes represent 'private' or recurrent tumor antigens and - in the case of recurrent antigens – (ii) whether these antigens are (sufficiently) tumor-specific.

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Engineering the stromal compartment of solid tumors circumvents resistance to immune checkpoint blockade by sparking anti-tumor immunity

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Conventional type 1 dendritic cells (cDC1) cDC1 infiltration into the tumor microenvironment (TME) and tumor-to-lymph node trafficking correlates with favourable outcomes and clinical responses to immunotherapy. Depletion studies in preclinical models have revealed that cDC1s play a key role in both CD8 + T-cell activation within the TME. FLT3L is a growth factor for cDC1s supporting their differentiation from HSCs, terminal differentiation within tissues, proliferation and survival. Here, we present a new immunotherapeutic approach based on local delivery of FLT3L within solid tumors. We show that intra-tumoral engraftment of autologous engineered mesenchymal stromal cells engineered to express membrane bound FLT3L (eMSC-FLT3L) efficiently stimulate cDC1 infiltration when combined with the TLR3 agonist poly(I:C). Engraftment of eMSC-FLT3L supports sustained infiltration by DCs, promotes lymph node trafficking and the cross priming of tumor-specific CD8+ T cells. As a result, eMSC-FLT3L+poly(I:C) therapy induces T cell-dependent tumor regression and circumvents resistance to CTLA4 and PD1 blockade characterizing the hard-to-treat B16 melanoma model. Altogether, these data support the immunotherapeutic potential of intra-tumoral engraftment of engineered, autologous mesenchymal stromal cells to induce anti-tumor immunity.

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CellFit: T cells fit to fight cancer

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The use of T lymphocytes in adoptive cell therapy (ACT) shows great promise for hard-to-treat cancers. Nevertheless, one of the largest challenges faced in cell-based cancer therapy is to provide an efficient and scalable production. The use of "living drugs" leads to the development of promising therapy but requires precise logistics at all stages of cell life: development, manufacturing, transport and finally the infusion to the patient.
Presently, only six chimeric antigen receptor (CAR)-based T-cell products have been approved for clinical use in haematological malignancies. Whilst the main focus of development has been on finding tumour targets and improving the genetic modifications of the immune cells, less effort has been made to efficiently produce the optimal subset of immune cells, which ideally is more **homogenous** and **stem-cell like,** for treatment. Indeed, it is now clear that the **quality, efficacy, and longevity** of T-cell immunity depend on the differentiation of naïve T cells (T_N) into phenotypically distinct subsets with specific roles in protective immunity. These include memory stem-like (T_{SCM}), central memory (T_{CM}), effector memory (T_{EM}), and highly differentiated effector (T_E) T cells. Less

differentiated cells like T_{CM} and T_{SCM} have been shown to respond more persistently against cancer cells. Currently, the *ex vivo* T-cell manufacturing is very different from physiological T-cell expansions occurring *in vivo* when T cells encounter antigen and does not generate the T-cell subsets that provide long-term therapeutic efficacy in solid cancers. To realise broader clinical applications for efficacious ACT and enhance the current therapeutic approach, the immunotherapy field has a great need to further improve therapeutic T-cell manufacturing methods.
In this project, we aim to define optimal growth conditions for improved manufacturing of adapted therapeutic T cells required for solid tumour treatment. The T-cell culture methods will be translated from manual to robotic set-ups where all pipetting, cell culturing and assay read-outs will be performed in 96-well plate formats with robots coupled to incubators, spectrophotometer and high-content confocal microscope. Moreover, we will test a panel of phenotypic markers using state-of-the-art technologies such as mass cytometry (CyTOF) and cellular metabolism (Seahorse analysis) to be able to compare different culture conditions and characterise the heterogeneity of the T-cell population. The data acquired by these high-throughput systems will be analysed with a customized pipeline designed for this project and including various modern computational cytometry techniques. We will present the pipeline of the project to establish an optimal T cell product and a high throughput screening platform for cellular therapies.

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Ex vivo modification of haematopoietic cell transplants in GvHD prevention: Revealing the mechanism of action of the anti-human CD4 antibody MAX.16H5 for induction of immune tolerance

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Haematopoietic cell transplantation is the only curative approach for many haematologic malignancies. However, over 30% of patients receiving an allogeneic transplant develop Graft-versus-Host disease (GvHD) as a secondary disease. Current therapeutic options against GvHD are limited and induce a broad immunosuppression that increases the risk of infections and cancer relapse. By *ex vivo* incubation of haematopoietic cell transplants with the anti-human CD4 antibody MAX.16H5, we investigated an alternative therapeutic approach that aims at inducing an antigen-specific immunological tolerance, while maintaining the integrity of the immune system.
Previous studies have shown that treatment with MAX.16H5 attenuates GvHD *in vivo*. In order to investigate the underlying molecular mechanism, primary CD4+ T cells were stimulated and characterized *in vitro* regarding proliferation and T cell activation using 3H-thymidine- and flow cytometry-based approaches, respectively. In addition, mRNA sequencing was performed on isolated CD4+ T cells post stimulation for differential gene expression analysis and subsequent bioinformatic evaluation.
Preincubation of CD4+ T cells with MAX.16H5 was able to reduce the proliferation by over 50% and lower the cell surface expression of the T cell activation marker CD25. Furthermore, mRNA

sequencing revealed a preferential regulation of cell cycle-related genes by MAX.16H5 and a strong correlation of gene regulation indicating impaired T cell receptor signalling.
By targeting cellular processes relevant for GvHD progression, we could demonstrate *in vitro* functionality of MAX.16H5 for the first time. Transcriptome analysis supported our *in vitro* findings and indicated impaired T cell receptor signalling as primary mechanism of action. Thus, MAX.16H5 could potentially be used as an effective agent not only in the treatment of GvHD, but also in the control of exaggerated T cell-mediated immune responses in a variety of indications (e.g. autoimmune diseases).

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Antigen-specific solutions for T Cell Therapy Development and Manufacturing.

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The antigen-specific interaction between the T cell receptor (TCR) and cognate peptide MHC (pMHC) complex is at the heart of the development of successful T cell-based therapies. Understanding and characterizing the TCR:pMHC interaction is key to being able to demonstrate efficacy and investigate potential side effects of CAR & TCR cell therapies. We demonstrate that Dextramer® technology can be applied as an assay for unbiased quantification of engineered T cells, as well as assessment of T cell identity and purity. We discuss how Clinical-Grade Dextramer® (GMP) reagents can be applied in the Quality Control and Lot Release Testing of CAR-T and TCR-T cell therapeutics, as well as in tracking infused T cells in patients. We propose a workflow to screen for TCR cross-reactivity and identify potential off-target reactivity in order to mitigate the risk of side effects and toxicity in subsequent clinical trials. Finally, we discuss tumor escape, and present the TCR Dextramer technology as a tool which could potentially address this challenge by investigating levels of the target pMHC in tumor tissue. We present results from a study of TCR target recognition using TCR Dextramer®. The study investigates the impact of TCR:pMHC Affinity on TCR binding using an *in vitro* cell surrogate system to examine the binding of three TCR candidates to the target NY-ESO1 in a T2 cell line.

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Clonotype and transcriptome analysis of adoptive TIL products allows insight into T cell behaviour to improve future processing approaches

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Adoptive cell therapy (ACT) using tumor infiltrating lymphocytes (TILs) has shown to be an effective treatment for some patients suffering from advanced melanoma. However, less than 50% of the patients are responding which partially depends on the ability of tumor-reactive T cells to proliferate and persist *in vivo*. T cells with early memory differentiation are therefore preferable for TIL therapy

as these cells might show increased proliferation and persistence potential. Not only the differentiation state but also the antigen specificity of T cells is crucial for a successful treatment. It has been shown that during *in vitro* expansion tumor-dominant T cells can be overgrown by newly emerged clones that are present only in low numbers in the initial tumors.
The aim of this study is to examine the behavior of tumor-reactive clonotypes during TIL therapy. For this purpose, single cell RNA sequencing was used to determine TCR sequences and gene expression profiles of tumor-infiltrating lymphocytes before and after the expansion as well as after cell transfer in peripheral blood or metastasis. Our data suggests that during expansion only a particular subtype of T cells expanded showing a more activated and naive expression profile compared to non-expanded cells. Moreover, it was possible to identify tumor-reinfiltrating clones after TIL transfer for a more comprehensive analysis. This indicates that tumor infiltration after ACT can inform about anti-tumor effector function of certain clones. To correlate this with *in vitro* anti-tumor effector function, an activation assay is being established, in which autologous tumor material is co-cultured with expanded TILs. Altogether, these findings can be used to determine factors to potentially predict response to TIL therapy and improve future TIL processing.

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The therapeutic potential and limitations of mesothelin-directed CAR T cells for the treatment of ovarian cancer

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Chimeric antigen receptor (CAR) T cell therapy targeting CD19 has caused a paradigm shift for the treatment of hematological malignancies. Translation of this success to solid tumors has proven to be troublesome with marginal clinical efficacy reported. Mesothelin (MSLN) has emerged as an attractive target for CAR T cells in solid malignancies, including ovarian cancer. The solid tumor niche imposes various hurdles for successful CAR T cell therapy including antigen heterogeneity, limited homing to the tumor site and the immunosuppressive tumor microenvironment. In this challenging environment, CAR design is crucial. Here, we evaluate the therapeutic potential of three different 2nd generation MSLN-directed CAR constructs and the mechanisms impacting CAR T cell functionality in relevant models of ovarian cancer.
Two conventional 2nd generation MSLN-CAR constructs were included, composed of a 4-1BB (MBBz) or CD28 (M28z) co-stimulatory domain followed by the CD3ζ chain, and a novel construct combines CD28 co-stimulation with tuned activation potential through mutations in the CD3ζ chain (M1xx). MSLN-CAR T cells were evaluated in various *in vitro* and *in vivo* models, both orthotopic and disseminated mouse models of ovarian cancer were used.
MBBz, M28z or M1xx CAR T cells evoked powerful anti-tumor responses in preclinical models of ovarian cancer. However, M1xx CAR T cells elicited superior tumor control as compared to the

conventional 2nd generation CAR constructs. In the orthotopic model, M1xx CAR T cell treatment induced persistent tumor clearance and long-term remissions in all mice. Treatment with M1xx CAR T cells delayed tumor progression significantly, but mice eventually relapsed in the disseminated disease model. *Ex vivo* analysis of tumor cells revealed an MSLN-CAR T cell driven loss in cell surface expression of the MSLN antigen. In parallel, we detected intratumoral MSLN+ trogocytotic M28z, MBBz and M1xx CAR T cells. These findings were confirmed *in vitro*, as MSLN surface expression decreased steeply on tumor cells following exposure to MSLN-CAR T cells while high level of MSLN+ trogocytotic CAR T cells were detected. Exhaustion marker expression was higher in trogocytotic than non-trogocytotic MSLN-CAR T cells. Importantly, the degree of trogocytosis negatively correlated with tumor cell lysis and MSLN-CAR T cells were capable of fratricide killing.
In conclusion, MSLN-CAR T cells and in particular M1xx CAR T cells demonstrated therapeutic potential for the treatment of ovarian cancer. Calibration of MSLN-CAR T cell activation resulted in enhanced functional persistence as compared to the conventional 2nd generation M28z and MBBz constructs. Tumor antigen escape poses a major risk for limited MSLN-CAR T cell persistence. Trogocytosis negatively affects functional persistence of MSLN-CAR T cells by promoting tumor antigen escape and fratricide killing.

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Studying the role of α2,6-linked sialic acid in human dendritic cells and the potential of genetically glycoengineered dendritic cells for cancer immunotherapy

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Dendritic cells (DCs) play key roles in the immunological network as mediators between innate and adaptive immunity and thus holds a, so far unrealized, potential for cancer immunotherapy. Based on studies using broad acting sialidases or metabolic inhibition, sialylation has been suggested to play regulatory functions in DC biology with reported effects on activation, maturation, and tumor antigen presentation.[1–3] Furthermore, DC maturation is associated with reduced expression of α2,6-sialyltransferase 1 (ST6GAL1)[2,4,5], suggesting that glycoengineering DCs to specifically lack α(2,6)-linked *N*-glycan sialylation through knockout (KO) of ST6GAL1 may improve their maturation and thus optimize the immunotherapeutic potential. The human acute myeloid leukemia cell line MUTZ-3 can be differentiated and matured in a cytokine dependent manner to fully functional human dendritic cells.[6,7]
Here we established a workflow enabling successful genetic glycoengineering of MUTZ-3 using CRISPR-Cas9 and report the effects of ST6GAL1 knockout (ST6GAL1-/-) on MUTZ-3 DC biology. Sanger sequencing and cell surface lectin staining confirmed a 90-99% knockout efficiency of ST6GAL1 as well as stability and continued functional knock-out of ST6GAL1-/-.
Transcriptional- and flow cytometric analysis show that ST6GAL1-/- precursors have a more stem cell like phenotype with increased CD34 expression and decreased CD14 expression compared to WT. Nevertheless, ST6GAL1-/- cells respond to cytokine-induced differentiation by upregulation of CD11c, CD209, CCR6, CD1a, CD80, CD40, and CD86, as well as downregulation of CD34, while maintaining an immature phenotype (iDC, $CD83^-$ and $CCR7^-$). Compared to WT, ST6GAL1-/- iDCs express higher levels

of CCR6 and migrate more efficiently towards the CCR6 ligand CCL20 *in vitro*. Furthermore, ST6GAL1-/- iDCs express more costimulatory and antigen presenting receptors including HLA-DR, CD1a, and CD80. A reduced expression of CD209 in ST6GAL1-/- compared to WT also correlates with reduced endocytic capacity. Upon activation of iDCs with a high-dose TNFα we surprisingly found, that ST6GAL1-/- exhibited fewer mature DCs (mDCs, CD83+ and CCR7+ cells, mDCs) and lower upregulation of the co-stimulatory receptor CD80. Furthermore, TNFα activated ST6GAL1-/- DCs secreted lower amounts of IL-6 and sustained a higher expression of CCR6 while failing to upregulate CCR7, resulting in a skewed migration pattern.
Thus, while ST6GAL1-/- MUTZ-3 DCs may not provide the expected phenotypical improvements, our data confirm important regulatory functions of α(2,6)-linked sialic acid in DC biology.

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Short amino acid linker in the extracellular antigen recognition domain of CD137-based anti-BCMA CAR for improved T cell therapy in multiple myeloma

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In multiple myeloma (MM) patients, B cell maturation antigen (BCMA)-directed chimeric antigen receptor (CAR) T cell therapy has emerged as a novel therapy option with potential for long-term disease control. Anti-BCMA CAR T cells with a CD8-based transmembrane (TM) domain and an intracellular co-stimulatory CD137 (4-1BB) domain have already been clinically approved. Clinical efficacy might be still reduced, even after initial responses. For other CAR, it has been reported that a short amino acid linker in the extracellular single chain variable fragment (scFv) region of CD137-based CAR can result in intermolecular CAR interactions promoting autonomous CD137 signaling which in turn leads to T cell priming and enhanced anti-tumor efficacy. Here, we investigated the impact of this short amino acid linker for anti-BCMA CAR. CD8TM.CD137-based CAR vectors with either a long or a short linker between the light (VL) and the heavy (VH) chain of the scFv part, CD28TM.CD137-based and CD28TM.CD28-based CAR vector systems were generated and used in primary human T cells. MM and B cell malignancies expressing the respective BCMA in different intensities were used as target cell lines for the experiments. Each CAR construct was transduced into human T cells with a mean transduction efficiency > 50%. Killing capacity against MM and B cell lymphoma cell lines showed a trend towards a superior cytotoxicity for CD28TM.CD28-based CAR T cells compared to the other anti-BCMA CAR constructs. There was no clear difference between CAR T cells with a short and a long linker. Whereas evaluation of *in vitro* cytokine production showed a trend towards higher IFN-gamma production for short-linker CAR T cells compared to long-linker CAR T cells. Expression of CD69, PD-1 and LAMP-1 (CD107a) upon activation by recombinant BCMA and

the *in vitro* proliferative capacity after one week of co-culture with tumor cells ($p < 0.02$) were superior for CD28TM.CD28-based anti-BCMA CAR T cells compared to the other anti-BCMA CAR constructs. There was no significant difference between CAR T cells with a short and a long linker. Likewise, in an *in vivo* xenograft mouse model, CD28TM.CD28-based anti-BCMA CAR T cells led to superior killing of MM cells (U-266) compared to the other constructs, whereas all constructs cleared the tumor. We currently conduct long-term co-culture experiments and re-challenge tumor-free mice with U-266 to investigate potential prolonged effects and persistence of short-linker CAR T cells. Shortening of the amino acid linker between the VL and VH chain of the scFv part of a CD8TM.CD137-based CAR is promising for CAR T cell therapy. However, the approach could not significantly improve the clinically approved anti-BCMA CAR construct. It is still unclear which CAR vector and target antigen profit from this strategy. This highlights the need for further development of new vector systems to optimize CAR T cell therapy.

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A basket approach for customized off-the-shelf CAR-Target-EBV: CAR-T cells armed via genome editing against tumors associated with EBV

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CAR-T cells targeting CD19 are approved for immunotherapy of relapsed lymphomas. Lymphomas caused by Epstein-Barr virus (EBV) display particular anti-apoptotic mechanisms and immuno-evasive pathways depending on the latency type. EBV^+ lymphomas are highly heterogeneous, can be very aggressive, and have not been extensively considered as indications for CAR-T cell treatment. Furthermore, EBV^+ lymphomas often are not $CD19^+$, such as in EBV^+ Hodgkin lymphoma (HL). We propose to develop modular non-viral CRISPR/Cas gene-editing to target CAR-T cells against different types of EBV^+ lymphomas: $CD19^+CD20^+$ Burkitt lymphoma (BL), EBV-$gp350^+CD30^+$ post-transplant lymphoproliferative diseases (PTLD), $CD30^+$ HL and $CD30^+$ peripheral T cell lymphoma (PTCL). We initially evaluated and compared the effects of CD19CAR.CD28z-T and gp350CAR.CD28z-T cells against BL (Braun, Pruene et al, in revision). We used CRISPR/Cas9 methods to knock-in (KI) the CD19CAR.CD28z or gp350CAR.CD28z into the T cell receptor (TCR) alpha chain (*TRAC*) locus. For both CARs, the KI efficacy was roughly 20%. Both types of CAR-T cells caused cytotoxic effects against EBV^+ BL lines *in vitro*, and showed upregulation of the activation / exhaustion markers PD-1, LAG-3 and TIM-3. $CD19^{KI}$CAR-T cell treatment of mice challenged with EBV^+ Jiyoye/fLuc-GFP cells and developing BL showed systemic partial therapeutic responses and accumulation of $CD4^+FoxP3^+$ Tregs in the bone marrow. We are currently developing gene-edited CAR-T cells targeting CD20 or CD30 in order to evaluate which 'basket' of combinatorial CAR-T cells can show the highest efficacy against different types of EBV^+ lymphomas. Further, multiplex editing will be developed to knock-out genes involved with EBV's immunosuppressive pathways in T cells. In parallel, we are developing xenograft and humanized mouse models recapitulating different types of EBV^+ lymphomas for *in vivo* testing. Taking in account the significant global impact of EBV-related cancers, we foresee a broad application of a basket approach for customized

off-the-shelf CAR-Target-EBV in order to satisfy the demand for a sustainable, cheaper and fast production of the novel cell therapy.

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Machine learning-based prediction of tumor-reactive T cell receptors from tumor-infiltrating T cells

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Personalized adoptive cell therapies (ACT) utilize autologous tumor-reactive T cell receptors (TCR) to target patient-specific (neo)antigens presented by human leukocytes antigen (HLA) molecules. Traditionally, suitable tumor (neo)antigens are first identified and used to expand and screen for antigen-specific T cells. This current state-of-the-art method, however, is limited to *ex ante* definition of the type and immunogenicity of tumor (associated) antigens. In addition, the process of screening for antigen-specific T cells is time- and cost-intensive and thus difficult to integrate in a clinical and therapeutic workflow.

To circumvent these limitations, we have developed an antigen-agnostic, TCR-centric and Machine Learning (ML)-based approach to identify tumor-reactive TCRs from tumor-infiltrating T cells (TIL) using combined single cell (sc) VDJ- and RNA-sequencing (SEQ). We generated training data from TILs extracted from a melanoma brain metastasis, cloning 88 TCRs from different transcriptomic clusters and experimentally testing for tumor reactivity in co-culture assays using autologous patient-derived cell lines.

Using the transcriptomic and reactivity data, we trained a classifier to identify tumor reactive TILs based on the transcriptomic profile. The classifier was then validated using six published external datasets consisting of 28 patients with diverse cancer types for which scRNA and scVDJ-SEQ as well as TCR reactivity data were available. Compared to canonical genes thought to distinguish tumor-reactive T cells such as CD69 (F1 Score: 0.32) and programmed death 1 (PD1) (F1 Score: 0.55), our classifier (F1 Score: 0.89) predicted tumor-reactivity with much higher accuracy.

Even though our classifier was trained using TILs derived from primary and secondary brain tumors, which are considered an immune privileged and immunologically cold organ, our results show that our classifier was able to predict tumor reactivity of TCRs with high accuracy in other tumor entities such as lung and metastatic colorectal cancers processed in other labs. In addition, despite training mainly on CD8 T cells, our data also show that our classifier accurately identified vaccine-induced, neoepitope-specific CD4 T cells from tumor-infiltrating T cells.

Taken together, our data show the potential of a ML-assisted TCR-centric approach to identify tumor-reactive TCRs from TILs. This universal classifier may provide a basis for the development of a personalized antigen-agnostic ACT using tumor-reactive TCRs.

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TGF-β1 receptor inhibitor SB-431542 inhibits proliferation of metastatic breast carcinoma cells and enhances anti-tumoral effects of Doxorubicin

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Transforming growth factor beta 1 (TGF-β1) inhibits the growth of tumor cells at the early stage, but once aggressiveness of the tumor increases, TGF-β1 enhances metastatic potentials of cancer cells. In accordance, we previously reported that breast cancer cells metastasize to visceral organs (4TLM and 4THM) and brain (4TBM) secretes markedly higher levels of TGF-β1 compared to non-metastatic cells. In addition, levels of several proteins that enhances TGF-β1 activity such as Fibulin-4, BMP-1 and LTBP-1 were markedly higher in metastatic cells (1). TGF-β1 inhibitors are in clinical trials for breast cancer treatment and results are promising. On the other hand, possible effects of TGF-β1 inhibitors on metastatic cells alone or in-combination with doxorubicin, a commonly used chemotherapeutic for breast cancer is not directly examined. SB-431542, a potent and selective inhibitor of the TGF-β1 receptor, was used to treat subsets of brain and liver metastatic subset of 4T1 breast cancer cells (4TBM and 4TLM respectively). In addition, human breast carcinoma cell lines (MDA-MB 231 and MCF-7) were also included in the study. Both acute and sub-chronic effects of SB-431542 alone and in combination with doxorubicin were determined using cell proliferation, colony and spheroid formation assays. SB-431542 treatment for 10-12 day enhanced the anti-proliferative effects of doxorubicin and decreased TGF-β1 secretion in metastatic breast carcinoma cells. TGF-β1 inhibitor also markedly suppressed colony formation in 4TBM cells while increased cell proliferation was observed 72 h following of the treatment. Similar changes were observed with MDA-MB-231 and MCF-7 cells such that chronic (10-12 days) but not acute exposure to SB-431542 inhibits cell proliferation and enhances anti-proliferative effects of doxorubicin. These results reveal marked differences of acute versus chronic effects of TGF-β1 inhibition and demonstrate that chronic low dose treatment with inhibitors of TGF-β might be effective in metastatic disease and may potentiate anti-tumoral effects of doxorubicin.

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Translating CAR-T cell therapy in the treatment of Cutaneous T cell Lymphoma

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Cutaneous T Cell Lymphoma (CTCL) is a heterogeneous subset of non-Hodgkin's lymphoma, comprising two disease compartments; circulating and skin infiltrated leukaemic CD4+T cells. The link between these two compartments is unknown. Current chemo- and radiation- therapies have failed to achieve long-term disease remission. Chimeric antigen receptor (CAR)-T cells have provided promise in the treatment of B cell lymphoma. However, their efficacy for treating T cell lymphoma remains challenging, primarily because of the lack of tumor-specific targets. Accordingly, given the disease heterogeneity of CTCL, we explored whether dual specificity CAR-T targeting multiple

antigens could universally deplete the CTCL tumor cells and minimize mutational escape while limiting off-target toxicity? We selected TAG-72 and CD30 as putative CAR targets, which were expressed at different levels in CTCL cell lines (HH and HuT78). We also successfully generated two single CAR constructs (TAG-72 CAR and CD30 CAR). CD30 CAR-T cells and TAG-72 CAR-T cells demonstrated a potent degranulation and strong anti-tumor killing against CTCL cancer cells *in vitro* in an antigen-specific manner, whereas the control group (non-transduced cells) did not. Furthermore, by pooling the two single CAR-T cells together, anti-tumor killing efficacy was improved, especially in tumor cells with low expression of the target antigen. Multi-antigen targeting by tandem CAR construct is currently under investigation for their potential benefits in the killing efficacy of CTCL cells compared to single CAR-T cells. Overall, our findings strongly support the implementation of tandem specificity CAR-T cell therapy to treat CTCL.

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Development of a personalized neoantigen specific TCR discovery platform

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Tumor-infiltrating lymphocyte (TIL) therapy can mediate tumor regression in a range of solid cancers, most notably in melanoma. However, its wider application and efficacy has been limited by the low frequency and exhausted phenotype of tumor-specific T cells in the final product. Here, we developed a personalized, neoantigen-specific TCR discovery platform that will enable engineering of multiple TCRs into autologous peripheral blood T cells. This allows for the generation of a fitter T cell product with a high frequency of tumor-reactive TCRs of defined specificity.
Our platform first identifies tumor-specific mutations and TIL-derived TCR repertoires from non-viable tumor specimens using next-generation sequencing (NGS), which are subsequently recreated using synthetic biology technology. The synthesized TCR libraries are expressed in reporter T cells, whereas neoantigen libraries are engineered in autologous APCs. Following coculture of these cells, activated and non-activated T cells are separated, followed by neoantigen-specific TCR identification using NGS-based analysis.
We validated the high sensitivity and specificity of this platform by successfully identifying multiple TCRs and their cognate neoantigens from high tumor mutational burden (TMB) cancers including melanoma. Notably, we show that the platform is agnostic to the type of mutation and HLA class restriction. Importantly, neoantigen-specific TCRs can also be isolated from a panel of low TMB microsatellite-stable colorectal cancers, underscoring the pan-cancer potential of this approach.
We commenced applying this approach in a clinical study in which patients will be treated with functional autologous T cells engineered with neoantigen-specific TCRs of defined specificity and composition.

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Exploiting cDC1 reprogramming for TIL-based adoptive cell therapy

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Adoptive cell therapy (ACT) with autologous tumor infiltrating lymphocytes (TILs) has achieved durable responses in patients with metastatic melanoma, even after failure of prior immunotherapies. Response to treatment is often mediated by activation of T cells targeting tumor antigens, including neo-antigens. Studies show that neo-antigen specificity abundance in the TIL infusion product correlates with survival, while their absence results in progressive disease. Indeed, a great number of TILs are injected with this procedure, but only a smaller fraction of these cells is capable of killing tumor cells and persist to mediate durable tumor regression. We hypothesize that augmenting the antigen presentation ability of the tumor cells can improve the expansion of autologous tumor-antigen specific TILs for ACT. Enforced expression of the transcription factors PU.1, IRF8 and BATF3 (PIB) was shown to convert fibroblasts and cancer cells into professional antigen-presenting conventional dendritic cells type 1 (cDC1s). Here, we show that the transduction of primary melanoma tumor cells from 8 patients with a lentiviral vector encoding PIB leads to the reprogramming of tumor cells into cDC1-like cells within 9 days of culture. Reprogrammed cells acquire a cDC1-phenotype characterized by the expression of CD45, CD11c, CLEC9A and CD141, and upregulate antigen-presenting machinery, including HLA-DR, HLA-ABC and the co-stimulatory molecules CD40, CD80 and CD86. Functionally, reprogrammed cells secrete the cDC1-specific cytokines IL12p70 and IL-29, present exogenous antigens, like CMV and MART-1, to HLA-matched $CD8^+$ T cells, inducing both memory and naïve T cell responses, and acquire the ability to process long-peptides and cross-present antigens. Interestingly, co-culture of cDC1-like melanoma cells with autologous TILs promoted TIL activation characterized by an increased reactivity higher expression of CD107a, CD137, IFN-γ, and TNF-α, and cytotoxic activity compared to non-modified tumor cells. Expression of TIM-3, LAG-3, PD-1 and BTLA was also upregulated in TILs co-cultured with cDC1-like melanoma cells, suggesting increased presentation of endogenous melanoma antigens. In summary, we have demonstrated that melanoma tumor cells can be efficiently reprogrammed into cDC1-like cells with increased stimulatory capacity of autologous TILs. Ultimately, this work will pave the way to develop a cDC1 reprogramming-based platform for enrichment of tumor-antigen specific TIL product to improve clinical outcome of TIL-based ACT.

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Novel combinatorial IGK-CD19 CAR efficiently and selectively targets malignant B cells.

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Chimeric antigen receptor (CAR) T cells have exhibited great potential for the treatment of hematological malignancies. The approved CD19 CAR T therapies are a powerful treatment for B cell leukemia and lymphoma, but indiscriminately kill both leukemic and healthy B cells, resulting in B cell aplasia which increases risk of infection. To prevent this, the immunoglobulin (Ig) kappa light chain (IGK) of the B cell receptor, highly expressed on malignant B cells, can be targeted to improve target cell-specificity. IGK CAR T cells demonstrated high efficacy in preclinical models, however, showed reduced efficiency due to their reactivity to serum Ig. We further observed that serum Ig – induced stimulation led to lower expansion during manufacturing and short-lasting CAR-T effect likely due to exhaustion. We therefore designed a new combinatorial CAR construct, composed of IGK single chain variable fragment (scFv) – CD3ζ (Kz) and CD19 scFv – 4-1BB (19BB) (Kz-19BB CAR). Splitting the first and co-stimulatory T-cell activation signals would provide two benefits: (i) reduction of the Ig – dependent stimulation of IGK CAR and (ii) restriction of specificity toward IGK^+ targets only, while maintaining the level of efficacy of CD19 CAR. Kz-19BB CAR transduced T cells displayed high efficacy and selectivity against malignant B cells. Here, we confirm these results using retrovirally transduced Kz-19BB CAR, seeking to improve its stability and long-term efficacy. Further, to study the therapeutic potential of Kz-19BB CAR, immunodeficient mice transplanted with IGK^+ B cell lymphoma and treated with Kz-19BBCAR T cells are being evaluated, with the aim of reducing tumor burden. Taken together, our results confirm the efficiency and selectivity of Kz-19BB CAR T cells, which appear to be a promising therapy for leukemia and lymphoma.

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Tumor microenvironment actuated GD2 CAR T cells prevent on-target off-tumor toxicities

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Chimeric antigen receptor (CAR) T cell therapy has demonstrated high response rates (70-90%) in clinical trials for B cell cancers, resulting in the FDA approval of five CAR T cell therapies. The same success has yet to be demonstrated in solid tumors, largely due to the lack of tumor-specific antigens. Since most solid tumor antigen targets are also expressed on healthy tissue, targeting these antigens can result in severe on-target, off-tumor killing of normal tissue. For example, CAR T cells targeting GD2, a disialoganglioside over-expressed in melanomas and neuroblastomas, delayed solid tumor growth in a preclinical neuroblastoma model, but caused fatal encephalitis resulting from CAR infiltration and activation in brain regions expressing low levels of GD2. As such, systemic targeting of tumor associated antigens also found on normal tissues poses significant risks to patients and is a major limiting factor in the translation of CAR T cell therapy for the effective treatment of solid tumors.

To overcome this problem, we engineered tumor microenvironment (TME) actuated T cells to restrict expression of the CAR to tumor tissue to improve the therapeutic index of CAR T cell therapy for the treatment of solid tumors. Specifically, we design an inducible on-switch ("if recognize A, then express B") against a cell adhesion target upregulated in solid tumor vasculature to restrict CAR expression to the tumor microenvironment. We generated TME actuated T cells that restrict the expression of the GD2 CAR to the solid tumor to prevent on-target, off-tumor toxicity in the brain. TME gated GD2 CAR T cells demonstrated specific CAR expression (actuation), activation and killing in vitro after exposure to both target antigens. A single infusion of logic gated GD2 CAR T cells in immunodeficient mice bearing K562 flank tumors demonstrated antitumor efficacy comparable to conventional constitutively expressed GD2 CAR T cells without detectable neurotoxicity, as shown by neither T cell infiltration in the brain, nor mouse weight loss. TME gated CAR T cells may expand the therapeutic index GD2 CAR T cells by preventing fatal neurotoxicity and maintaining cytotoxicity. This modular system has the potential to expand to other solid tumor targets also expressed on healthy tissues, thus providing a general strategy to improve the safety of CAR T cells for solid tumors in the clinic.

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Capturing T cells with light: A novel strategy for the identification of myeloma-specific T cells in single cell interaction assays

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Innovative immunotherapy approaches such as adoptive transfer of chimeric antigen receptor (CAR) T cells or tumor infiltrating lymphocytes (TILs) have shown great success in the treatment of solid tumors and hematological malignancies. Although treatment of multiple myeloma with CAR T cells can induce deep responses, relapses frequently occur due to antigen escape and limited CAR T cell persistence. TCR-engineered T cells may show prolonged persistence *in vivo* and could mediate sustained antitumor effects. A further benefit of TCR transgenic T cells is the ability to target intracellular antigens that are inaccessible to CAR T cells, expanding the range of potential targets for immunotherapy. In our project, we propose to identify T cell receptors (TCRs) specifically targeting autologous myeloma cells. Tumor-reactive T cells were identified using the Berkeley Lights Lightning platform, allowing simultaneous functional analysis of up to 1500 individual T cell/target cell interactions on a chip. Reactive T cells were identified upon detection of secreted cytokines (IFNγ, TNFα, IL-2) and measurement of CD137 surface expression. Tumor-reactive T cells showing various cytokine secretion patterns and CD137 expression profiles were detected in each myeloma patient (7 to 26 cells of approx. 1400 cells tested per patient). Individual tumor-reactive T cells have been isolated and their TCRs were sequenced. TCR genes will be cloned and overexpressed in autologous T

cells for functional validation and analysis of tumor derived neoepitope specificity. In summary, we present a pipeline allowing identification of myeloma-recognizing T cells and recovery of *bona fide* tumor-reactive TCRs eligible for patient-individualized T cell therapy.

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Discovery and functional validation of high affinity Rpl18 neoantigen-specific TCRs

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Discovery of neoantigens and induction of neoantigen-specific T cell responses have gained significant importance mainly due to unique expression of neoantigens by tumor cells making them attractive targets for cancer immunotherapy. Both our preliminary work and literature revealed neoantigen specific T cells might possess anti-tumoral activities especially for tumors with high mutational burden. One of the most frequently used murine colorectal cancer tumor model MC-38 was revealed to possess neoantigens presented by MHC class I, which triggered strong $CD8^+$ T cell responses. Among different neoantigens tested, Ribosomal protein L18 (Rpl18) neoantigen was shown to dominate $CD8^+$ T cell responses. We established a platform for rapid and efficient isolation of neoantigen-specific murine T cells, cloning of neoantigen-specific T cell receptor (neoTCR) sequences and *ex vivo* reprogramming of primary mouse T cells with desired TCR sequences. Following *in vivo* priming and expansion, Rpl18 neoTCR sequences were identified via 10X V(D)J sequencing, cloned and evaluated for their functionality based on IFNγ secretion upon antigen specific stimulation. Functionally top ranked TCRs were used to reprogram T cells for further investigation of anti-tumoral activities both *in vitro* and *in vivo*. In addition, biodistribution, expansion and persistence as well as tumor infiltration of adoptively transferred Rpl18 neoTCR engineered T cells upon repeated antigen specific vaccination were examined *in vivo* using MC-38 tumor bearing C57BL/6 mice. Rpl18 neoTCR sequences we identified and validated might contribute to the development of more effective therapeutic strategies against widely used MC-38 tumor model.

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Neo-Scan a flexible MS-immunopeptidomics approach to unbiasedly discover shared neoantigens

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Tumors accumulate genetic alterations some of which may generate “non-self” epitopes recognized by T cells, known as neoantigens. Although the vast majority of cancer neoantigens are private, seldom T-cell responses targeting selected hot-spot driver mutations in specific HLA molecules have been reported and adoptive transfer of TCR-transduced T cells targeting $KRAS_{p.G12D}$ and $TP53_{p.R175H}$

have shown clinical activity in patients with pancreatic and breast cancer[1,2]. Yet, few public neoantigens are known, limiting the development of T-cell therapies targeting recurrent neoantigens. Here we exploited Neo-Scan a versatile MS-immunopeptidomics based approach to discover neoantigens derived from 34 frequently-occurring hot-spot driver mutations across cancers presented on the 12 top most frequent HLA class-I alleles. We first generated HLA-I null cells by knocking out the B2M gene using CRISPR/CAS9 in a tumor cell line. B2M-KO tumor cells were co-electroporated with *in vitro* transcribed (IVT) RNA encoding for one B2M-HLA class-I allele chimera and one of three of tandem minigenes (TMGs), each expressing up to 15 frequently occurring hot spot driver mutations. Next, MS-based immunopeptidomics was performed on cells expressing a total of 36 B2M-HLA & TMG combinations (B2M-HLA_{1-12} & TMG_{1-3}) to identify HLA-I bound peptides. Using this strategy, we detected more than 50 mutant peptide-HLA pairs derived from 21 shared cancer neoantigens and presented across 11 HLA alleles. We captured 10 of the 16 hot spot-derived minimal epitopes previously described that were encoded in our TMGs and, more importantly, we detected 41 novel shared cancer neoantigens presented on HLA-I. By leveraging the low turn-around time and high transient expression obtained using RNA, Neo-Scan enabled high-throughput HLA-I screening of presented neoepitopes while avoiding intrinsic bias for peptides prioritized based on HLA-I binding prediction. Targeted mass spectrometry is being conducted to validate all neoepitope-HLA combinations. The immunogenicity and therapeutic potential of these candidates warrants further investigation.

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Multiple human dendritic cells subsets express CD169 and can be targeted by ganglioside-liposomes for anti-cancer vaccination strategy

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CD169 is a sialic acid binding receptor with a variety of functions including cell-to-cell communication and pathogen binding. CD169 expression was initially only found in cells from macrophage and monocyte lineage, however, we and others have previously shown that a subset of human blood dendritic cells (DCs), Axl$^+$ DCs, constitutively express CD169 and take up sialic acid-containing liposomal cancer vaccines. Here, we explored the expression of CD169 in other types of human blood and spleen DCs, and analyzed the binding of sialic acid-containing liposome vaccines. Analysis of single cell RNA-sequencing of human blood DC subsets showed high expression of *SIGLEC1* encoding CD169 in Axl$^+$ DC, and *SIGLEC1* transcript was also found in DC1, DC2, and DC3, but not pDC. Using 27-color Cytek spectral flow cytometry analysis, CD169 could be detected in almost all Axl$^+$ DC, and to a lesser extent (5-40%) in DC1, DC2, and DC3, but not pDC. Similar to our observation with monocytes, these CD169$^+$ DCs showed increased expression of HLA-DR, HLA-ABC, CD40, CD80, and CD86, indicating a higher maturation status. Furthermore, in DC3, CD169 expression could be enhanced by IFN-I treatment. Next, we investigated whether sialic acid-containing ganglioside-liposomes could bind to CD169$^+$ DCs. Indeed, ganglioside-liposomes could target these CD169$^+$ DCs, with GD1a as the strongest binder. Similar findings were found in human spleen DCs. Moreover, CD169$^+$ DCs were present in patients with pancreatic ductal adenocarcinoma, hepatocellular carcinoma, colorectal liver metastasis, and melanoma, and ganglioside-liposomes were selectively taken up by CD169$^+$ DCs. To conclude, next to Axl$^+$ DC, CD169 can be detected in DC1, DC2, and DC3, but not pDC, and these cells exhibit a higher maturation status and can be selectively targeted with ganglioside-liposome nanovaccines. Further studies will be necessary to delineate the individual contribution of different DC subsets for T cell priming.

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From CIGB-247 to HEBERSaVax: The road to the clinic of a VEGF-specific active immunotherapy.

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CIGB-247 is a cancer active immunotherapy, recently branded as HEBERSaVax. The treatment is based on the combination of a recombinant antigen representative of human vascular endothelial growth factor (VEGF), and clinically tested adjuvants. So far, these adjuvants have included mainly VSSP (Very Small Size Particles obtained from Neisseria Meningitides outer membrane) and Alum phosphate. Pharmacological tests in several species in both adjuvants earlier demonstrate the therapy's positive impact on inhibiting metastases and tumor growth, by inducing specific VEGF-blocking antibodies and specific T-cell responses, also concomitant with an excellent safety profile. Recent studies have unveiled the immune-restoring effects of this immunotherapy in both, systemic and tumor counterparts, where the treatments induce a significant increase in effector cells, and a decrease in the regulatory counterpart, parallel to the downregulation of pro-angiogenic proteins and the induction of CD8 activation-related cytokines.
Two sequential phase I clinical trials in solid tumors were conducted and concluded from 2011 to 2014, to assess safety, tolerance, and immunogenicity, at different antigen doses, and combined with two distinct adjuvants. HEBERSaVax was found to be safe and tolerable, with mainly low-grade local adverse effects. Both VEGF-specific cellular and humoral neutralizing responses were detected in patients receiving the immunotherapy and, positivity in more than one test was associated with increased survival. Patients surviving week 16 in the trials received voluntary off-trial monthly re-immunizations with HEBERSaVax, until death, intolerance, marked disease progression, or the patient’s withdrawal of consent. No additional onco-specific treatment was administered. After up to 9 years of vaccinations, some individuals experience clinical benefits and 3 of them currently exhibit a complete response: two with originally advanced ovarian adenocarcinoma and one with an advanced metastatic NSCLC. In the meantime, the compassionate use of the vaccine was authorized in concomitancy with the therapies recommended for the specific malignancies that include surgery, immunomodulatory agents, and chemo- and radiotherapy. Of notice, more than 20% of these patients suffer from Ovarian related malignancies (OvCa) and showed some clinical benefits, and a patient suffering from an advanced Hepatocellular Carcinoma (HCC) that starts on HEBERSaVax after tumor relapse, exhibited a complete response. These facts combined with the similarities shared between the mechanisms of HEBERSaVax-induced responses, and the antiangiogenics recommended for OvCa and HCC treatments, encourage the design and start of phase II/III clinical trials in June 2018 in advanced OvCa and a phase I/II trial in advanced HCC. The design of these trials will be presented as well as the current evolution up to 2022 and perspectives for further development of this active immunotherapeutic strategy.

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A Vaccine Targeting The Recurrent Driver Mutation H3K27M Induces Mutation Specific T- and B-Cell Responses In Patients with Diffuse Midline Gliomas

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A recurrent point mutation at position 27 in the histone-3 gene (H3K27M) defines a distinct subtype of highly aggressive diffuse midline gliomas (DMG) characterized by high mortality and morbidity rates. Despite the high clinical need and several clinical trials focusing on novel treatments, effective treatments remain limited. A vaccine targeting the neoepitope H3K27M has been shown to induce a mutation-specific $CD4^+$ T-cell response and to control H3K27M-mutated syngeneic tumors in an MHC humanized mouse model.

We have developed a protocol to expand neoantigen-reactive T cells from patients with H3K27M-muteted diffuse midline gliomas not eligible for the currently ongoing Phase 1 clinical trial (ClinicalTrials.gov Identifier: NCT04808245) and vaccinated with a mutant long peptide vaccine (H3-vac) to recover T-cell receptors (TCR) using combined single cell RNA and VDJ sequencing. We established a pipeline to clone TCRs from expanded populations of T cells and test their neoepitope specificity. Applying a novel DNA assembly process, we significantly decreased costs, allowing us to test more than 200 TCRs, derived from three different patients, for neoepitope specific reactivity using a co-culture assay with immortalized patient-derived B cell lines. Employing a panel of different healthy-donor-derived immortalized B cell lines and by establishing CRISPR/Cas9-mediated HLA knock-out lines we determined the HLA-restrictions of all identified H3.3K27M reactive TCRs.

Additionally, we confirmed the presence of vaccine-induced mutation-specific $CD4^+$ TCRs in a cerebrospinal fluid (CSF) sample from a vaccinated patient. We also identified activated B cells in the CSF and validated these B cells to encode H3K27M-specific antibodies. Cloning and testing two B cell receptors (BCRs) recovered from single B cells revealed mutation-specific binding of H3K27M peptide as well as full length protein.

Taken together, our data show that a neoepitope vaccine targeting H3K27M not only induces a $CD4^+$ specific T-cell response across various HLA alleles but also induces a mutation-specific B-cell response with development of H3K27M-targeting antibodies.

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Unlocking Pancreatic Cancer to NanoImmunotherapy

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Introduction: Among immunotherapeutic approaches, immune checkpoint inhibitors (ICI), have revolutionized the treatment of several cancers. However, clinical trials have shown PDAC as one of the most ICI-resistant cancers. PDAC has proven to be refractory to the most conventional anti-

neoplastic therapies, like chemotherapy, but also to the most modern, as targeted- and immuno-therapies.[1] Looking into PDAC, the unique dense stroma, the highly immunosuppressive tumor microenvironment (TME), and the immune evasion mechanisms have limited the infiltration by immune cells and therapeutics. Here we show the synergistic therapeutic effect of a nanotechnology-based system in combination with ICI *ex vivo* and *in vivo*.
Methods: PDAC antigens were selected using immune-bioinformatic analysis. Poly(lactic acid) (PLA) and Poly(lactic-co-glycol) (PLGA)-based nanoparticles (NP) incorporating PDAC antigens and immune regulators were formulated following methods already established[2]. The anti-tumor immune-mediated effect was evaluated *ex vivo* and *in vivo* in patient-derived organoids and PDAC-bearing mouse models (KPC), respectively.
Results: NP presented similar average hydrodynamic diameters, despite the entrapment of large or short peptides, with low polydispersity index. AFM showed spherical particles with a slight roughness surface. NP showed entrapment efficiency (EE) of 70-90%. Cy5.5-labeled NP were extensively internalized by DC and triggered their activation in vivo. NP-promoted CD4 and CD8 T-cell migration into the tumor site and successfully induced a potent immune-mediated anti-tumor response. Synergistic anti-tumor effects were observed when NP was combined with ICI.
Conclusions: The developed nanotechnology-based system showed the induction of a strong antigen-specific immune response and unlocked PDAC to standard immunotherapeutic approaches, as immune checkpoint modulators.
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A STING agonist potentiates a C1 lipid-based mRNA cancer vaccine through promoting TNFα secretion in dendritic cells.

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We previously developed a mRNA cancer vaccine based on a C1 lipid nanoparticle with self-adjuvant activity through engaging TLR4 signaling in antigen presenting cells. Although C1-mRNA nanovaccine exhibited significant antitumor efficiency, it could not completely eliminate tumors with low immunogenicity. Here, to further improve the therapeutic efficacy of C1-mRNA nanovaccine, we screened the combination of a TLR4 agonist with different innate immune receptor agonists for boosting dendritic cell activation. We identified ADU-S100, a STING agonist, when used in combination with the TLR4 agonist or C1, effectively promoted the secretion of type I interferon and proinflammatory cytokines including IL-12、IL-6 and TNFα by dendritic cells. Such C1-mRNA nanovaccine combined with ADU-S100 effectively promoted the activation of antigen presentation cells and T cells, and more importantly, demonstrated significantly enhanced therapeutic efficacy on serval tumor mouse models. Dendritic cell-specific knockout of Sting in host mice largely abolished the therapeutic efficacy of such mRNA cancer vaccine, verifying that dendritic cell-intrinsic STING signaling is essential for the antitumor efficacy of ADU-S100-adjuvanted mRNA vaccine. Notably,

antibody blockade of TNFα, but not type I interferon or IL-12, abolished antitumor efficacy of such vaccine. Together, by incorporating a STING agonist with C1-mRNA nanovaccine, our work provided a new means for developing safe and effective mRNA cancer vaccine, and uncovered dendritic cell-derived TNFα as a key cytokine for mRNA nanovaccine-induced antitumor efficacy.

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NeoAnts – A pipeline to identify NEOantigens from AlterNaTive Splicing

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In recent years, cancer immunotherapy has revolutionised the treatment of various indications of cancer, such as melanoma and breast cancer. Personalised cancer vaccines are promising immunotherapeutics designed to trigger de novo T cell responses against neoantigens. However, the identification of suitable neoantigen targets remains challenging and initially has been focused on the exploration of somatic non-synonymous single-nucleotide variants (SNVs). As the number of SNVs strongly varies between patients and tumour entities, cases with low SNV-burden could benefit from an expansion of the repertoire of neoantigens. Alternative splicing of mRNA alters the proteomic diversity of many cancers and the resulting neoepitopes have been proposed as promising targets for cancer vaccines. To harness this class of potential new targets, we have developed NeoAnts, a pipeline that combines machine learning and next-generation sequencing approaches for the detection of tumour-specific alternative splicing variants present in the transcriptome and caused by somatic mutations in human cancer samples. NeoAnts can identify multiple different alternative splicing events such as exon skipping, alternative 3’ and 5’ splice sites, intron retentions and mutually exclusive exons that were likely induced by somatic mutations in the tumour. To prevent reporting new targets that might also be expressed in healthy tissues, a large collection of RNA-sequencing data derived from more than 600 healthy tissue samples from 34 different organ sites has been analysed. Splice junctions detected in this healthy tissue dataset as well as junctions that have previously been found in GTEx samples are excluded from the pool of identified neoantigen targets. By applying NeoAnts to large cancer datasets, such as TCGA, and developing experimental confirmation strategies for internal samples, we plan to build a high-quality ground truth dataset to assess the precision and sensitivity of the method. Taken together, NeoAnts will identify neoantigen candidates derived from tumour-specific alternative splicing and thereby provide new targets for personalised tumour vaccines.

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The evolution of neoantigens in tumor progression in patient cases with low TMB malignancies

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A lack of consensus in the screening of immunogenic neoantigens has led to concerns regarding the potential of neoantigen-driven immunotherapies. Different input materials, protocols, and

computational methodologies continue to provide diverging results, and only a small fraction of identified tumor neoantigens appear to be immunogenic. In addition, sub-clonality and tumor heterogeneity as well as low tumor mutational burden (TMB) malignancies further complicate neoantigen selection. Here we report on several patient cases suffering from low TMB malignancies, for which we use a combination of AI language models dedicated to optimal neoantigen selection. We also showcase the importance of using deep whole genome sequencing (WGS) combined with RNA sequencing (RNAseq) as often very few or no actionable targets are found with the state-of-the-art (SOTA) workflows due to mutation sparsity. For each patient, a formalin-fixated or fresh frozen tumor biopsy was sequenced (WGS 90X, 200M rRNA depleted seq) as well as a blood sample (WGS 30X). As a prime example, we were able to assess the evolution of neoantigens using two biopsies (primary and recurrent) from a given patient suffering from glioblastoma, a very low TMB tumor. As expected, only a limited number of actionable small variants (SNVs and indels) were observed. Moreover, none of these were present in both samples, indicating that small variants were not conserved during tumor progression. Hence, at first sight there were no neoantigens with enough potential as immunotherapy targets to deliver a strong long-lasting tumour-killing effect. Nevertheless, deep investigation of the non-canonical variants using the WGS data revealed new, additional tumor alterations. Based on novel filtering methodologies, a subset of these was selected to be holding most clinical promise. Indeed, most of the filtered alterations were found in both biopsies, where some even had a higher coverage in the relapsed sample. We show that combining WGS and RNA seq is essential to find sufficient and novel actionable targets. We elaborate on the exploration of novel neoantigen types that have a higher immunogenic potential and go beyond the standard SNVs and indels commonly focused on with the whole exome sequencing (WES) SOTA workflows. We show the importance of functionally relevant driver mutation selection with high allelic fractions and the prioritization of antigens that are representative for the whole tumor and its metastases. Furthermore, we focus on multi-epitope selection with broad HLA coverage to increase the therapy efficacy while lowering the impact of tumor evolution. Altogether, the presented results highlight the superiority of these next-generation immunotherapy technologies as it clearly points to the importance of being able to identify non-canonical variants, especially in a low TMB setting. They appear to be an important additional neoantigen source shown to be conserved during tumor progression.

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Establishment of a validated human papillomavirus T cell epitope repertoire map via targeted immunopeptidomics enables vaccine design with broad population coverage

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High-risk types of human papillomavirus (HPV), including HPV16 and HPV18, cause more than 600,000 anogenital and oropharyngeal cancer cases worldwide per year. While prognosis for HPV-

driven cancers in the early stages is favorable, late stages are often resistant to standard therapy. The existing prophylactic vaccinations against HPV prevent infection but are not able to cure infection or neoplasia. For the development of a therapeutic vaccine against HPV16, we aimed to identify viral epitopes of the oncoproteins E6 and E7 that are *bona fide* presented on the surface of HPV16-transformed cells using a highly sensitive targeted liquid chromatography mass spectrometry (LC-MS) assay.
As the presentation of epitopes is specific to human leukocyte antigen (HLA) molecules, an epitope-specific vaccine needs to cover any patient's HLA types. The number of epitopes that are required to achieve broad population coverage can be reduced by exploitation of HLA supertypes, each of which covers HLA types with similar binding preferences. Accordingly, we have experimentally verified binders for HLA supertypes HLA-A01, A02, A03, A24, B07 and B62 which in total allow for > 99% population coverage.
To verify the true HLA presentation of the close to 300 HPV16 E6- and E7-derived HLA-binding peptides on the tumor cell surface, we established a targeted immunopeptidomics assay for the sensitive detection of every candidate. Target peptide LC-MS characteristics, including fragmentation, retention time and precursor charge state, were acquired and detection parameters optimized accordingly to achieve a sensitivity exceeding that of untargeted MS methods. The assay was run with peptides eluted from immunoprecipitated HLA-complexes derived from HPV16-transformed cell lines of different HLA backgrounds and cancer entities. This lead to the identification of more than 20 peptides and at least one peptide per probed HLA supertype. Recently, the addition of a data-independent acquisition (DIA) step for the HPV cell lines gave insight into the relative yield per HLA-type of the cells and indicated a small number of additional epitope candidates. In parallel, the immunogenicity of identified peptides is characterized in terms of cytokine induction and mediation of $HPV16^+$ target cell lysis by cytotoxic T cells.The definition of validated HPV16 E6- and E7-derived epitopes establishes a HPV16 epitope repertoire map and will enable the rational design of a therapeutic HPV16 vaccine or other T cell epitope-focused immunotherapies as a treatment option for persistent HPV16 infections and HPV16-driven malignancies.

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A long peptide vaccine targeting the clonal driver mutation H3K27M in adult patients with diffuse midline glioma

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Clonal and mutually exclusive substitution of lysine 27 to methionine in histone H3-3A (H3K27M) defines an aggressive subtype of diffuse glioma. These diffuse midline gliomas (DMG) are aggressive, incurable primary brain tumors that occur predominantly in a midline location in children and young adults. Surgical treatment options remain limited, response to chemoradiation is poor and palliative radiotherapy remains the only intervention with proven benefit[5], resulting in a median overall survival between 10 and 15 months after initial diagnosis. Previous studies have shown that a H3K27M-specific long peptide vaccine (H3K27M-vac) induces mutation-specific immune responses capable of controlling H3K27M$^+$ tumors in a major histocompatibility complex (MHC)-humanized mouse model.
Here we describe the first-in-human treatment with H3K27M-vac of eight adult patients with recurrent H3K27M$^+$ DMG. Repeat vaccinations with H3K27M-vac were safe and induced mutation-specific immune responses in five of eight patients after a median of two vaccinations as determined by Interferon-γ enzyme-linked immunosorbent spot. Median progression free survival after H3K27M-specific immune response was 6.2 months and median overall survival was 12.8 months.
In vitro restimulation of peripheral CD4$^+$ and CD8$^+$ T cells with H3 mutant peptide or wild type peptide from patients with mutation-specific peripheral immune response revealed that H3K27M specific immune responses are CD4$^+$-mediated and can be suppressed by blocking antibodies against MHC II, but not MHC I. Furthermore, proximity ligation assay of formalin-fixed paraffin-embedded primary tumor tissue revealed that H3K27M neoepitope co-localizes with HLA class II-DR.
One patient showed radiographic pseudoprogression (PsPD) according to iRANO criteria within six weeks after first detection of mutation-specific peripheral immune response. Following PsPD, 3 out of the top 10 vaccine-induced, K27M-expanded CD4+ T cell receptor clonotypes from peripheral blood were detectable in the cerebrospinal fluid of this patient who went into sustained complete remission for > 31 months.
In conclusion, H3K27M-vac is safe and induces H3K27M-specific CD4+ T cell responses in patients with H3K27M$^+$ DMG. An ongoing multicenter, phase 1 clinical trial for adult patients with newly diagnosed H3K27M$^+$ DMG will assess the safety and immunogenicity of H3K27M-vac in combination with atezolizumab and standard-of-care radiotherapy (NCT04808245).

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Therapeutic vaccination against the vascular immune checkpoint extracellular vimentin – a clinical trial in client owned dogs with urothelial cancer of the bladder

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Urothelial carcinoma (UC) of the bladder is associated with high morbidity and mortality and new treatment modalities are required. Targeting extracellular vimentin (eVim), a tumor vascular marker and vascular immune checkpoint common to solid tumors, has been shown safe and effective in preclinical models. Here we report a bridge study in spontaneous canine bladder cancer, which resembles human bladder cancer. The objective of the study was to demonstrate safety and efficacy of CVx1, an iBoost technology-based vaccine targeting eVim.
A single-arm prospective phase 1/2 study with CVx1, in 20 client-owned dogs with spontaneous UC, was performed. Dogs received 4 vaccinations at 2-week intervals with CVx1 subcutaneously for induction of antibody titers, followed by maintenance vaccinations at 2-month intervals. Additionally, standard daily cyclooxygenase (COX)-2-inhibition with meloxicam was given. Response was assessed by antibody titers, physical condition, abdominal ultrasound and thorax X-ray. Primary endpoints were the development of antibody titers, as well as overall survival compared to a historical control group receiving carboplatin and COX-2 inhibition with piroxicam. Kaplan-Meier survival analysis was performed.
All dogs developed antibodies against eVim after induction vaccinations with CVx1. Titers waned in time but were adequately kept up by maintenance vaccinations for the duration of the study. A median overall survival of 374 days from the first vaccination was observed, as compared to 196 days for the historical control group ($p<0.01$). Short-term grade 1-2 toxicity at the injection site and some related systemic symptoms, but no toxicity related to the induction of an eVim antibody response was observed. A limitation of the study is the single-arm prospective setting.
In conclusion CVx1 plus meloxicam consistently induced efficient antibody titers, was well tolerated and showed prolonged survival. The results obtained with CVx1 immunotherapy in this study in dogs with bladder cancer merits further development for human clinical care.

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Design and ex vivo testing of hepatitis B virus-based synthetic long peptides to treat chronic infection and hepatocellular carcinoma

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Therapeutic vaccination with synthetic long peptides (SLP®) is a promising treatment strategy for chronic hepatitis B virus infection (cHBV) and hepatocellular carcinoma (HCC) expressing (pieces of) integrated viral proteins.
We designed 17 SLPs for three viral proteins based on viral protein conservation, functionality, predicted and validated binders and epitopes for prevalent HLA supertypes and chemical producibility. We investigated SLP processing by dendritic cells (DC) using mass spectrometry-based

immunopeptidomics and the ability of SLPs to induce T cell responses by ELISpot and in vitro SLP-expansion followed by flow cytometry and Luminex cytokine analysis.
Immunopeptidomics using DCs from 8 healthy donors revealed HLA presentation of 26 unique SLP-contained HLA-peptides of which several in multiple donors. Individual SLPs each activated both CD8+ and CD4+ multi-functional T cells in one or more HBV resolvers and cHBV patient sample(s). Importantly, pools of SLPs induced T cell responses in all 11 individuals tested indicating subjects with different HLA types can be covered by selecting a suitable mix of SLPs for therapeutic vaccine design. In conclusion, designed SLPs are important components for a novel therapeutic vaccine to support the patient's failing immune system in curing cHBV and/or preventing and potentially curing HCC.

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A retrospective analysis of risk factors associated with immune checkpoint inhibitor induced-pneumonitis in cancer patients

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Background: Despite the recent success of immune checkpoint inhibitors (ICPIs) in treating cancers, immune-related adverse events (iRAEs) remain a topic that requires further attention. Pneumonitis is one of the recognised iRAEs of ICPIs that could lead to poor clinical outcomes. Here we analysed the baseline characteristics of the patients to identify potential risk factors for developing pneumonitis.
Method: Firstly, clinical records of new patients who were receiving ICPIs at Cambridge University Hospital Foundation Trust between January 2020 and July 2022 were identified to calculate the risk of pneumonitis on ICPIs. Patient data from December 2014 to July 2022 were then retrospectively analysed. 51 patients who developed pneumonitis were identified. Patient's baseline characteristics such as age at receiving the first ICPI treatment, gender, ICPI treatment regime, BMI, smoking history, previous radiotherapy to lungs, pre-existing lung disease and history of COVID-19 were examined to look for the potential association with ICPI-induced pneumonitis. The data were then compared against the 102 randomly identified drug-matched controls who did not develop any iRAEs.
Result: Between January 2020 to July 2022, the overall risk of pneumonitis on ICPI was 5.9% (24/403). The risk for patients on combined treatment ipilimumab/nivolumab was 6.3% (7/112). The risk on single-agent PD1 e.g. pembrolizumab and nivolumab was 8.4% (15/178) and 2.9% (3/106) respectively. In the matched cohort study, a history of smoking (OR 2.05, 95%CI 1.06-4.07) and pre-existing lung disease (OR 2.83, 95%CI 1.18-6.75) were identified to be associated with ICPI-induced pneumonitis ($p<0.005$). Of the 13 patients with pre-existing lung disease who developed ICPI-induced pneumonitis, 53.8% (7/13) had asthma and 30.8% (4/13) had COPD. There was no association between ICPI-induced pneumonitis and the other factors mentioned.
Conclusion: We have identified a history of smoking and pre-existing lung disease as potential risk factors for developing ICPI-induced pneumonitis.

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Modelling immune responses utilizing a broad range of features

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Initial results from clinical studies of personalized neoantigen-based vaccines have demonstrated tumor--specific immunogenicity. As the cellular immune response towards an antigen is a result of a complex machinery, various efforts have been undertaken from the computational perspective to evaluate neoantigen targets on behalf of their potential to evoke an immune response. It is known that tumor cells that present mutated peptides via the major histocompatibility complex (MHC) molecules can in general be recognized by T cells. However, not all neoantigens elicit an immune response, and identifying which ones will do so remains a challenge.
Improvements on the selection of targets to finally advance the efficacy of therapy have been continued. This started with efforts to predict MHC binding, then being extended by other features such as antigen abundance and processing features to obtain the presentation of the antigenic peptide on MHC molecules, as well as additional features that are assumed to contribute to the recognition by antigen-specific T cells.
Various prediction algorithms for MHC presentation have been developed by training artificial neural networks and other machine learning models on MHC ligands. However, other factors such as biochemical, biophysical determinants, positional and sequence based-features as well as similarity to self and pathogens have been previously described.
In this approach, we present an immune response predictor that considers the above described features, but also RNA and peptide location information, abundance, as well as energy and accessibilities derived from simulated molecular binding models of the complex, to define the immune response to an antigen. Further, we have used additional biophysical and biochemical features and a large dataset collection of immune response data that enabled us to address additional critical determinants of T cell responses. With this approach, we were able to significantly improve the immune response prediction compared to using binding and presentation only and developed an advanced approach to identify neoantigens that are likely to elicit a T cell response in cancer patients.

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Identification and characterization of vaccine-induced neoepitope-specific T cell receptors in patients with IDH1-mutant glioma

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More than 80% of diffuse astrocytomas and oligodendrogliomas carry the R132H driver mutation in isocitrate dehydrogenase type 1 (IDH1). The IDH1R132H neoantigen was shown to be presented on major histocompatibility complex class II (MHC II) molecules and induces a T helper cell response. These findings provided the basis for the first-in-human NOA16 clinical trial, in which both safety and immunogenicity of a long IDH1R132H peptide vaccine was demonstrated.
Following NOA16, NOA21 (AMPLIFY-NEOVAC) was initiated as phase 1 “window-of-opportunity” clinical trial, in which safety, tolerability and immunogenicity of the long IDH1R132H peptide vaccine in combination with the anti-PD-L1 (programmed death-ligand 1) antibody Avelumab is being assessed. To characterize the intratumoral adaptive immune responses in this ongoing trial, patients with clinically warranted re-resection of a recurrent IDH1R132H-mutated glioma are recruited, allowing for single cell analysis of immune infiltrates from post-treatment tumor tissues.
Here, we focus on characterizing the peripheral and intratumoral anti-IDH1 T cell response in detail. Peptide-based expansion assays were used to identify a total of 104 vaccine-induced IDH1R132H reactive T cell receptors (TCRs) from the blood of seven representative patients. Analysis of HLA-restriction of reactive clonotypes showed recognition of the IDH1R132H neoepitope on DR, DP and DQ MHC II alleles, with reactive TCRs having a broad range of higher and lower degrees of affinity against IDH1R132H presented on the same MHC II allele in peptide titration experiments. IDH1-specific T cell responses could be tracked over time in blood as well as in tumor tissue resected after 3 vaccination doses, showing a strong vaccine-induced increase in clone frequency. Moreover, a distinct signature of IDH1R132H-vaccine induced tumor-infiltrating lymphocytes was observed within tumor tissues, with an upregulation of markers such as CD40LG (CD40 Ligand) in IDH1R132H-reactive clones. While no IDH1R132H-reactive T cells could be detected by ELISpot in blood withdrawn before onset of vaccination, for at least one patient, enrichment of a reactive clonotype was found in the previously resected tumor tissue prior to any study treatment. This clone was shown to expand in blood after receiving the IDH1R132H long peptide vaccine.
TCRs identified in the scope of this study may be used to build a TCR warehouse of IDH1-reactive TCRs with known HLA-restriction, which could be used to offer patients with IDH1-mutant tumors off-the-shelf adoptive T-cell therapies.

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Therapeutic vaccines consisting of cancer germline antigen-based synthetic long peptides are immunogenic in human hepatocellular carcinoma patients

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Background and Aims: In melanoma, cancer germline antigen (CGA)-directed vaccination has shown to induce objective clinical responses accompanied by strong anti-tumor immune responses. As CGAs are immunogenic and highly expressed by hepatocellular carcinoma (HCC) tumor cells, these have demonstrated to be attractive targets to be implemented in therapeutic anti-liver cancer vaccination as well. Synthetic long peptide (SLP) vaccination has proven to elicit efficient anti-tumor $CD4^+$ and $CD8^+$ T cell responses and to have promising clinical effects. We aimed to develop an SLP vaccine targeting HCC-restricted CGA-epitopes covering at least five different HLA super types that are highly prevalent globally.
Methods: We applied an integrative pre-clinical approach of *in silico* epitope prediction, immunopeptidomics, and *in vitro* tools to select GSAs and validate CGA-SLPs in HCC patient-derived tumor infiltrating lymphocytes (TILs) and peripheral blood mononuclear cells (PBMCs).
Results: Out of a set of 13 CGAs, previously shown to be expressed in primary human HCC tissues, two CGAs (i.e., CGA-A and -B) demonstrated no healthy tissue expression and covered >75% of HCC patients collectively (N = 55). Immunopeptidome analysis of human HCC-derived hepatocytes (N = 12), together with *in silico* CGA-related epitope predictions according to epitope immunogenicity, enabled identification of 196 and 220 potential epitopes for CGA-A and -B, respectively. HLA-A*02:01 binding of these epitopes was validated *in vitro* using a HLA-A2 stabilization assay and ranked accordingly. Six SLPs were designed incorporating 54 HLA-A*02:01, 25 HLA-A*01:01, 24 HLA-A*03:01, 27 HLA-A*24:01, and 15 HLA-B*07:02 predicted and/or validated CGA-A- and -B-related epitopes. Top three-ranked epitopes were selected to validate ex vivo intra-tumor immune reactivity using corresponding peptide-HLA-A*02:01 dextramers in human HCC-derived TILs. Tumors of 8 / 11 patients contained CGA-A- and CGA-B-specific TILs that were characterized by a tumor reactive phenotype. Upon *in vitro* enrichment, SLP immunogenicity was demonstrated through Interferon gamma ELISPOT in 2 / 3 of human HCC-derived PBMCs using an *in vitro* co-culture system with autologous antigen presenting cells.
Conclusion: Here, we describe the intelligent design of a set of immunogenic SLPs comprising CGA-related epitopes for the global population that can be further exploited for the development of an off-the shelf anti-cancer vaccine to treat HCC.

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ODI-2001 personalized immunization platform shows antitumoral effectiveness across different syngeneic mouse tumor models.

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The impressive achievements of immune checkpoint inhibitors (ICI) has revolutionized cancer treatment over the last decade. However, the proportion of responding patients remains below expectations as their effectiveness largely rely on pre-existing neoepitope-specific immune responses. By combining a synthetic DNA vector coding multiple tumor-specific neoepitopes, a live MVA vaccinia virus as physiologic adjuvant, and an anti-CTLA-4 antibody to ensure a potent priming of the immune response, ODI-2001 aims to both increase the pre-existing and induce *de novo* host immune responses against tumors. Several mouse cohorts were inoculated with B16F10 melanoma cells, CT26 colorectal carcinoma cells, or 4T1 breast adenocarcinoma cells. Animals were treated by repeated subcutaneous injections of ODI-2001, alone or combined with anti-PD1 in B16F10-bearing mice. In all models, neoepitopes were chosen from the literature, and additional alternative neoantigens were identified in 4T1 tumor cells using a state-of-the-art dedicated neoantigen prediction algorithm (myNEO, Ghent, Belgium). Mouse survival was followed as primary outcome. Tumor volume and immune cell infiltration were determined. B16F10- and CT26-bearing mice presented a significantly improved survival upon ODI-2001 treatment. In a metastatic 4T1 model with primary tumor resection, ODI-2001-treated mice showed a higher proportion of tumor-free individuals over an 80-day observation period. Therapeutic immunization with ODI-2001 induced an increased $CD8^+$ cell infiltration in B16OVA tumors and combination of ODI-2001 with anti-PD1 performed better than either of the treatment alone. In the 4T1 tumor model, newly predicted and prioritized neoantigens outperformed those extracted from the literature. No signs of toxicity were observed after ODI-2001 treatment. These data demonstrate the significant activity of ODI-2001 as a neoantigen therapeutic immunization platform across different preclinical cancer models, both in monotherapy and combined with anti-PD1 and underline the importance of appropriate neoantigen selection. Thus, ODI-2001 represents a promising option to induce *de novo* cancer-specific immune responses, potentially increasing the proportion of patients responding to immunotherapy treatments. Based on this preclinical program a phase I with ODI-2001 in advanced solid tumors is under preparation.

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Applying Geometric Deep Learning to structure-based peptide-MHC binding affinity predictions

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Here we present a geometric-deep-learning-based approach to improve generalization capability of peptide-MHC binding predictions. In silico peptide-MHC binding predictions are crucial to reduce the costs of therapeutic cancer vaccine design. Nowadays, these predictions are mainly provided by sequence-based methods, while 3D-structures natively contain more information than sequences and could therefore be a more complete source of feature for deep learning approeaches. Here we combine 3D-modeling and geometric deep learning to tackle the p-MHC binding affinity prediction task.

For each p-MHC class I case with associated binding affinity, we generated 20 3D-models using our modelling package PANDORA v2.0. This information-rich data was processed using Deeprank into 3D-grids and 3D-graphs to calculate and map multiple structural features (charge, van der Waals energy, energy of desolvation, etc.) in addition to a sequence embedding. We then trained different types of geometric deep learning networks (3D-CNNs and 3D-GNNs) and compared their results with a purely sequence-based network mimicking the State of The Art softwares.
Due to the nature of this proof-of-principle study, the data used include only 100,315 peptide-MHC binding affinity samples taken from the MHCflurry2.0 database and it does not include yet mass spectrometry samples. The samples span accross 114 HLA-I alleles and 56% of them are considered strong binders (IC50 <= 500 nM).
Our preliminary experiments show competitive performance compared to state-of-the art methods (AUC 0.85~0.89). It is important to note that we trained on a relatively small dataset and did not optmize much the network architectures. Our structure-based method is capable of exploiting a wide range of biochemical features, which could significantly help improving binding affinity predictions. Finally, by comparing very different data representations, network architectures and features, we can provide insights on the learning capacity of several networks on the p-MHC and the impact of different features on the complex's binding affinity, to increase the community's knowledge on this system.

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PANDORA: A pan-allele modeling package for peptide-MHC class I and II structures

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Structures of peptide:Major Histocompatibility Complexes (pMHC) can provide fundamental molecular insights into T-cell mediated immune responses, and can aid in the identification of neoantigens for cancer immunotherapies. Here we present PANDORA-II, a fast anchor-restrained algorithm to model pMHC class II complexes. PANDORA-II extends PANDORA-I, our previously published fast, configurable and robust pipeline to model pMHC class I complexes. PANDORA (both PANDORA-I and -II) generates reliable pMHC complexes by using an integrative homology modeling approach that exploits highly conserved binding pockets of MHCs. By restraining the four defined peptide-binding pockets of MHC-II, our approach can generate low-energy pMHC-II models with high accuracy in minutes.
We have benchmarked PANDORA-II on 136 pMHC-II cases with experimentally determined structures covering 105 allele types, achieving a median backbone ligand root-mean-squared-deviation (L-RMSD) of 0.42Å for the binding core, and 0.88Å for the whole peptide. In addition, we compared PANDORA's performance to AlphaFold and other software; PANDORA outperforms both in terms of accuracy and speed. PANDORA also supports several Post Translational Modifications (PTMs), which modulate antigen presentation and recognition.
Unlike other modeling software, PANDORA covers both MHC class I and MHC class II, making it an incredibly versatile tool for researchers in the field of immunology. By providing a more detailed and accurate understanding of the structure of peptide:MHC complexes, PANDORA helps researchers to

identify potential targets for cancer immunotherapies. One of the key advantages of PANDORA is its computational efficiency (~5 minutes/case on one CPU core), making it now feasible to enrich large accumulated sequence-level binding affinity data with 3D structure information. This makes it an invaluable tool for AI (artificial intelligence) researchers to design powerful software for identifying neoantigens for cancer vaccine design, superantigen design and TCR therapy design.
PANDORA is user-friendly, easy to install, and highly-configurable, which makes it an ideal choice for preparing input structures for subsequent molecular dynamics analysis.
PANDORA-II is freely available for academic use athttps://github.com/X-lab-3D/PANDORA

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Glioblastoma immunotherapy: challenges and immune correlates of response

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Glioblastoma (GBM) is the most frequent and aggressive primary brain tumour in adults with a median survival of only 12–15 months. Several immunotherapeutic strategies are under investigation in GBM, but have not yet shown convincing clinical efficacy.
We previously performed a clinical trial investigating dendritic cell (DC)-based vaccine targeting cancer stem cells (CSC) in GBM (NCT00846456). mRNA from autologous CSCs in addition to mRNA encoding universal tumour antigens (telomerase and survivin) were transfected into autologous DCs and then injected intradermally.
Vaccine-induced T-cell responses were detected in the majority of the patients and data from first seven patients have shown prolonged progression-free survival and overall survival in the vaccinated GBM patients, and three were extreme long term survivors (more than 6 years). To link survival with immune response, we investigated the T-cell repertoire at different locations in one of the long term survivors. We detected both survivin- and telomerase-specific T-cell proliferation in peripheral blood post-vaccination and survivin-specific CD4+ T cell clones were isolated. The antigen specificity was confirmed in functional assays and we analyzed T-cell composition by T cell receptor (TCR) high-throughput sequencing (TCR-seq). TCR-seq analysis revealed substantial overlap between the TCR repertoire in blood samples and leukapheresis product pre-and post-vaccination. Tumour infiltrating lymphocytes (TILs) demonstrated high clonality and overlap with post-vaccination samples whereas T cells from CSF showed minimal overlap. Of note, TILs from a later biopsy taken upon tumour progression showed increased TCR diversity and phenotypic heterogeneity indicating reduced tumour specificity. Additionally, we isolated T cells with tissue-resident memory phenotypes, which interestingly, demonstrated reactivity against HLA-matched CSC from GBM patients. Work is still ongoing to identify the target antigens and TCRs of these TILs.
In summary, our data suggest that tumour-specific T cells were induced by vaccination and could be detected both in the periphery and intra-tumourrally. Following these encouraging data, a

randomized study (NCT03548571) comparing DC vaccine and standard treatment is now ongoing. Additional CSF samples and biopsies were obtained post-vaccination and are currently analysed to confirm specific clonal expansion of T cells. This will provide insight into how a successful anti-tumour response is constituted in GBM and guide the design of more efficient immunotherapy approaches.

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Induction of circulating antitumor specific CD8+ T cells in patients with non-small cell lung cancer treated with an allogeneic plasmacytoid dendritic-cell based cancer vaccine with or without anti-PD-1 treatment

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We have developed a therapeutic cancer vaccine (PDC*lung01) based on an irradiated plasmacytoid dendritic cell line loaded with HLA-A*02:01 restricted peptides (NY-ESO-1, MAGE-A3, MAGE-A4, Multi-MAGE-A, MUC1, Survivin and Melan-A) able to prime and expand peptide-specific CD8+ T cells *in vitro* and *in vivo*, and is synergistic with anti-Programmed Death (PD)-1 for the treatment of non-small cell lung cancer (NSCLC) patients (NCT03970746). Stage II/IIIA (cohort A) or stage IV with TPS≥50% (cohort B; +pembrolizumab) patients received weekly intravenous and subcutaneous injections of PDC*lung01 for 6 times at two dose levels. We report here the analysis of immune response of the first 3 cohorts of patients at four timepoints: at baseline, one week, four weeks and ten weeks post last injection of PDC*lung01.
We monitored several circulating immune parameters at different times before and after vaccination using different assays we have developed: leukocyte count and determination of circulation peptide-specific CD8+ T cells by flow cytometry using multimer tools without prior *in vitro* culture. The assay made on purified CD8+ T-cells allowed us to define a limit of quantification (LOQ) to better assess the fold changes of the cell expansion. In addition, tumor microenvironment (TME) was analyzed by immunochemistry.
Twenty three of the 25 patients included in the study received at least 4 doses and were evaluable. No major changes in circulating main lymphocyte frequencies (B cells, NK cells, CD4+, CD8+, or Treg T cells) were observed during treatment. In addition, no major cell activation (CD25+, CD54, or DR+) was noted. In contrast, in a significant number of patients (60.9%), PDC*lung01 induced circulating peptide-specific CD8+ T cell expansion in all 3 cohorts at different levels and time points against at least one out of seven peptides used.

The intensity of the immune response was proportional to the dose of PDC*lun01 and to the combination with pembrolizumab. When possible, total and peptide-specific CD8+ T cells were sorted for analysis of TCR repertoire, illustrating the modelling and dynamics of the immune response.

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Liver tissue-resident T cell generation upon liposomal cancer vaccination

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Tissue resident memory T (Trm) cells mediate local immunity and prevention of cancer metastasis. Since the liver is one of the major sites of metastasis formation, we compared two liposomal cancer vaccines for their capacity to induce liver Trm. In both vaccination approaches we used liposomes containing the ganglioside GM3 to facilitate targeting to CD169-expressing macrophages in spleen and liver (Kupffer cells). The vaccines contained either liposomal α-galactosylceramide (αGC) or a strong soluble adjuvant (the combination of poly I:C and activating anti-CD40 antibody) to stimulate immunity. Since the liver is rich in invariant natural killer T (iNKT) cells, we hypothesized that liposomal αGC would elicit intrahepatic inflammation and stimulate liver Trm formation. Intravenous injection of both vaccines resulted in uptake by and maturation of splenic and liver CD169-expressing macrophages, but only the αGC-containing liposomes stimulated iNKT cells in spleen and liver. While both vaccination strategies elicited potent effector T cell responses at day 7 in spleen and liver, only αGC-containing liposomes induced formation of liver Trm at day 28. By using Batf3KO and CD169-DTR mice, we established that cDC1 are essential for the activation of liver T cells, but that $CD169^{+}$ macrophages are not necessary for liver iNKT cell activation and liver Trm generation. Further studies will evaluate the efficacy of liver Trm to prevent metastasis formation. In conclusion, vaccine design impacts liver Trm formation and vaccination may be one of the tools to combat or prevent liver malignancies.

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Simultaneous gene-based delivery of tumor antigen and cytokines to liver macrophages unleash a potent immune response against liver metastases

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Liver metastases arising from colorectal cancer are associated with unfavorable prognosis and are often the cause of death in cancer patients. Whereas invasive primary tumors are often surgically resettable, liver metastases are not, and classical anti-cancer treatment are not effective. The liver immune environment is naturally programmed towards hypo-responsiveness, and it is believed that the general immune-suppressed state of this organ favors metastatic seeding and cancer progression. Notably, it has been shown that liver metastases promote tumor antigen-specific T cell apoptosis, which in turn results in systemic loss of T cells and consequently diminished efficacy of immunotherapy. This evidence highlights the need to develop novel therapeutic approaches aimed at enabling the activation of anti-tumoral immune responses directly within the liver.
To this aim we developed a lentiviral vector (LV) platform, which, upon a single well tolerated dose, transduces selectively liver resident macrophages (Kupffer cells, KC). We leverage on this platform to develop a new type of LV-based tumor vaccine, which allows simultaneous delivery of tumor antigens (TA) and immune activating cytokines selectively to KCs (hereon Combo LV). Therapeutic efficacy of Combo LV was investigated in mice hosting established liver metastases, obtained by intrahepatic injection of MC38-OVA cells, a colorectal cancer cell line engineered to express ovalbumin (OVA), a surrogate tumor antigen. We found that systemic delivery of Combo LV resulted in complete response in most mice. In the liver parenchyma as well as in residual liver metastases from Combo LV-treated mice, we observed enhanced infiltration of CD8 T cells, including OVA-reactive CD8 T cells, which displayed phenotypic features associated with memory/effector-like functions (CD8 Tem-like cells), and reduced numbers of T cells displaying a terminally exhausted-like phenotype. Of note, the percentage of OVA-reactive CD8 Tem-like cells positively correlated with enhanced clearance of liver metastases.
In summary, we found that simultaneous gene-based delivery of tumor antigens and cytokines to KCs in mice hosting established liver metastasis enabled the generation of tumor-antigen specific T cells leading to complete tumor eradication in most mice. Further studies are ongoing to investigate the therapeutic efficacy of this platform in distinct syngeneic liver metastasis mouse models and the mechanisms whereby simultaneous tumor antigen presentation and immunomodulatory molecule production by KCs modulate the liver immune environment.

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An innovative LC-MS methodology for the GMP-compliant quality control of peptide vaccines in individualized immunotherapy settings

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Multiple studies have demonstrated antigen-specific immune responses induced by peptide-based vaccination. Even though peptide cocktails promise highly personalized treatments of various diseases, clinical responses remain so far limited to single patients and broad clinical applicability was not yet achieved. To induce effective immune responses, peptide-based vaccines rely on the combination with effective and strong adjuvants. Recently, the novel water-soluble adjuvant XS15 consisting of a synthetic TLR 1/2 binding Pam_3-Cys-derivative, which is covalently linked to a non-vaccine 9-mer peptide, showed its efficiency in inducing strong and long-lasting $CD4^+$ and $CD8^+$ T cell responses in first-in-man proof-of-concept experiments as well as in the setting of a Phase I clinical trial evaluating the SARS-CoV-2-derived multi-peptide T cell activator CoVac-1.
To translate the research behind peptide-based vaccines into clinical trials, precise and practical analyses are required to meet Good Manufacturing Practice (GMP) quality standards. Thus, we have implemented an in-house GMP-approved production and quality control of peptide vaccines. The quality of late-stage development of the peptide vaccines is confirmed and validated via liquid chromatography (LC) and mass spectrometry (MS) analyses; validated according to International Conference on Harmonization (ICH) of technical requirements for registration of pharmaceuticals for human use. However, so far, the quality control was performed in several different time-consuming steps, separately for the vaccine peptides and the adjuvant XS15.
To further optimize the bench-to-bedside process and timeline, this study aims to improve the existing methods and to establish a much faster process for the quality assurance of the peptide-based therapeutics in a single analysis. Therefore, we developed a novel method for Waters ultrahigh-pressure liquid chromatography (UPLC™) linked to the Time of Flight (ToF) mass spectrometry (MS) BioAccord system with the aim of obtaining chromatography and mass spectra information via a single sample run with cut-in-half measurement and analysis time for the GMP-approved production of vaccine cocktails. Various gradients and different columns were investigated to optimize the LC separation of peptides. In addition, optimal MS settings were defined to determine reliable and precise qualification of the vaccine peptides and cocktails.
Using the optimized methodology, we are able to overcome the multiple steps of long and expensive analyses previously developed to detect the lipopeptide XS15, which is a well-known and significant obstacle in biopharmaceutical methodologies. The here developed innovative workflow, which combines LC and MS quality control of peptide vaccines and the adjuvant XS15 in one single run, represents a robust and reproducible method that meet the quality criteria of GMP with optimized time and cost invested all-along the process of peptide-based cocktails.

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Development of a Pan-Peptide Checkpoint Inhibitor in the Treatment of Central Nervous System Tumors

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Despite decades of research, malignant brain cancer remains a devastating disease, currently affecting nearly 140,000 people nationwide. The first line of treatment for these patients consists of complex surgery, chemotherapy, and radiation, all of which may have additional complications and

lead to poor quality of life, with a 5-year survival < 10%. In 1995 James Allison pioneered immunotherapy employing the inhibition of "immunological brakes" allowing the immune system to respond to cancer cells. It is now well established that these immune checkpoint inhibitors directed against PD-1, PD-L1 and CTLA4 significantly enhance an anti-tumor response. However, immunotherapy, especially the use of multiple checkpoint inhibitors, results in serious immune-related adverse events including mortality. We developed a novel peptide checkpoint ligand (CD200AR-L) that binds to a complex of CD200 activation receptors (CD200AR). In contrast to current immune checkpoint inhibitors, the CD200AR-L stimulates antigen-presenting cells (APCs) and triggers an anti-tumor response by upregulating CD80/86, eliciting cytokine release and enhancing APC differentiation into immature dendritic cells. In addition, the CD200AR functions through a specific signaling pathway that intersects multiple immune checkpoints. We discovered that CD200AR-L signals a complex of CD200ARs which simultaneously downregulate the inhibitory CD200 receptor (CD200R1), PD-1 and PD-L1. Recently, we conducted a clinical trial in dogs with high-grade glioma using canine-specific CD200AR-L combined with an autologous tumor lysate (TL) vaccine, which was administered after tumor resection. Adding CD200AR-L to TL vaccine significantly improved median overall survival from 196 days to 414 days in dogs receiving TL alone compared with dogs that were treated with TL + CD200AR-L. We are the first to develop a peptide that simultaneously modulates the inhibitory CD200, PD-1 and PD-L1 checkpoints. We demonstrated the capacity of the peptide to modulate these checkpoints *in vitro* and *in vivo* in mice and dogs. We also have encouraging early data from an ongoing clinical trial in humans with recurrent glioblastoma (WHO grade IV astrocytoma). In this trial, a subgroup of patients receiving the CD200AR-L in combination with an allogeneic tumor lysate had a decrease of the inhibitory CD200R1, PD-1 and PD-L1 on CD14, CD8- and CD4 T cells. In addition, we observed a decrease in MDSC and PMN-MDSCs. RNAseq analysis of pre- and post-treatment biopsies in a single patient showed increases in caspase 3, 6, 7, 8 and M0 macrophages after treatment**.** Currently, we have a 51.2 (n=9) week OS and 77.8% (n=9) OS-6 compared to historic results of 30.7 week (n=1661) and 54% (n=796) respectively. In addition, two-year survival of the patients that received the lowest dose is 33% (2/6). This early data is encouraging and suggests that targeting the CD200 immune checkpoint may be useful for the treatment of human cancer.

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Adenoviral-based vaccine promotes neoantigen-specific CD8$^+$ T cell stemness andtumor rejection

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Upon chronic antigen exposure, $CD8^+$ T cells become exhausted, acquiring a dysfunctional state correlated with the inability to control infection or tumor progression. In contrast, stem-like $CD8^+$ T progenitors maintain the ability to promote and sustain effective immunity. Adenovirus (Ad)–vectored vaccines encoding tumor neoantigens have been shown to eradicate large tumors when combined with anti–programmed cell death protein 1 (aPD-1)
in murine models; however, the mechanisms and translational potential have not yet been elucidated. Here, we show that gorilla Ad vaccine targeting tumor neoepitopes enhances responses to aPD-1 therapy by improving immunogenicity and antitumor efficacy. Single-cell RNA sequencing demonstrated that the combination of Ad vaccine and aPD-1 increased the number of murine polyfunctional neoantigen-specific $CD8^+$ T cells over aPD-1 monotherapy, with an accumulation of Tcf1+ stem-like progenitors in draining lymph nodes and effector $CD8^+$ T cells in tumors. Combined T cell receptor (TCR) sequencing analysis highlighted a broader spectrum of neoantigen-specific $CD8^+$ T cells upon vaccination compared to aPD-1 monotherapy. The translational relevance of these data is supported by results obtained in the first 12 patients with metastatic deficient mismatch repair (dMMR) tumors vaccinated with an Ad vaccine encoding shared neoantigens. Expansion and diversification of TCRs were observed in post-treatment biopsies of patients with clinical response, as well as an increase in tumor-infiltrating T cells with an effector memory signature. These findings indicate a promising mechanism to overcome resistance
to PD-1 blockade by promoting immunogenicity and broadening the spectrum and magnitude of neoantigen-specific T cells infiltrating tumors.

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NKG2A blockade oppositely impacts intra-tumoral and peripheral CD8 T cell response induced by KISIMA – VSV-GP heterologous vaccination

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KISIMA™ platform allows the production of recombinant chimeric protein vaccines composed of three elements: a cell penetrating peptide for cytoplasmic delivery, a multi-antigenic domain and a TLR2/4 agonist conferring self-adjuvanticity. Vaccination with KISIMA derived protein vaccines was shown to elicit both CD8 and CD4 T cell immune responses resulting in control of tumor growth in preclinical models. Vesicular stomatitis virus (VSV)-GP is a modified chimeric variant of VSV in which the surface glycoprotein G was substituted by the LCMV surface glycoprotein (GP) to avoid neurotoxicity. We have previously shown that therapeutic heterologous prime-boost vaccination with KISIMA protein vaccine and VSV-GP oncolytic virus enhances tumor infiltration of antigen-specific cytotoxic CD8 T cells and inflames the tumor microenvironment (TME) in different mouse tumor models, resulting in increased anti-tumoral efficacy. However, tumor infiltrating CD8 T cells are often found to be exhausted upon cancer vaccination, resulting in tumor escape or relapse. In this study, we investigate the impact of NKG2A blockade on antitumoral efficacy of heterologous prime-boost vaccination KISIMA – VSV-GP in three murine tumor models: TC-1, lung epithelial cell line transfected with HPV16 E6/E7 and c-H ras oncogene, and MC-38 or CT-26 colorectal carcinoma.

Tumor bearing mice were primed subcutaneously (s.c.) with KISIMA and boosted intravenously (i.v.) with VSV-GP followed by intravenous treatment with NKG2A blocking antibody. We found that KISIMA – VSV-GP prime-boost vaccination promoted the expression of NKG2A on tumor infiltrating but not circulating antigen-specific CD8 T cells. Combination treatment with NKG2A blocking antibody resulted in delayed tumor growth in all tumor models, in particular in TC-1 tumor-bearing mice, where it prolonged survival and increased number of complete responders. Mechanistically, NKG2A blockade therapy did not improve tumor infiltration of multifunctional antigen-specific CD8 T cells induced by heterologous prime-boost vaccination, but significantly reduced their exhaustion highlighted by a decreased frequency of PD-1^{+}Tim-3^{+}NKG2A^{+} cells. Surprisingly, despite a positive impact on anti-tumoral efficacy, combination treatment reduced the magnitude and activation of circulating antigen-specific CD8 T cells elicited by KISIMA – VSV-GP vaccination. Complete responders were only partially protected against tumor rechallenge and analysis of memory response showed reduced number of antigen-specific effector memory CD8 T cells in secondary lymphoid organs. Taken together, these data suggest that NKG2A blockade modulate the phenotype of tumor antigen-specific CD8 T cells induced by KISIMA-VSV-GP vaccination, with diametrically opposed effects on intra-tumoral and peripheral response.

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Multicomponent adjuvantation of antigen-based nanocapsules using site-directed click chemistry crosslinking for the treatment of melanoma

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Nanocarrier-based antigen delivery is a promising vaccination approach in the context of tumor therapy. Formulating polymeric nanocapsules (NCs) out of tumor antigens in combination with vaccine adjuvants enables efficient targeting and maturation of dendritic cells (DCs), essential prerequisites for the induction of vigorous cellular immune responses. Aim of the present study was the synthesis of polymeric protein nanocapsules composed exclusively of vaccine antigens and encapsulated with combinations of adjuvants, as well as the evaluation of their potential to induce antigen-specific immune responses.
The NCs consisting of ovalbumin (OVA), were bioorthogonally crosslinked by copper-free azide-alkyne Click-Chemistry using the inverse miniemulsion technique. This method ensures integrity and processability of crosslinked antigens leading to effective epitope presentation by dendritic cells. The inverse miniemulsion approach led to polymeric nanocapsules with a spherical morphology that were efficiently ingested by DCs. In addition, a combination of the vaccine adjuvants Resiquimod (R848) and diABZI (STING agonist) were encapsulated to efficiently trigger strong DC activation analyzed by costimulatory surface marker expression and the secretion of pro-inflammatory cytokines. The induction of robust antigen-specific T cell proliferation was observed in DC-T cell co-cultures. The high biocompatibility and effectiveness of this vaccination platform was shown using the OVA-NC formulation as a therapeutic vaccine in different B16 melanoma models.
In conclusion, multiadjuvant-functionalized protein nanocapsules are a promising delivery system for the combined delivery of antigens and vaccine adjuvants to dendritic cells promoting T cell-based

immune responses. This novel anti-tumor vaccination strategy avoids the use of structural compounds, increases the antigen load of DCs and bears the potential to overcome tolerance and to induce vigorous antigen-specific anti-cancer immunity.

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DCOne-derived mature DCs opsonized with anti-PD-L1 antibodies as potential intratumoral immune primers.

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Introduction: One factor associated with the outcome of immune checkpoint blockade (ICB) therapy is whether the tumors are “hot” or “cold”. In a recent study an essential role for functional Fc-gamma-receptors (FcγR) on intratumoral NK cells and macrophages in the induction of an “hot” tumor was shown. Notably, this inflammatory response led to a self-sustaining systemic tumor-specific T cell activation driven by ICB.
Mature dendritic cells derived from the leukemic cell line DCOne (DCOne mDC) strongly activate cocultured allogeneic T cells, while expressing PD-L1. We therefore decided to evaluate how opsonization of these cells with anti-PD-L1 antibodies could affect the activation of FcγR expressing NK cells and monocytes within cocultured allogeneic PBMCs.
Material and methods: PBMCs from healthy CMV^+ donors were co-cultured with DCOne mDCs +/- different anti-PD-L1 antibodies for 4-7 days without stimulating cytokines. Proliferation (CFSE), cytotoxicity (granzyme B; GrB) and immune cell phenotype was monitored with flow cytometry and cytokine/chemokine production with Luminex.
Results: Addition of anti-PD-L1 antibodies with high FcγR affinity, but not low affinity antibodies, led to prominent NK cell activation (CD25 expression). When analyzing immune cell phenotypes after 7 days co-culture the relative frequency of $CD56^+CD3^-$ NK cells among cocultured PBMCs was dramatically increased, particularly the subgroup of NK cells co-expressing the adaptive/memory markers NKG2C and CD57. As to functionality, the tumor killing capacity (GrB-expressing K562 target cells) was strongly enhanced. Finally, addition of anti-PD-L1 to the mDC/PBMC co-culture led to strongly enhanced production of several proinflammatory cytokines, including TNF-α and IFN-γ, and chemokines, including CCL-3, 4, and 5. Notably, the production of IL-1β was by far highest when anti-PD-L1 antibodies were present, indicating an Fc-receptor-mediated activation of monocytes.
Conclusion: Taken together, our data indicate that DCOne mDCs opsonized with anti-PD-L1 antibodies with high Fc-receptor affinity induce a strong activation of co-cultured NK cells and monocytes from CMV+ donors. If administered intratumorally these opsonized “off-the-shelf” leukemia-derived mDCs may potentially induce Fc-gamma-receptor dependent activation of intratumoral macrophages and NK cells (as well as activation of alloreactive T cells) that may initiate critical steps including local tumor cell killing and recruitment of immune cells, including cross-presenting DCs, that could sensitize for concomitant ICB therapy.

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Molecular mimicry and cancer vaccine development

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Background: tumor-associated antigens (TAAs) are shared wild-type cellular self-epitopes highly expressed on tumor cells and are used to develop off-the-shelf cancer vaccines appropriate to all patients affected by the same malignancy. However, given that they may be also presented by HLAs on the surface of non-malignant cells, they may be possibly affected by immunological tolerance or elicit autoimmune responses. In order to overcome such limitations, analogue peptides with improved antigenicity and immunogenicity able to elicit a cross-reactive T cell response are needed. To this aim, non-self-antigens derived from microorganisms (MoAs) may be of great benefit.
Indeed, data suggest that MoAs share sequence homology with TAAs ("molecular mimicry") and elicit a cross-reacting CD8+ T cell response.
Methods: We looked for homology between published TAAs and non-self epitopes derived from viruses as well as microbiota species of the Firmicutes and the Bacteroidetes phyla, which together account for 90% of gut microbiota (MoAs). Blast search for sequence homology was combined with extensive bioinformatics analyses. Cross-reactive T cells were evaluated by tetramer staining as well as IFNg EliSpot assay.
Results: Several pieces of evidence for homology between TAAs and MoAs have been found. Strikingly, 100% homology between paired sequences has been identified. The predicted average affinity to HLA molecules of MoAs is very high (< 100 nM). The predicted structural conformation of the MoAs is, in general, highly similar to the corresponding TAA, and, in some cases, contact areas with both HLA and TCR chains are indistinguishable. Moreover, the spatial conformation of TCR-facing residues can be identical in paired epitopes, with exactly the same values of planar as well as dihedral angles. T cells cross-reactive with the paired TAAs and MoAs have been identified by tetramer staining as well as IFNg EliSpot assay, confirming the predicted sequence and conformational homology.
Conclusions: The data reported in the present study show for the first time a comprehensive homology analysis between published TAAs and peptides derived from viruses as well as microbiota species. Cross-reacting CD8+ T cell responses confirm the possibility of eliciting an anti-tumor immunity by non-self peptides derived from viruses as well as microbiota species. This may have a two-fold relevance: 1) the natural T cell memory elicited by MoAs during the lifetime may turn out to be an anti-cancer T cell memory, able to control the tumor growth; 2) such non-self MoAs may be included in preventive/therapeutic cancer vaccines with a more potent anti-tumor efficacy compared to those based on TAAs.

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Toll-like receptor 3 agonist immunoconjugates eradicates EGFRvIII-positive experimental glioma

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Glioblastoma, a WHO grade IV glioma, represent the most common primary adult brain tumor. Despite multimodal treatment, glioblastomas ultimately recur. The interplay between tumor cells and tumor-associated stroma, the latter mainly consisting of myeloid cells, drives tumor progression and contributes to immunologically “cold” glioblastomas. Treatment of glioma cells and programming of the tumor stroma from “cold” to “hot” with agonists for Toll-like receptors (TLRs) represents a promising therapeutic strategy. However, the treatment with TLR agonists in either unmodified form or encapsulated in nanoparticles bears the risk of an unwanted systemic activation of the innate immune system. To achieve selective tumor treatment and to avoid adverse effects, we developed a targeted delivery system for TLR3 agonist Riboxxol (a serum stable 50bp dsRNA), designated “Rapid Inducer of Cellular Inflammation and Apoptosis” (RICIA). For treatment of epidermal growth factor receptor variant III (EGFRvIII)-positive glioma, RICIA consists of biotinylated Riboxxol and biotinylated EGFRvIII-specific single chain antibodies (scFv) both conjugated to neutravidin. We hypothesized that accumulation and uptake of EGFRvIII-RICIA in glioma should reprogram the tumor stroma and in parallel lead to the release of neo-epitopes from dying tumor cells capable of inducing an adaptive immune response. We show that treatment with EGFRvIII-RICIA results in a receptor-mediated endocytosis of Riboxxol in EGFRvIII-positive human U87vIII and Bs153resE glioblastoma as well as in murine SMAvIII astrocytoma cells, which was not observed when employing isogenic control cells devoid of EGFRvIII or when using RICIA coupled to unspecific scFv control. In all tested glioma cell lines uptake of Riboxxol was accompanied by a type-I interferon response and increased cell death. CRISPR/Cas9-mediated knockout of TLR3 in SMAvIII cells resulted in a loss of type-I interferon response of EGFRvIII-RICIA-treated cells, confirming that endosomal delivery of Riboxxol specifically activates TLR3 signaling. Furthermore, intraperitoneal application of EGFRvIII-RICIA lead to significantly reduced s.c. tumor burden and to a significant improved survival of mice. In addition, mice showing stable complete remission of tumors completely rejected newly s.c. transplanted SMAvIII tumor cells. In line with this, RNA-seq analysis of s.c. gliomas showed an inflammatory expression pattern and a response to virus signature. Stereotatic injection of EGFRvIII-RICIA in orthotopic glioma led to, a significant total mean regression of tumor mass, prolonged overall survival and in 20% of cases to a complete and stable tumor remission when compared to controls. In conclusion, targeted delivery of EGFRvIII-RICIA induces tumor regression in experimental glioma and was accompanied by the induction of innate and adaptive immune responses.

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T cell inducing SLP vaccine against SARS-CoV-2 for protection of patients with spontaneous or therapy-induced B cell deficiencies

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Approved SARS-CoV-2 preventive vaccines act through induction of neutralizing IgG antibodies against conformational B cell epitopes on the Spike (S) protein of the virus. These vaccines are insufficiently protective against COVID-19 in patients with hereditary or acquired B cell deficiencies or patients receiving B cell-depleting anti-CD20 monoclonal antibodies, associated with deficient antibody formation. We hypothesize that robust T cell responses against T cell epitope-rich regions of not only the S, Nucleocapsid (N) and Membrane (M) proteins of SARS-CoV-2 can be elicited by adjuvanted synthetic long peptide (SLP®) vaccination. In the absence of protective neutralizing antibodies these T cells are expected to confer protection against SARS-CoV-2, even in persons with defective B cell immunity.
Our vaccine, called ISA106, consists of multiple SLPs derived from the S, N and M proteins of SARS-CoV-2. Proprietary algorithms were used to identify immunogenic hotspots containing HLA class I and HLA class II epitopes and predict manufacturability of the SLPs. Subsequently, a selection of SLPs was screened in biological assays. The impact of adjuvant, administration route, vaccination interval and SLP dose on vaccine immunogenicity was investigated by vaccination of C57BL/6 mice with a murine equivalent (mISA106) and subsequent characterization of SLP-specific T cell responses. C57BL/6 mice were vaccinated with SLPs emulsified in Montanide ISA-51, mixed with CpG or Amplivant ® (TLR1/2 ligand). Early data show that vaccination with even a single SLP can protect against a lethal dose of SARS-CoV-2 in ACE2-receptor transgenic mice. Furthermore, PBMCs were collected from 11 human convalescents, recovered from proven SARS-CoV-2 infection, as well as from 4 healthy control subjects and tested for memory responses against the SLPs. SLP-specific T cell responses of PBMCs were measured by IFNγ ELISpot assay and Luminex.
Many SLPs were immunogenic in C57BL/6 mice, eliciting both CD4+ and CD8+ T cell responses. Intradermal vaccination with SLPs in combination with Amplivant induced T cell responses comparable to subcutaneous Montanide-51 formulations. All SLP vaccine candidates contain epitopes that elicit T cell responses in the IFN-γ ELISpot assay with PBMC from convalescent persons. These clearly represent memory T cell responses elicited by natural SARS-CoV-2 infection.
Thus, the ISA106 adjuvanted SLP vaccine is capable of eliciting robust T cell responses in mice and reactivating robust memory T cell responses ex vivo in PBMC from convalescent donors recovered from SARS-CoV-2 infection. This vaccine warrants further development to provide better protection against SARS-CoV-2 virus-induced disease, to persons with spontaneous or therapy-induced B cell deficiencies, by inducing adequate anti-viral T cell immunity.

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Real world data on individualized multimodal immunotherapy as part of first-line treatment for patients with glioblastoma multiforme

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Glioblastoma multiforme (GBM) is an orphan disease with a poor prognosis. We explored the added value of individualized multimodal immunotherapy (IMI), including treatment adaptations during treatment, in patients treated at the IOZK as “individueller Heilversuch”. With pre-defined criteria, 50 adults with first-line GBM, both MGMT promoter-unmethylated (unmeth) and –methylated (meth), were selected. Unmeth (10 f, 18 m) and meth (12 f, 10 m) patients were treated between 27/05/2015 and 01/01/2022 and retrospectively analysed with 01/07/2022 as cut-off. IMI consisted of 1/ immunogenic cell death (ICD) immunotherapy (injections of Newcastle disease virus and sessions of modulated electrohyperthermia) during maintenance chemotherapy, followed by 2/ Dendritic Cell vaccination and modulatory immunotherapy after all chemotherapy, and finally 3/ maintenance ICD immunotherapy. Median age was 48y, median Karnofsky performance index was 80. More than half of the patients had less than complete resection. Dynamics in the tumor biology and immune response were demonstrated during treatment (like mRNA expression for PDL1 in circulating tumor cells), and required treatment adaptations during treatment (like the addition of checkpoint inhibitors). The median OS of unmeth patients was 22m (2y-OS: 42%). OS of meth patients was significantly better (p=0.0414) with 38m (2y-OS: 81%). There were no major treatment-related adverse reactions. These real world OS data were compared with reported data in randomized clinical trials with tumor treating fields (TTF) or DC vaccine (DCvax®-L) alone. The (external) control arms in these trials, which reflect the current state of the art results, showed median OS and 2y-OS of 15m / 21% in unmeth and 21m / 42% in meth patients. TTF, which is now recognized as ICD treatment) improved the OS in both unmeth and meth patients (17m / 27% and 32m / 59%) while DCvax®-L improved OS only in meth patients (15m / 19% and 30m / 58%). The addition of IMI during/after standard of care and the role of treatment adaptations during treatment should be prospectively explored.

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Identification and functional characterization of HLA-restricted HPV16 E6- and E7-derived epitopes

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Persistent infections with high-risk human papillomaviruses (HPVs) cause anogenital cancers as well as head and neck cancers. The malignant transformation of the infected cells is driven by the viral oncoproteins E6 and E7, which are expressed at all disease stages. Therefore, HPV-driven malignances are ideal model systems for therapeutic vaccination; and peptides derived from the E6 and E7 proteins are considered optimal candidates for the design of an epitope-specific therapeutic vaccine. However, as the set of presented T cell epitopes is specific to the individual human leukocyte antigen (HLA) molecules, short peptide-based vaccines usually need to match a patient’s HLA type. By defining peptides, binding to HLA supertypes, i.e. groups of HLA molecules that can present similar peptide repertoires, the number of epitopes that are required to achieve a broad population coverage can be reduced. Based on this, a vaccine formulation combining epitopes binding to the six HLA supertypes A1, A2, A3/A11, A24, B7 and B15 could provide a population coverage of approximately 99%.

Within our workflow of identifying suitable HPV16 E6- and E7-derived epitopes, possible target peptides for these HLA supertypes were preselected by state-of-the-art *in silico* HLA binding predictions, and validated regarding their HLA binding by competitive *in vitro* cellular binding assays. The identified 294 peptide-HLA complexes are currently investigated regarding their immunogenicity in interferon-γ ELISpot assays, in which PBMCs of HLA-matched heathy donors are tested for T cell memory responses. Additionally, a flow cytometry-based phenotyping for CD3, CD4 and CD8 and an intracellular cytokine staining (ICS) for IFNγ and TNFα are performed to gain a deeper insight into the immune response. So far 42 HLA-A2-, 8 HLA-A1-, 14 HLA-A3- , 11 HLA-A11, 16 HLA-A24, 11 HLA-B7 and 29 HLA-B15-restricted epitopes were identified in ELISpot assays. The ICS data proved that at least one of the identified epitopes for each HLA supertype specifically induced CD8 T cell activation. For each HLA type at least 8 donors have been analyzed, and the analysis of additional donors is ongoing.
Promising candidate epitopes will be further characterized in a live cell imaging-based cytotoxicity assay, which is currently established. In this assay, the capability of peptides to induce specific killing of HPV16-transformed cell lines will be validated. Additionally, the live cell imaging setup will enable the analysis of the time course of the cytotoxic activity.
The combined data will contribute to an HPV16 E6/E7 epitope repertoire map, which will serve as a rational basis for the development of epitope-centric HPV16 immunotherapies.

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Phase I/II clinical trial targeting alternative shared neoantigens (TEIPP) in checkpoint-resistant non-small cell lung cancer.

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Immune checkpoint inhibitors (ICI) have significantly improved the outcome of various solid tumours by reinforcing the activity of tumour-reactive CD8+ T cells. Still, the majority of patients develop secondary resistance and succumb to disease. A frequently observed immune escape mechanism is the down-regulation of the intracellular peptide transporter TAP1/TAP2, resulting in impaired presentation of conventional (neo)antigens in HLA class I, evasion from tumour-reactive CD8+ T-cell mediated control, and a negative impact on prognosis and efficacy of checkpoint inhibition. We discovered that tumour cells with TAP defects instead present a new class of ubiquitously expressed, nonmutated HLA-restricted neoantigens, called T-cell epitopes associated with impaired peptide processing (TEIPP). Furthermore, we showed that TEIPP-targeting to control TAP-impaired cancers via adoptive cell transfer (ACT) or therapeutic vaccination was safe and effective in preclinical models. In a first-in-human ongoing phase I/II dose escalation study (20, 40, 100 ug of peptide) with an extension cohort, the latter in which the vaccine will be combined with pembrolizumab (EudraCT

2020-005427-36), we determine the safety, tolerability, immunogenicity and efficacy of the LRPAP1$_{7\text{-}30V}$-SLP (TEIPP24) admixed with Montanide ISA51 in a total of 24 patients with checkpoint blockade-resistant non-small cell lung cancer (NSCLC). Currently, 12 patients have been vaccinated and tumour growth control associated with the presence of a TEIPP vaccine-induced T-cell response was obtained in 7 patients (1 partial response, 5x stable disease, and 1 mixed response). No dose limiting toxicities were observed. After one round of stimulation the highest frequencies of LRPAP1-specific CD8+ T cells were found in clinical responding patients. These LRPAP1-specific CD8+ T cells express memory (CD45RO) and activation (PD-1) markers and produce multiple cytokines and importantly, they specifically recognized LRPAP1 expressed by tumor cells lacking TAP1. In addition, expansion of activated polyfunctional LRPAP1-specific CD4+ T cells producing GM-CSF, IFNy, TNFa, IL2 and/or CCL4 was observed upon vaccination merely in the clinical responding patients. In conclusion, our preliminary data indicate a proof-of-principle on the safety and potential of TEIPP-targeted T cells as salvage therapy for immune-escaped tumours progressive on immune checkpoint therapy.

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Induction of mucosal immunity by modulation of therapeutic HPV16 vaccination approaches

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Many therapeutic vaccine approaches against HPV16 (human papillomavirus 16)-associated malignancies have already been advanced to the preclinical and clinical stage. Even though they were successful in inducing high levels of HPV-specific T cells and also in curing mice from HPV16-positive tumors, most vaccines were not efficacious in patients. One of the likely reasons is that circulating T cells are exempt from entering the mucosal tissues, especially of the female genital tract, from which HPV-caused lesions arise. We therefore developed an orthotopic (vaginal) HPV16-dependent tumor model in MHC-humanized mice that allows testing of vaccines containing HLA-A2-restricted epitopes against mucosally located tumors. The goal of the present study was to induce a vaginal HPV16-specific CD8$^+$ T cell response in these mice. To this end, a validated target epitope, E7$_{11\text{-}19}$, was tested in different vaccine formulations including liposomes, nanoparticles and amphiphilic constructs, in intravaginal vaccination approaches. Additionally, the systemic induction of T cells by subcutaneous vaccinations was combined with interventions leading to the migration of lymphocytes to the genital mucosa. These were application of a booster vaccination to the mucosa after having primed subcutaneously and chemotactic attraction of the induced HPV16-specific T cells into the tissue by

induction of an inflammatory response. The efficacy of the different methods to establish a specific anti-tumor response was then determined in a therapeutic tumor vaccination experiment. Although attracting HPV16-specific T cells to the vaginal mucosa was successful in non-tumor-bearing mice, we did not yet observe therapeutic efficacy in the new tumor model. Further vaccination approaches (changed timelines, combination schemes) are currently under investigation. Results obtained with the new model can thus provide important insights for future clinical trial design.

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Spontaneous antigenic mimicry arises from acquired mutations in cancer giving rise to TIL responses in vitro

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The adaptive immune system is a refined network of cells and mechanisms that recognize and clear foreign antigens to protect an organism against pathogen infections. Mutations in tumour cells may lead to the generation of neoantigens which display various degrees of homology to common microbial antigens. We therefore set out to investigate whether the efficacy of cancer immunotherapy, observed in some patients, might be contributable to the boosting of an existing immunological memory to previously encountered microbial antigens. We sought to identify tumour infiltrating lymphocytes (TILs) specific to both tumour and microbial antigens through a next generation sequencing approach, assessing the homology between tumour-specific mutations and known microbial antigens. In this study, we use matched tumour cell lines, TILs and serum samples collected from 12 responding patients with malignant melanoma treated at our center (CCIT-DK) with expanded TILs. We assessed the reactivity of TILs to autologous tumour cell lines by measuring the surface expression of CD107a, and measured TNF and IFNg with intracellular staining. TILs of all patients showed high reactivity to the autologous tumour. We next performed next generation sequencing on the tumour samples to identify minimal epitopes that could bind to HLA. The identified mutations were mapped onto a broad database of selected bacterial peptides that the Danish population has most likely been exposed to. For each patient, our analyses identified a large number (47-2392 peptides) of mutated short peptides (9aa) with different degrees of homology. Mutated peptides for each patient were aligned and further selected to obtain a shorter array of potential targets, with a comparison to the wild type peptide. Peptides were subsequently screened in ELISpot assays with TILs to assess reactivity to microbe-homologous peptides. Moving forward, serum samples of patients with peptide-reactive TILs will be analyzed to confirm previous exposure to the specific pathogen with high homology to the predicted mutated peptide. This data will provide insight into how the microbial repertoire can influence cancer progression in patients and shed a light on the heterogeneity observed in clinical responses. This knowledge has the potential to improve responses and prepare the stage for developing new next generation personalized immunotherapies.

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Bovine meat and milk factor protein expression in pancreatic ductal adenocarcinoma: implications on cancer development

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Over 20% of the cancer cases worldwide are expected to arise as a result of previous infections. Bovine Milk and Meat Factors (BMMF) are plasmid-like, infectious agents isolated from cow's milk and serum. BMMF have been previously described to be causatively involved in indirect carcinogenesis of colorectal cancer after decades-long latency via expression of a conserved BMMF protein (Rep) and induction of increased levels of chronic inflammation. Despite first evidence of BMMF in pancreatic ductal adenocarcinoma (PDAC) based on immunohistochemistry (IHC) and electron microscopy, the presence and role of BMMF in PDAC development has not been analyzed specifically, yet. In this study, pancreatic tissues of PDAC (n=22), chronic pancreatitis (CP) (n=6) patients and healthy donors (n=18) were subjected to IHC, immunofluorescence (IF) and immunoblotting using anti-Rep monoclonal antibodies and *in silico* BMMF analysis, allowing quantification of BMMF DNA and protein. IHC analyses of pancreatic tissue stained with 4 individual Rep antibodies showed BMMF Rep expression both in exocrine and endocrine compartments of pancreas. The intensity and spread of BMMF Rep staining in Langerhans islets were significantly increased in PDAC patients when compared to CP patients and healthy donors. Moreover, both intensity and spread of Rep staining were negatively correlated with preoperative glucose level of PDAC patients. Analyses by IF showed Rep expression in insulin producing β-cells in islets, suggesting a possible regulation of β-cells by BMMF. Indeed, variation of insulin secretion levels was observed after the treatment of β-cell lines (EndoC-βH5) with BMMF Rep protein (MSBI1.176; 1 µM) depending on the level of glucose in glucose-stimulated insulin secretion assay. In exocrine part of tissue, IHC and IF analyses revealed anti-Rep staining mainly in $CD68^+$ macrophages. Cell-based quantification by IF with Rep, CD68 and CD163 antibodies showed a significantly increased number of M2-like ($Rep^+CD163^+CD68^+$) macrophages in PDAC tumor tissues when compared to tumor-adjacent or healthy donors' tissues. In addition, BMMF Rep expression was supported by detection of bands in immunoblotting and validation of Rep-specific peptides by mass spectrometry. *In silico* analysis allowed to detect BMMF DNA reads in 62 patients (41,6%; PCAWG data; Canadian cohort) and BMMF RNA reads in 24 patients (20%; DKFZ-HIPO). Most of the hits showed similarity to one representative isolate of BMMF group 1. In conclusion, the study showed that BMMF can be identified in pancreatic cancer tissue in both nucleic acid and protein level. Moreover, we demonstrated that BMMF might be involved in tumorigenesis via accumulation in macrophages, mediating them into pro-tumorigenic M2-like polarization and/or via affecting β-cells of pancreatic islets. These data provide novel insights on the possible contribution of BMMF to PDAC development and might enable to discover new approaches for intervention.

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Antibody therapeutic development with an integrated platform combines immunized libraries, synthetic libraries, AI/ML guided leads optimization and HT IgG production

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Antibody drugs are a promising and rapidly growing component of the therapeutic landscape. The success of antibody therapeutics is largely driven by its specificity and selectivity in contrast to the off-targets liabilities of small molecule drugs. To advance antibody drug discovery, Twist integrated multiple techniques and platforms including synthetic and immunized libraries, artificial intelligence and machine learning (AI/ML), and High Throughput IgG Production (HT IgG) to discover, optimize, produce, and characterize antibody drug candidates against a wide range of inflammatory and immune-oncology targets to assist in generating leads for downstream pre-clinical and clinical campaigns. Wnt signaling pathway has been known to play important regulatory roles in tumorigenesis. Dickkopf-1 (DKK1) is an endogenous protein known to over-express in tumor micro-environments and can block Wnt/β-catenin signaling by interrupting Wnt-FZD-LRP interactions. Previous studies have shown the anti-tumor effect of anti-DKK1 antibodies in multiple different cancers including esophagogastric cancer, lung cancer, prostate cancer, etc. Here, we would like to show how Twist uses our integrated platform to identify functional anti-DKK1 antibody drug candidates through screening of hundreds of hits enriched from phage display and AI/ML affinity maturation. This platform can also be easily expanded to any other “difficult to target” drug targets.

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Genome-scale in vivo CRISPR screens identify Erap1 as a non-classical MHC-I-dependent immunotherapy target

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Cancer immunotherapy with immune checkpoint blockade (ICB) has revolutionized the treatment of several cancer types, but many patients do not respond to treatment and others develop resistance to treatment over time. To systematically discover novel genetic targets that can sensitize tumors to ICB, we conducted genome-scale, in vivo pooled genetic screens across tumor models treated with ICB and identified endoplasmic reticulum aminopeptidase 1 (Erap1) as a candidate immunotherapy target. Using several in vivo and in vitro tumor models, we confirm that loss of Erap1 sensitizes tumors to T- and NK-cell mediated anti-tumor immunity. Using syngeneic murine models of renal, pancreatic, colorectal, and melanoma cancers, we confirm that Erap1-null tumor cells are strongly sensitized to ICB in vivo. We used depletion of CD8+ T cells and NK cells to identify the cytotoxic immune subsets that are responsible for killing Erap1-null tumors and found that both subsets were necessary in vivo. Similarly, primary CD8+ T cells and NK cells isolated from mice selectively killed

Erap1-null tumor cells in vitro. To probe the possible mechanism of sensitization, we conducted an independent sub-genome-scale in vivo CRISPR screen in Erap1-null tumor cells treated with ICB. Although sgRNAs targeting the non-classical MHC-I Qa-1b (H2-T23) were top depleted hits in the genome-scale screens, loss of H2-T23 had no synergistic effect in Erap1-null tumor cells, suggesting an epistatic interaction between Erap1 and H2-T23. In an in vivo competition assay using tumor cells transduced with an Erap1-targeting sgRNA or a control sgRNA on a control, H2-T23-null, or B2m-null background transplanted into WT mice treated with ICB, Erap1-null cells were depleted in the control background but not in the H2-T23-null and B2m-null backgrounds. Immunopeptidomic analysis revealed dramatic loss of Qdm peptide intensity and marked increase in novel peptide diversity/intensity on Qa-1b isolated from Erap1-null tumor cells. Blockade of the NKG2A/CD94 checkpoint using a monoclonal antibody against NKG2A was sufficient to completely reverse the Erap1-null phenotype in vivo and in an in vitro NK cell killing assay. In summary, we demonstrate that loss of Erap1 sensitizes tumors to ICB by inhibiting proper trimming/loading of Qdm onto Qa-1b to disrupt self-tolerance and increase the cytotoxicity of NKG2A+ CD8+ T cells and NK cells. Our results support further investigation of Erap1-directed therapeutic strategies and provide functional validation of our screening platform for identification of novel immunotherapy targets.

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Identification of actionable neoepitopes for personalized cancer immunotherapy by MS-based immunopeptidomics

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Presentation of tumor-specific peptides by HLA class I molecules to $CD8^+$ T cells is the foundation of epitope-centric cancer immunotherapies such as therapeutic vaccines or TCR-transgenic T cells. Currently, the only available technique to provide a direct proof of actual peptide presentation at the tumor cell surface and thus of actionable targets is mass spectrometry (MS)-based immunopeptidomics.
Here, we used our recently established targeted MS workflow for the analysis of tumor biopsies. For all samples analyzed, HLA class I:peptide complexes were purified by immunoprecipitation and peptides were separated by solid phase extraction. Whole exome and RNA sequencing was performed to identify somatic mutations derived from single nucleotide variations, insertions/deletions and gene fusions and HLA binding prediction was performed for the resulting mutated protein sequences. Up to 50 candidate peptides per patient were selected for systematic parameter optimization based on HLA binding predictions as well as mutated allele frequency and

expression data. Employing our targeted MS workflow for the analysis of tumor biopsies with limited amounts and from different entities, we were able to detect 5 mutation-derived neoepitopes in 3 of the 5 analyzed samples. In parallel, peptide-specific T cell populations were identified for one of the identified neoepitopes by multimer staining. Importantly, these T cell populations were expandable by *in vitro* stimulation highlighting their clinical potential.
Albeit being highly sensitive, targeted MS analysis is limited to a relatively small set of pre-defined peptides. To circumvent this limitation, we have evaluated data-independent acquisition (DIA) MS as a complimentary alternative. DIA MS uses sequential and systematic fragmentation of all detectable peptides to generate a digital map of the sample. We combined DIA MS with improved high-throughput sample preparation and ion mobility separation to gain deeper sampling of the immunopeptidome. Using library-free analysis, we consistently detected more than 10,000 unique peptides from as little as $2.5x10^7$ cells input material. Combination of different ion mobility separation parameters further boosted these numbers by up to 50%. Importantly, these identifications included multiple neoepitope candidates originating from somatic mutations as well as low abundant viral proteins derived from human papilloma virus. In the future, the newly established DIA workflow will be applied in combination with the targeted workflow for the analysis of clinical samples.
Taken together, we have established two complimentary workflows for the identification of actionable targets for the development of neoepitope-centric cancer immunotherapies with DIA MS allowing for unbiased discovery and targeted MS enabling ultra-sensitive discovery.

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Investigation of the antitumor immune response of optimized multidrug combinations in colorectal cancer model

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Colorectal carcinoma (CRC) is one of the major causes of cancer-related mortality worldwide. CRC is widely treated with combinations of chemotherapeutics and/or with targeted strategies. Nevertheless, current chemotherapy regimens need improvement, as their efficiency and selectivity are limited, especially in late-stage disease. Harnessing the immune system to fight cancer is a field of research that received major attention over the last decades. Our lab aims to develop a strategy where optimized drug combinations (ODCs) of targeted agents' prime cancer patients for responsiveness to immunotherapies. We developed a panel of ODCs composed of tyrosine kinases inhibitors (TKI) and anti-angiogenic drugs, known to have the capacity to normalize the tumor microenvironment and to enhanced tumor immunogenicity. We validated the efficacy and selectivity of these low-dose multidrug combinations in CRC organoids. Furthermore, we developed an *in vitro* 3D co-culture model that includes CRC organoids, endothelial cells and a subset of immune cells derived from the same host species. Using this platform, we showcased the potential of our optimized ODCs to overcome endothelial cell anergy by increasing expression of the endothelial

adhesion molecules involved in leukocyte extravasation such as ICAM. Finally, we highlight that the ODC can boost anti-cancer immunity *in vivo* in a syngeneic mouse model of CRC by a significant reduced tumor growth and an increased anti-tumoral immune cells infiltration. Our findings highlight the potential of low-dose multidrug combinations in stimulating the immune response and paves the way for new combination strategies with immunotherapy in CRC.

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Decipher novel cytotoxic T cell resistant mechanisms in lung cancer.

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To this day, non-small cell lung cancer (NSCLC) is still the leading cause of cancer related deaths worldwide. In the past few years, immunotherapies that aim to stimulate the cancer killing potential of cytotoxic T lymphocytes (CTLs), have transformed the field of cancer therapies. Unfortunately, response rates remain <25% in unselected NSCLC patients, indicating the need to uncover and tackle CTL-killing resistant mechanisms (CRMs) installed by the tumor. These CRMs can be tumor cell extrinsic, installed within the non-malignant tumor microenvironment or tumor cell intrinsic. Typical examples of the latter are PD-L1 upregulation, loss of β2-microglobulin (β2M) and insensitivity to IFNɣ.
Here we aimed to unravel novel tumor cell intrinsic CRMs by generating β2M+ CTL-killing resistant tumor cells, still responsive to IFNɣ. To that end, we used the KrasG12Dp53-/- KP line to generate an eGFP and ovalbumin+ KP-eO line. In vitro we subjected this line for 12hrs to OT-I CTL mediated killing (T:E of 1:10). Next, leftover KP-eO cells were expanded and subjected to OT-I CTLs again, for 6 rounds in total, until the completely CTL-resistant KP-eO-R6 was generated. In vivo, 5x10e5 KP-eO cells were injected intravenously. Four weeks later, eGFP+ cells were isolated from the lungs, subjected to an OT-I killing assay in vitro after which the killing resistant KP-eO-Rx line was generated. After FACS sorting of β2M and IFNɣ responsive KP-eO-R6 and KP-eO-Rx cells, we used RNAseq to decipher transcriptional differences between them and the KP-eO CTL-killing sensitive (wild type) line. Upon GSEA and subsequent KEGG pathway analysis we could define two significantly upregulated genes; GSTA2 in KP-eO-R6 and GSTM3 in KP-eO-Rx and -R6. Both have previously been linked to evasion of apoptosis but not resistance to immunotherapy. Currently we are evaluating if we can erase the resistance of KP-eO-R6 and/or KP-eO-Rx to OT-I mediated killing by knocking out GSTA2 and GSTM3 (alone or both). Moreover, we are deciphering if the KP-eO wild type, KP-eO R6 and KP-eO Rx lines differently grow and mold the tumor microenvironment and ability to stimulate T cells in the periphery upon re-engraftment in mice. Our results can reveal valid biomarkers for patient selection prior to immunotherapy as well as novel bullseyes to target on the battlefield against cancer.

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NF-kappaB c-Rel is a master regulator of CD8+ T-cell function in cancer

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Context and objectives: Cancer progression is greatly influenced by a delicate balance between tolerance to and immune response against tumors. The recent success of immunotherapies such as checkpoint-blockade therapies, demonstrates that this balance can be shifted effectively towards enhanced anti-tumor immunity, to delay or even stop cancer progression, particularly by enhancing the function of CD8+ T cells. However, only a fraction a patients respond to these regimens, and the molecular mechanisms that drive the response and resistance to immunotherapies are largely unknown. The transcription factor NF-kappaB (NF-kB) has been implicated in the regulation of T-cell function. However, NF-kB is in fact a family of transcription factors composed of 5 distinct subunits; due to the lack of suitable tools, the contribution of individual subunits of NF-kB to CD8+ T-cell biology has never been addressed. We hypothesized that the activation of selective NF-kB subunits orchestrates CD8+ T-cell biology and underlies the clinical response to checkpoint-blockade therapies.
Results: We used novel models carrying conditional ablation of canonical NF-kB subunits RelA and c-Rel, in mature CD8+ T cells. We found that RelA had a prominent role in shaping the transcriptome and function of CD8+ T cells at steady-state. Conversely, ablation of c-Rel -but not RelA- accelerated cancer growth in a model of melanoma. scRNA-seq and spectral flow cytometry experiments revealed a role of c-Rel in the activation and maintenance of T cells in the tumor microenvironment. In addition, c-Rel was required for the response to anti-PD-L1 therapy in mice. Accordingly, enrichment of a c-Rel-dependent gene signature correlated with good prognosis in melanoma patients treated with checkpoint-blockade therapies. Finally, c-Rel-deficient human primary T cells displayed reduced production of inflammatory cytokines and cytotoxic function against melanoma cells.
Conclusion and perspectives: Our results highlight a novel molecular mechanism orchestrating T-cell function in cancer, and paves the way toward NF-kB-targeted agents in cancer immunotherapy.

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Link between bovine meat and milk factor protein expression and inflammation as possible cause of colorectal cancer after decades-long latency

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Bovine Meat and Milk Factors (BMMFs) are small, circular DNA molecules and part of a group of infectious agents with both bacterial and viral features. BMMFs were initially isolated from bovine serum and milk by EM de Villiers et al. in 2014. The systematic search for these zoonotic pathogens in cow's products started after identification of a geographic association of the worldwide meat and milk consumption with the incidence of colon and breast by H zur Hausen. The model that zoonotic risk factors contribute to cancer, in particular of colorectal cancer (CRC), subsequently after decades-long latency based on indirect carcinogenesis, was outlined in 2001 and 2009 by H zur Hausen and refined later. In CRC tissues, BMMF DNA and high levels of expression of a conserved BMMF protein (Rep) have been identified by antibody staining in the close vicinity of the tumor, but not in the tumor itself. In the tumor-adjacent tissues, Rep expression was linked with macrophages and an increased detection of a marker for radical formation and DNA mutation in the Rep expressing inflammatory foci. This supported the previously proposed model of BMMF-induced chronic inflammation resulting in increased radical formation, which in turn induces random DNA mutations in neighboring replicating crypt cells as precursors of dysplasia and tumors.
To verify Rep expression and test causative and prognostic relevance based on larger tissue cohorts, tissue sections with paired tumor-adjacent mucosa and tumor tissues of CRC patients (tissue microarrays, TMAs, n = 246) and individual cohorts for low-/high-grade dysplasia (LGD/HGD) and mucosa of healthy donors were used for quantification of Rep and CD68/CD163 (macrophage) expression by co-immunofluorescence microscopy and immunohistochemical scoring (TMA).
Rep was expressed in the tumor-adjacent mucosa of 99% of CRC patients (TMA) and was histologically associated with CD68+CD163+ macrophages. Macrophage-linked Rep expression was increased in CRC patients when compared to age-matched healthy controls supporting a causative contribution to CRC. Rep was also expressed in low grade dysplasia and LDG/HGD-adjacent tissues indicating a role as early risk factor. Albeit not significant, incidence curves for CRC-specific death were increased for higher Rep expression. Recently, structural analyses by immuno electron microscopy identified pleomorphic vesicular bodies of 50-200 nm in tissues of colon, lung and pancreatic cancer.
The association between Rep and CD68 expression supports the previous model that BMMF-specific inflammatory regulations, including macrophages, are involved in the pathogenesis of CRC and might open new avenues for BMMF-specific preventive or therapeutic intervention. The presence of antibody-accessible BMMF targets in different types of cancer might represent a marker and early risk factor for cancer and target for intervention.

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GEN1046 (DuoBody®-PD-L1x4-1BB) in combination with PD-1 blockade potentiates anti-tumor immunity

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GEN1046 (DuoBody®-PD-L1x4-1BB), an investigational, potential first-in-class, immunomodulatory bispecific antibody, is designed to elicit an anti-tumor immune response by simultaneous and

complementary PD-L1 blockade on tumor or immune cells and conditional 4-1BB stimulation on T cells and NK cells. Encouraging preclinical and early clinical activity observed with GEN1046 treatment has been published (Muik, et al, 2022, *Cancer Discovery*). We hypothesized that GEN1046 in combination with PD-1 blockade would further potentiate anti-tumor activity via distinct and complementary immunomodulatory effects. The addition of a PD-1-blocking agent could help maintain complete PD-1 pathway blockade by inhibiting interactions with both PD-L1 and PD-L2. Furthermore, it could potentially free up PD-L1 for binding to GEN1046, therefore promoting PD-L1-dependent 4-1BB conditional agonism. Here we report preclinical evidence supporting the therapeutic synergy of GEN1046 when combined with an anti-PD-1 agent and describe the mechanisms of enhanced anti-tumor immunity elicited by the combination. In mixed lymphocyte reaction assays using either unstimulated T cells or T cells exhausted by repeated CD3/CD28 co-stimulation, the combination of GEN1046 with an anti-PD-1 agent potentiated cytokine release. In antigen-specific proliferation assays, enhanced T-cell expansion and cytokine secretion were observed with the combination versus each single agent. In mice bearing syngeneic subcutaneous MC38 tumors, combination of anti-mouse PD-L1x4-1BB bispecific antibody with anti-mouse PD-1 resulted in therapeutic synergy, with significantly enhanced survival ($P \leq 0.001$) and durable, complete tumor regressions (CR) in 7/10 mice versus no CR observed with either single agent. Animals with CR were protected from tumor outgrowth upon rechallenge with MC38 cells, indicating that a long-lasting immune memory response was achieved with the combination. Furthermore, animals that received the combination showed a trend for ≥1.5-fold increase in the average density of $CD3^+$ and $CD4^+$ tumor-infiltrating lymphocytes (TILs) and proliferating ($Ki67^+$) and cytotoxic ($GZMB^+$) $CD8^+$ TILs versus each single agent, which is consistent with an amplified anti-tumor immune response. These preclinical findings suggest that GEN1046-induced conditional 4-1BB stimulation combined with complete PD-1 blockade may improve the anti-tumor immune response via distinct and complementary immunomodulatory effects. Ongoing clinical studies are evaluating GEN1046 in combination with pembrolizumab in patients with advanced NSCLC who are treatment-naive (NCT03917381) or who have progressed on prior checkpoint inhibitor–containing therapy (NCT05117242).

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Enhancement of anti-tumor T-cell immunity by means of an oral small molecule targeting the intracellular immune checkpoint MAP4K1

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Immune checkpoint blockade using antibodies targeting the cell surface expressed proteins CTLA-4, PD-1 and PD-L1 has revolutionized cancer care and its clinical impact in several indications has prompted a search for complementary immunostimulatory approaches that can further increase the efficacy of these drugs.

Mitogen-activated protein kinase kinase kinase kinase 1 (MAP4K1; HPK1), a serine/threonine kinase expressed exclusively in hematopoietic cell lineages, mediates a negative feedback signal downstream of T-cell receptor stimulation. Its activity is enhanced by PGE2 and TGFβ, factors commonly present in the tumor microenvironment. Mice deficient for MAP4K1 or expressing a kinase-dead variant of MAP4K1 exhibit enhanced T-cell function, including increased anti-tumor immunity.
We developed the small molecule inhibitor BAY-405 that displays potent nanomolar MAP4K1 inhibition in biochemical and cellular assays, good kinase selectivity, and in vivo exposure after oral dosing. Pharmacological inhibition of MAP4K1 enhances T-cell immunity and overcomes the suppressive impact of PGE2, TGFβ and CD4+ T-regulatory cells. Single agent treatment of tumor-bearing mice results in suppression of tumor outgrowth in several syngeneic models. This is accompanied by an increase in the anti-tumor T-cell response, dependent on an intact T-cell compartment, while not involving direct anti-tumor cytotoxicity. Inhibition of MAP4K1 in conjunction with PD-L1 blockade results in further suppression of tumor outgrowth. Moreover, we found that MAP4K1 is expressed in both PD-L1-high and PD-L1-low human cancers.
In summary, our data show that selective inhibition of MAP4K1 by means of small molecule drugs may be used to expand the patient population responding to immune checkpoint inhibition.

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Bovine Meat and Milk Factors protein expression coincides with peritumor alveolar macrophages in lung cancer tissues

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Although up to 90% of lung cancer cases are linked to smoking, exposure to additional environmental and dietary factors and chronic inflammation are regarded as risk factors. Increased cancer incidence was found for butchers and meat workers; conversely, decreased incidence was shown for people with lactose intolerance or regularly taking non-steroidal anti-inflammatory drugs. Global epidemiology points towards an association between lung cancer incidence and consumption of bovine products. A search for infectious risk factors led to the isolation of plasmid-like DNA molecules termed Bovine Meat and Milk Factors (BMMF), which replicate in human cells and express a conserved replication protein (Rep). Although vesicular BMMF structures were identified in lung cancer tissues by immune electron microscopy and immunohistochemistry (IHC), the presence of BMMF was not systematically tested in lung cancer. In this study, Rep expression was quantified in paired tumour and peritumour tissues from lung cancer patients as well as cancer-free individuals (control) by IHC co-immunofluorescence microscopy (IF) and immunoblot based on monoclonal anti-Rep antibodies and mass spectroscopy. In addition, publicly available single cell RNA sequencing (scRNA-seq) data was assessed to monitor $BMMF^+$ cells and BMMF-specific expression patterns. 4 of 7 antibodies applied in IHC showed tissues staining, with strongest staining observed in peritumor tissue (20 of 23 patients tested positive). Tumor and control tissues showed less staining. In peritumour tissues, a congruent staining of Rep and $CD68^+$ cells was observed in IHC and IF in

alveolar macrophages (MFs). IF triple staining with Rep, CD68 and CD163 (marker of M2-like macrophages) revealed congruent staining of Rep in $CD68^{+}CD163^{+}$ alveolar macrophages. Cell-based quantification indicated a significantly increased number of $Rep^{+}CD68^{+}CD163^{+}$ cells in peritumour when compared to tumor and control tissues. About 40% of $Rep^{+}CD68^{+}$ MFs in lung peritumour tissues are also $CD163^{+}$ (22% of $CD68^{+}$ MFs were Rep^{+}). The presence of Rep in lung peritumour tissues was supported by detection of bands by immunoblotting of tissue lysates and BMMF (Rep-specific) peptides validated by mass spectroscopy. Screening for BMMF reads in a publicly available scRNA-seq dataset from lung adenocarcinoma revealed $BMMF^{+}$ cells in 83% of patients (n=30), mostly in MFs and T-cells. In conclusion, BMMF antibody staining revealed Rep expression in $CD68^{+}$ (and $CD163^{+}$) MFs of lung peritumour tissues and was enriched in case vs. control suggesting a causative contribution. The identification of Rep^{+} MFs in scRNA-seq, with the addition of further scRNA data, might enable to study expression changes induced by BMMF. This is of particular interest as alveolar MFs have been described as drivers of early tumorigenesis and target for therapeutic intervention.

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A Phase I/II trial of NOX66 in combination with nivolumab in patients with advanced cancer.

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Background: Immunotherapy in oncology has demonstrated significant improvements in survival in multiple tumour types, but over time, cancers will develop resistance and patients ultimately progress whilst on treatment. NOX66 (idronoxil) targets ENOX2 and suppresses STING-mediated inflammatory pathway reducing IFN and NFκB driven cytokine production by cancer cells. Adding NOX66 to nivolumab may help to overcome resistance mechanisms to immunotherapy and reinvigorate responses in patients.
Methods: IONIC-1, is an open label Phase I/II dose escalation trial evaluating safety and preliminary efficacy of increasing doses of NOX66 (one week of suppositories followed by one week rest) combined with a set nivolumab dose (240mg IV every 2 weeks). Objectives include safety, tolerability, clinical and radiological responses, as well as patient's immune functional state after treatment with the combination therapy at set time points. Two groups are being studied: 1) Patients who have experienced progression on prior immunotherapy; 2) Patients naïve to immunotherapy.
Results: 12 patients have been treated to date; 4 in Group 1 and 8 in Group 2 . Most patients have received at least 3 or 4 cycles of combination treatment with NOX66 and nivolumab; 2 patients completed 8 cycles of treatment and 2 patients are ongoing in the study. Tumour types include lung, prostate, gastric, pancreatic, nasopharyngeal and metastatic squamous carcinoma. Early signals of tumour response based on RECIST 1.1 criteria include stable disease in 2 patients (SD at 8 weeks and at 16 weeks); partial response in 1 patient at 16 weeks; complete response in 2 patients, both with

ongoing CR at 24 weeks. Using a 32 multiplexed immune cytokine assay to evaluate the single-cell functional states of CD4+ and CD8+ T cells in patients' PBMCs at pre-treatment, and at Cycle 3 Day 1, we found a significant increase in polyfunctionality in both effector CD4+ and CD8+ve T cells compared to baseline in responder patients. Six patients experienced disease progression, and 1 patient was withdrawn due to suspected immune mediated myositis and hepatitis resulting from nivolumab and was not included in the response analysis. The most common adverse event (AE) was local perineal irritation that was readily managed with topical corticosteroid cream. Two patients developed an erythematous rash over the trunk and limbs after 2 cycles, suspected to be related to the combination treatment.
Conclusions: Preliminary analysis shows promising tumour responses from the combination therapy which appears to be well-tolerated with no safety signals evident to date. This study is ongoing.

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Colorectal cancer cells induce a unique transcriptome signature in eosinophils and primes their responses to IL-3-induced activation.

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Eosinophils are evolutionary conserved innate immune cells that have been mainly studied in the context of allergic diseases and parasitic infections. Nonetheless, recent data demonstrate that eosinophils accumulate in a variety of solid tumors including colorectal cancer where their presence is associated with improved prognosis. Eosinophils can promote anti-tumor immunity by at least two non-mutually exclusive mechanisms, promoting T cell activation and direct cytotoxicity towards tumor cells. Yet, how tumor cells regulate eosinophil activities is largely unknown. Herein, we characterized the interactions between eosinophils and colorectal cancer cells using an unbiased transcriptional and proteomics analysis approach. Human peripheral blood eosinophils, isolated from different donors, were stimulated with colorectal cancer cell conditioned media, containing tumor cell-secreted factors from multiple cancer cell lines. Analysis of the RNA sequencing data identified a "core" shared signature consisting of 101 genes that characterize a transcriptional program baseline for the response of human eosinophils to colorectal cancer cells. Among these, the increased expression of IL-3Ra and its bc chain was identified and validated at the protein level. Subsequently, we demonstrated that secreted factors from tumor cells potentiated IL-3-induced expression of the adhesion molecule CD11a in eosinophils.
Combining proteomics analysis of tumor cell-secreted factors, with the RNA sequencing of eosinophils, revealed potential ligand-receptor pairs between tumor cells and eosinophils, as well as the potential involvement of the adhesion molecule CD18. These data provide important insight into the interactions between eosinophils and colorectal cancer cells, Identifying mechanisms by which eosinophils interact with tumor cells could potentially be used as targets for future immunotherapy.

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Fast and easy: isolation of functional immune cells straight from mouse spleen

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Introduction: The isolation of lymphocytes and myeloid cells from the spleen of mice is a key technique in many fundamental, translational and applied studies. Research using magnetically isolated cells contributes not only to the understanding of the immune system, but also to the development of many new vaccines and immunotherapeutic approaches. Isolated cells are often used in an *in vitro* studies for the target identification, genetic manipulation, expression profile analysis or *in vivo* adoptive cell transfers. The key to ensuring consistent and reproducible results is to limit any unnecessary manipulation of the cells and to ensure that isolated cells retain their functionality. Therefore, strategies to simplify and accelerate cell isolation are highly desirable to save time, ensure reproducibility and enable complex workflows to be carried out.
Methods: Spleen dissociation and simultaneous magnetic labelling of cells (CD4, CD8, CD90.1, CD90.2, CD19, and CD11c) was performed using the GentleMACS dissociator. Subsequent automated cell isolation was done using autoMACS NEO Separator. In order to compare our innovative StraightFrom Spleen cell isolation with a competitor's untouched cell isolation approach, CD4 and CD8 T cell separation was performed in parallel for both workflows. Target cell purity, yield and viability were assessed, and activation status and functionality were assessed using *in vitro* assays.
Results: Isolated cells were stained with 7-AAD and REAfinity antibodies and acquired on MACSQuant Analyzer. Purity and viability of CD4 and CD8 T cells were superior for the StraightFrom Spleen protocol compared to the competitor protocol. The yield of isolated CD4 T cells was higher for the StraightFrom Spleen protocol, while similar numbers of CD8 T cells were obtained in both workflows. No expression of CD25 or CD69 was observed when unstimulated CD4 and CD8 T cells were cultured overnight in complete medium at 37°C, and similar induction of CD25 and CD69 was observed when cells were activated with the T cell Activation/Expansion Kit. Cells isolated with both protocols showed similar proliferation and expansion in cell culture.
Conclusions: We have developed a simple and fast cell isolation method with minimal hands-on time. Significantly accelerated isolation protocols enable rapid isolation of cells of interest with excellent viability, purity and yield, reducing the time required for downstream analysis. Importantly, our StraightFrom Spleen protocol did not induce cell activation and did not affect cell functionality, such as proliferation and expansion of polyclonally stimulated T cells. The workflow presented here can be used to isolate pure and functional cells in an express time for a wide range of *in vivo* and *in vitro* applications and offers an attractive alternative to the laborious multi-step protocols commonly used in academic and industrial laboratories.

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Long-lasting response to anti-TIM3 immune checkpoint inhibitor therapy in acute myeloid leukemia patient with large oligoclonal T cell expansion

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Immuno-Oncology (IO) therapy is a promising and innovative avenue of treatment, which aims to shift the imbalance and lack of immune recognition in cancers to engage the body's immune system to fight against cancer cells. Previous IO studies with anti-CTLA4 and anti-PD1 treatments in acute myeloid leukemia (AML) have shown modest outcomes. An ongoing phase Ib clinical trial (NCT03066648) is assessing the efficacy of anti-TIM3 treatment (sabatolimab) in combination with hypomethylating therapy (decitabine) in patients with refractory AML. An elderly patient with therapy related AML participating in the trial achieved an exceptional long-lasting complete remission and interestingly, also had a sizable concomitant large granular lymphocyte (LGL) clone.
In this project, we aim to understand the antigen target of the LGL clone and whether it can participate in the anti-leukemia immune response and long-lasting remission. We analysed 5 longitudinal bone marrow samples from the patient before and during the sabatolimab + decitabine combination therapy with paired single-cell RNA and T cell receptor (TCR) sequencing (scRNA+TCRαβ-seq). At the start of therapy, the LGL clone comprised up to 36.6 % of the total TCR repertoire and remained stable during the remission. Based on scRNAseq, the clone had a CD8+ T_{EMRA} phenotype with co-expression of Natural Killer cell receptors, high cytotoxic activity, and the highest expression of *TIM3* out of the studied immune populations. At 23 months post-remission, the patient relapsed and at that time the LGL clone had contracted to 5% of TCR repertoire.
To study the antigen specificity of the LGL clone, we Gibson cloned paired TCRαβ sequences into retroviral plasmids and transduced them into a gene edited TCR knockout Jurkat reporter T-cell line. First, we created two reporter cell lines carrying cytomegalovirus (CMV) specific TCRs as a positive control and they showed high specificity to dominant CMV antigens. Using a similar design, we next generated Jurkat reporter T-cells expressing the LGL clone TCR based on the scRNA+TCRαβ-seq data. Co-culture experiments with human leukocyte antigen matched AML cell lines and primary patient leukemia cells are ongoing to determine the target recognition of the LGL clone.
Our results will provide clarity to the possible immune targets and drivers of long-lasting therapeutic responses in AML.

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Application of the microbiome-based prediction test BiomeOne quantifies antibiotic, geographical and health-related effects on response to cancer immunotherapy in a large European cohort

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The outcome of cancer immunotherapies is deeply influenced by the crosstalk between the intestinal microbiome and the immune system. However, several factors associated with the lifestyle and genetics of each individual are known to modulate and even modify the gut microbiome. As such, we can expect that not everyone can benefit from these therapies. In order to understand which factors can modify response to immune checkpoint inhibitors (ICIs), a comprehensive dataset consisting of microbiome profiles and self-reported health data was screened with BiomeOne, a microbiome-based algorithm that predicts response to ICIs prior to therapy initiation.
A diverse, European microbiome dataset consisting of 9,691 participants aged 18-90 years was screened. Each sample was classified by BiomeOne as a potential responder (R) or non-responder (NR) to ICIs and attributed a probability of response. Additional self-reported metadata of the study participants included age, sex, ethnicity, country of residence, antibiotic and probiotic usage, diet, and occurrence of gastrointestinal diseases. Groups were subsetted in order to achieve a balanced number of observations in each group and perform statistical analysis. Statistical analysis was performed via Wilcoxon, Kruskal-Wallis or two-sample t tests, and statistical significance was defined at $P < 0.05$.
A total of 7,000 samples were classified as Rs, while 2,691 were classified as NRs. Probiotic usage did not seem to impact response ($P = 0.92$), contrarily to antibiotics ($P < 0.01$). Study participants claiming to have used antibiotics in the last 3 months had a significantly lower number of Rs than those who have not taken antibiotics for over a year ($P < 0.01$). Additionally, participants reporting antibiotic usage in the last year had a significantly lower Rs than those not having taken antibiotics for over a year ($P = 0.01$). Interestingly, also participants that reported suffering from inflammatory gastrointestinal diseases had lower probabilities of response to ICIs than those who reported not experiencing inflammatory disturbances ($P < 0.01$). Females seemed to have higher response rates than males ($P = 0.01$). Significant differences in responder abundance was found across Czech Republic, Belgium and Italy ($P = 0.029$). No significant impact of age, ethnicity, or diet on the probability of response was identified.
Our preliminary data suggests that the administration of antibiotics up to a year prior initiation of ICI therapy can modify microbiome composition and lead to a negative outcome. Therefore, therapies aiming to modify the microbiome in order to influence therapeutic outcomes should refrain from using antimicrobials. Further multivariate analysis will be conducted to better understand the impact of other self-reported characteristics on ICI response.

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Under embargo

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Profiling pathogenicity of Bovine Meat and Milk Factors in different cancer types via analysis of high-throughput sequencing data

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Bovine Meat and Milk Factors (BMMF) are circular DNA molecules proposed as zoonotic pathogenic agents contributing to cancer development by indirect carcinogenesis. The BMMF-encoded Rep protein has been shown to colocalize with peritumour CD68$^+$ macrophages in colorectal cancer (CRC) tissues via immunohistochemistry staining and was expressed at a higher level in CRC patients when compared to healthy donors. Thus, BMMFs are perceived to contribute to carcinogenesis by causing the accumulation of mutations due to increased tissue inflammation after decades-long latency. BMMF DNA and Rep was identified in CRC tissues, yet the presence of BMMF DNA and RNA in different cancer types was not assessed so far. In this project, the presence of BMMFs in different cancer types is investigated by analysing high-throughput sequencing data of publicly available cancer sequencing projects. The *D-ViSioN* pipeline (Detection of Integration of Virus[s] by SingletoN[s]) was used to examine RNA-seq samples of more than 900 patients and whole genome sequencing data of tumour and normal tissue samples of about 1500 patients of the PCAWG study of the International Cancer Genome Consortium. For this, a library of BMMF sequences of more than 100 BMMF genomes assigned to four different BMMF groups was applied. The analysis of the PCAWG RNA sequencing cohort identified BMMF-positive patients in 10 of 25 cancer types, with positivity being defined as a detection of at least three BMMF reads for at least one BMMF group per patient. Across the entire RNA-seq cohort, 2.5% of the samples were BMMF-positive, whereas e.g. 19% of ovarian serous cystadenocarcinoma and 16% of gastric adenocarcinoma samples were BMMF positive. In the PCAWG WGS sequencing data cohort, 9.5% of the tumour samples were found to be positive for BMMF DNA, with the Canadian prostate cancer cohort showing the highest positivity rate with 40%. In addition, 5.6% of the normal tissue samples of the whole genome sequencing data were BMMF positive, with the German early onset prostate cancer cohort exhibiting the highest positivity rate with 23%. For all analysed cohorts, the detected reads are distributed over the entire sequence of the respective BMMF genomes, which indicates that the detected BMMF reads most likely do not result from contaminations or artefacts. Differences in the distribution of the BMMF subtypes in the different cancer cohorts are currently under investigation and might help to improve tools for BMMF detection. In conclusion, BMMF sequences can be detected in tumour samples as well as in normal

tissue samples of different cancer cohorts albeit – with exception of a few patients – with low read numbers per patient.

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Tumor-associated macrophage-targeted immunotherapy by intravenous injcetion of TLR agonist-loaded, DC-SIGN-selective nanoparticles

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Immunosuppressive M2-like tumor-associated macrophages (TAMs) have been attracting great interest, as they are one of key underlying mechanisms for tumor immune resistance. Various therapeutic approaches to deplete or inhibit TAMs have been tested in the clinical settings; however, they have not yet been approved due to the limited efficacy and safety arising from the lack of TAM-specificity.
We previously reported that pullulan nanogel, a pullulan polysaccharide-based hydrogel nanoparticle, is selectively incorporated into TAMs after intravenous injection into the mice bearing highly immune-resistant tumor. Delivery of tumor antigen using pullulan nanogel to TAMs effectively activates T cell immunity within the tumor microenvironment, leading to the eradication of the resistant tumor.
In this study, we identified C-type lectins human DC-SIGN (CD209), human L-SIGN (CD209L) and mouse SIGN-R1 (CD209b) as specific targets for pullulan nanogel. Importantly, there is accumulating clinical evidence showing that the high expression of human DC-SIGN in TAMs well correlates to poor prognosis and higher tumor stages across various solid tumors including brain tumors. On the other hand, the expression of DC-SIGN in normal tissues is limited to only the lymphoid organs and adipose tissue. Taking these advantages, we newly generated the pullulan nanogel formulations for intravenous injection to deliver TLR agonist to TAMs and evaluated their efficacy using in vivo models of mouse syngeneic tumor as well as in vitro cultured human PBMC-derived M2-like macrophages. As a result, these nanoparticulate immunotherapies induced inflammatory responses in vitro and in vivo and efficiently inhibited in vivo tumor growth as monotherapy or in combination with anti-PD-1 antibody. The use of pullulan nanogel also mitigated the side effects of TLR agonists in the treated mice. These findings indicate that pullulan nanogel would be a useful drug delivery system (DDS) to enhance the efficacy and safety of TAM-targeted immunotherapies. A first-in-human clinical trial of a novel TAM-targeted immunotherapy using pullulan nanogel for intravenous injection is planned in early 2025.

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Sphinganine membrane-anchors TLR4 adapters in macrophages to promote inflammation

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In both, sepsis and cancer, macrophages are key players initiating, regulating and driving helpful or harmful inflammatory processes. Sphingolipids have been associated to positively or negatively regulate inflammatory Toll-like receptor (TLR) 4 signaling and vice versa, with the underlying mechanisms remaining uncertain.
Sepsis induced expression of Sptlc2, the key enzyme in sphingolipid biosynthesis, in human and murine macrophages. Myeloid cell-specific deficiency of *Sptlc2* reduced sphingolipid levels, attenuating the pro-inflammatory macrophage phenotype. Mechanistically, this study identifies Sptlc2-derived sphinganine to interact with TLR4 adaptor proteins MyD88, TIRAP and Tollip, initiating downstream signaling. Overexpression of membrane-anchored MyD88 in *Sptlc2*-deficient BMDMs rescued the M1-like phenotype. In sepsis, *Sptlc2*-deficient mice showed attenuated cytokine levels, less M1-like macrophages and weakened symptoms, but in a different context impaired anti-tumor macrophage activity, increasing tumor burden.
These results indicate that sphinganine is an indispensable regulator of pro-inflammatory signaling, opening new possibilities to pharmacologically interfere with inflammation through sphingolipid modulation in the future.

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A new membrane protein library for the identification of novel protein/protein interactions to different immune cell populations and to discover novel modulators of phagocytosis

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Immune cells communicate and physically connect to their environment and neighboring cells via transmembrane proteins. Due to the exposed nature of extracellular domains, membrane proteins are a prime target for immunotherapy such as therapeutic antibodies or immune cell-based therapies. Despite their therapeutic potential and the crucial roles of transmembrane proteins in health and disease, for many membrane proteins their functions and interactions remain often poorly understood. Here, we seek to identify a) novel transmembrane protein interactions with immune cells and b) previously unidentified extracellular domains of proteins which modulate phagocytosis or antigen cross-presentation (for the latter, please see abstract from Marta Puig-Gamez, Boehringer Ingelheim). To this end, we designed a recombinant protein library based on canonical Uniprot annotations consisting of the extracellular domains of type I, type II, and multipass human transmembrane proteins, as well as GPI-anchored proteins and proteins found in the ER-Golgi-lysosomal systems. The constructs were tagged to enable protein dimerization, capture and detection and then expressed as soluble proteins following transient transfection. a) To identify

novel interactions of our library proteins with different immune cell populations, we set up two high-content flow cytometry-based immune cell binding screens which allow us to discriminate either between different immune cell populations found in blood of healthy human donors. In addition, we examined their binding to differently polarized human monocyte-derived macrophages. Along with many known interactions, we identified some new interactions of transmembrane proteins and immune cells with potential implications for the immune system. b) We also developed a phenotypic image-based screen to discover novel phagocytosis modulators within our library. Here we provide proof-of-concept data for the screening assay. In summary, we present a new protein library that we used for interaction and functional screens providing great potential for the discovery of unidentified membrane protein functions and for the identification of potential novel targets for immunotherapy.

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Shining a light on GPR65 for cancer immunotherapy

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Whilst the advent of immune checkpoint blockade has revolutionised the management of cancer, many patients do not respond to these therapies due to the hostile solid tumor microenvironment (TME), dominated by immunosuppressive tumor associated macrophages (TAMs). Our data suggest that the acidic TME, resulting from key hallmarks of cancer, plays a critical role in driving immune suppression via the activation of the pH sensing GPCR, GPR65.

Genetic deletion of *Gpr65* in mice prevents the pro-tumorigenic polarization of macrophages and reduces the growth of B16.F10 tumors. Here we demonstrate that myeloid cells within healthy human PBMCs are particularly sensitive to changes in pH, exhibiting the most pronounced changes in gene expression in response to acidity, as assessed by single cell RNA sequencing. TCGA data revealed that GPR65 is expressed mostly on myeloid cells from a variety of solid cancers and it is ubiquitously co-expressed with the *CREM* gene that encodes one of GPR65 key downstream signalling partners, the immunosuppressive transcriptional modulator ICER (inducible cAMP early repressor). This suggests that the GPR65 pathway is active across a range of solid malignancies. Critically, patients homozygous for a hypomorphic coding variant in *GPR65* (I231L) show increased overall survival, providing compelling genetic validation for the clinical potential of GPR65 inhibition. Pathios has developed potent and selective small molecule inhibitors of GPR65 that block proximal signalling in recombinant cells and primary macrophages exposed to low pH. These inhibitors dose-dependently reverse the acid-induced immunosuppressive features of human and murine macrophages by dramatically altering gene expression and cytokine release. The desirable PK properties of our inhibitors have enabled *in vivo* studies in murine syngeneic cancer models, which showed potent and dose-dependent single agent tumor growth inhibition. At the cellular level, oral dosing in tumor-bearing mice results in significant modifications of the TME such as the elevation of

CD8+ T cells and the recruitment of NK cells. Additional transcriptomic analysis of tumor-infiltrating leukocytes reveals increased signatures associated with anti-tumor macrophages and cytotoxic lymphocytes.
In fresh primary human tumor histo-cultures from clear cell renal cell carcinoma patients, our compounds markedly decrease the levels of IL10 whilst elevating pro inflammatory chemokines. Gene expression changes consistent with those seen in human macrophage cultures were also observed.
In summary, activation of GPR65 on TAMs is a fundamental tumor-promoting process in both humans and mice and its inhibition with small molecules restores an effective anti-tumor immune response. GPR65 antagonism therefore represents a compelling approach for cancer immunotherapy in a range of solid tumors.

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Immune resistance genes prevent immune rejection of pancreatic ductal adenocarcinoma (PDAC)

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Pancreatic ductal adenocarcinoma (PDAC) is an aggressive and prevalent exocrine tumor. An elucidation for the low survival rate associated with PDAC is the desmoplastic tumor microenvironment critical for its progression, metastasis, and drug resistance. Advances in cancer treatment were achieved with the development of immunotherapies that exploit the immune system's ability to recognize aberrant cells. Specific immune checkpoint molecules, that are upregulated by tumor cells resulting in the evasion of anti-tumor immunity, are therapeutically targeted to restore the immune response. Although nanovaccines show great potential for the improvement of cancer vaccine efficacy, long-term immunity to PDAC has proved elusive. By broadening the potentials of immunotherapy to PDAC, we hypothesize that this elusiveness is due to the presence of hitherto unknown immune resistance genes (IRGs) expressed by PDAC. To systematically identify such genes, we performed high-throughput RNA interference screens of tumor-T cell co-cultures and secondary extensive validations on human primary PDAC cells and a number of other tumor entities. The expression profiles of 220 validated IRGs were explored in bulk and single-cell transcriptome analyses where we evaluated primary PDAC cell transcriptomes from multiple patients, investigating the degree of heterogeneity between tumor subjects and in comparison to control pancreases. By merging multiple single-cell RNA-seq datasets, we obtained four major clusters of malignant ductal cells reflecting distinct biological states and characterized by a differential co-expression of groups of IRGs. Ten candidates confirmed to be significantly upregulated in PDACs and associated to worse survival rates. These were selected for deeper functional analyses, all of which proved to protect against the immune rejection mediated by tumor-specific cytotoxic T lymphocytes. The tested IRGs were shown to mediate tumor intrinsic resistance to T cell-derived cytotoxic molecules such as TRAIL, FasL and TNFa with differential impacts on distinct death-receptor downstream signaling pathways. Downregulation of some IRGs resulted in

elevated death receptor expression on tumor cells sensitizing them towards immune rejection. An ongoing selection of PDAC patient-derived organoid cell lines representative of the immune resistance profiles of malignant ductal clusters is critical for further in vitro validations of relevance. Obtained results do not exclude extrinsic impacts of IRGs on the T cell functionality. Yet, our ultimate aim is to elucidate the regulation of IRG co-expression and their respective molecular pathways before therapeutically inhibiting them in a PDAC vaccination mouse model for an enhanced treatment of pancreatic cancer.

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Detecting somatic HLA loss from genomic and transcriptomic tumor sequencing data

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Cancers evolve under selective pressure from the immune system and treatments. Somatic loss of class I human leukocyte antigen (HLA) is a common mechanism of immune evasion, occurring in 4% to 40% of patients, depending on their tumor entity. Detecting and considering loss or allelic imbalance of individual HLA alleles might improve neoantigen selection for personalized tumor vaccines and other therapies. Germline HLA alleles can be assessed from blood samples or sequencing data with high accuracy. However, detecting a genomic loss or down-regulation specific to tumor cells is challenging due to low tumor purity in clinical samples, tumor heterogeneity, the high variability of classic HLA genes between individuals, and the high homology with other HLA genes and pseudogenes. The currently available methods to predict HLA loss from sequencing data have only been evaluated using few samples and with custom sequencing platforms. Furthermore, they only consider the genomic loss of HLA alleles and do not detect down-regulation at the RNA level.

We developed and implemented a novel computational pipeline, *HLAhound*, to detect somatic loss and allelic imbalance of HLA alleles at the genomic and transcriptomic level from whole-exome sequencing (WES) and RNA-sequencing data. First, we construct personalized HLA reference sequences based on germline HLA alleles, to which we align sequencing reads with stringent mapping criteria. Next, we compute and visualize characteristic features for HLA loss. We applied *HLAhound* to WES data from 14 pairs of matched tumor and normal samples. Based on manual evaluations of presumed HLA losses, we trained a random forest classifier to predict individual HLA allele losses. We applied the model to a previously published independent test data set of three cell lines with experimentally confirmed somatic loss HLA loss. The features were highly predictive, and in this small test dataset, we detected all known events without any false-positive prediction. We are currently evaluating the performance with respect to sequencing depth and simulated tumor purity.

We are establishing a targeted experimental confirmation approach to enlarge the evaluation data set and include more clinical tumor samples and more diverse HLA alleles. We implemented *HLAhound* as a NextFlow pipeline and a custom Python package. The computational efficiency and scalability of *HLAhound* will allow analyses of larger patient cohorts. However, validation studies are

required to enable the integration into clinical sequencing and analysis pipelines. Together, the initial evaluation results of *HLAhound* indicate accurate detection of HLA losses in individual tumors and suggest fine-tailored therapy approaches, such as individualized neoantigen vaccines, which consider and restrict this common form of immune evasion.

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YB-200, a novel IgG1 antibody targeting CEACAM1/5, induces complete response in syngeneic liver Hepa1-6 tumor-bearing mice and modulates the immune response

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CEACAM1 is expressed on a variety of immune cells and acts as a cell–cell communication receptor with both co-stimulatory and co-inhibitory effects. In the pathophysiological stage, CEACAM1 and 5 are dysregulated in various cancers. YB-200, is a novel IgG1 antibody targeting CEACAM 1/5, which has shown to preserve the direct immune agonistic function of CEACAM1 on leukocytes in addition to potentially inhibiting both the homophilic and heterophilic checkpoint blockade of CEACAM1 and 5 on tumor cells. Previous studies (presented at SITC2022) confirmed that YB-200 led to significant anti-tumor activity in syngeneic CEACAM1^{POS} liver Hepa1-6 tumor model. In addition, consistent with the role of CEACAM1 as cell-cell communication molecule, the anti-tumor activity of YB-200 was demonstrated to be correlated with significant increase in B-cells, CD3+ and CD4+ T-cells in the Hepa1-6 tumor microenvironment, while granulocytes decreased (SITC 2022). The present studies were designed to further elucidate the mechanism of action and to test the potential *in vitro* anti-proliferative activity of YB-200 in a panel of human tumor cells, and YB-200 anti-tumor effect (compared to immune checkpoint inhibitor, aPD-1 antibody) either as single agent or in combination with aPD1, in Hepa1-6 tumor-bearing immuno-competent mice (syngeneic tumor model), and potential stimulation of animal immune response. Taken together data confirm that: (i) YB-200 does not have a direct effect on tumor cell proliferation consistent with the postulated MOA as immune agonist; (ii) treatment of Hepa1-6 tumor-bearing mice with YB-200, at well-tolerated doses, induced significant anti-tumor effect compared to PBS, and similar to that observed with aPD1 antibody (or YB/aPD1 combination treatment regimen); and (iii) upon subsequent challenge of the treated animals with injection of Hepa1-6 cells in the opposite flank (challenge), animals treated previously with YB-200 show a stronger anti-tumor immunity compared to anti-PD-1. In conclusion, the data confirm the anti-tumor effect of YB-200 is mediated via modulation of the immune response and may provide a complementary modality to current immune checkpoint inhibitor treatment modalities.

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Early de-risking of IMA402 TCER® by a unique preclinical safety program

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Immatics' bispecific T cell-engaging receptors (TCER®) are TCR-based biologics that redirect T cells towards tumor tissues expressing the target peptide-HLA complex. IMA402, a PRAME-specific TCER®, is Immatics' second TCER® program to enter the clinical phase. Here we describe the comprehensive preclinical safety program for IMA402 that accompanied its development. Using the XPRESIDENT® target discovery and validation platform which combines quantitative mass spectrometry, transcriptomics and bioinformatics, presence of the target peptide-HLA in several cancer indications and its absence in relevant human normal tissues was confirmed. The XPRESIDENT® technology was also utilized to identify potentially relevant off-targets on normal tissues. From more than 150 identified potentially relevant off-targets, a weak binding to IMA402 could be confirmed in only three cases. For those three cases, the risk for unwanted cross-reactivity was assessed to be minimal based on the low binding affinity and the comparative functional assessment of on-target and off-target reactivities at physiological pHLA copies per cell. Finally, an unbiased safety screen against 20 primary cultured normal cell types revealed no relevant reactivities against any of the tested cell types covering a broad range of toxicologically relevant tissues. To assess the risk of alloreactive responses, IMA402 was screened against a panel of 60 B-lymphoblastic cell lines representing the vast majority of HLA class I alleles of the prospective patient population. The study did not reveal any signs of alloreactivity for IMA402. Thus, exclusion of patients with certain HLA alleles is not required. To assess the risk to induce cytokines in absence of target, IMA402 was tested in a whole blood cytokine release assay which showed no release of IL-6, IL-10, TNFa or IFNg suggesting a low risk of causing target-independent CRS in patients. Finally, the potential of IMA402 to give rise to anti-drug antibodies is expected to be low based on *in silico* (Epibase® scoring) and experimental testing. In summary, IMA402 has passed a very thorough safety assessment and the suggest low safety risks for treated patients.

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Association of PD-L1 expression and autophagy in patients with ovarian cancer

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Background: Ovarian cancer (OC) is one of the most common malignant tumors in women with the highest mortality rate of all gynecological tumors. OC cells are able to upregulate PD-1/PD-L1 checkpoint and autophagy which could be in close and significant interaction. The aim of the study was to analyze the expression of PD-L1 immunosuppressive and autophagy markers p62, LC3 and Beclin1 in OC and evaluate their prognostic potential. **Methods:** The study has included 122 subjects with OC. The expression of PD-L1, p62, LC3 and Beclin1 was analyzed in central and invasive tumor parts by immunohistochemistry combined with tissue microarray. Expression levels of analyzed markers were correlated with histopathology parameters. **Results:** High grade serous carcinoma (HGSC) showed significant level of PD-L1, p62 and LC3 expressions, compared to all other histology types. Advanced FIGO (International Federation of Gynaecology and Obstetrics) stages were associated with incresed levels of PD-L1, p62 and LC3 expressions. Beclin1 expression was not show significant correlation with analyzed parameters. Between central and invasive cancer segments there was not significant difference in expressions of all analized markers, considering histological type and FIGO stage. **Conclusion**: HGSC type shows significant PD-L1, p62 and LC3 expression in higher FIGO stages. The combined presence of PD-L1, p62 and LC3 expressions indicates simultaneous activation of immunosuppressive and autophagy mechanism in ovarian cancer cells. It could suggest synergistic therapy with PD-L1 and autophagy inhibitors in OC treatment.

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First-in-class inhibitors of ERAP1, generating cancer antigens as novel targets for MHCI-targeted therapies

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Immune checkpoint inhibitors (ICI) have changed the cancer treatment paradigm, yet significant unmet need remains. Clinical data shows that increasing antigen presentation diversity, genomic instability, tumor mutational burden and HLA diversity, as well as preventing permanent exhaustion of pre-existing cytotoxic T cells, caused by chronic cancer antigen stimulation, are all factors that improve clinical response to ICI. Additionally, increasing presentation of cancer-specific target peptides on cancer cells will enhance T cell receptor (TCR)-mediated therapeutics such as ImmTACs or TCR-Engineered T cells. Endoplasmic reticulum aminopeptidase 1 (ERAP1) trims peptides loaded into classical and non-classical MHC Class I. In cancer, knockout or inhibition of ERAP1 changes a proportion of the antigen repertoire, generating and upregulating cancer antigens. This leads to the activation of *de novo* anti-tumor T cell responses, thereby overcoming key resistance mechanisms to current immune-oncology therapy such as poor tumor recognition by T cells and T cell exhaustion. We report the preclinical development, characterization and mechanistic analysis of the first-in-class ERAP1 inhibitor, GRWD5769. Extensive analysis of the immunopeptidomes of diverse cancer cell lines robustly showed that GRWD5769 modulates the cancer-related antigen repertoire across genotypes and cancer-type backgrounds. Novel or upregulated neoantigens generated by ERAP1 inhibition are

conserved across cancer cell types and genetic backgrounds. ERAP1 inhibition in combination with an ICI (anti-PD1 mAb) diversified the TCR repertoire, upregulated prognostic immune gene markers in the tumor, including markers for recently activated (thus non-exhausted) T cells, and drove infiltration of T cells into syngeneic mouse model tumors. This combination is efficacious across syngeneic models, including different mouse strains and the effects of ERAP1 inhibition on the T cell response correlate with efficacy. Further, *ex vivo* human T cell co-cultures with cancer cell lines demonstrate that novel and upregulated neoantigens generated by ERAP1 inhibition drive tumor cell killing. In addition, these studies showed enhanced tumor cell killing when an ImmTAC was used in combination with ERAP1 inhibition to enhance presentation of the cancer-specific target peptide. In conclusion, the first-in-class, ERAP1 inhibitor clinical candidate, GRWD5769, drives novel anti-tumor T cell responses through neoantigen creation and circumventing T cell exhaustion. GRWD5769 has demonstrated a good safety profile in GLP toxicology studies and robust proof of mechanism and proof of principle biomarkers have been developed to provide a clear path to establish the activity and efficacy of GRWD5769 in patients in 2023.

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GEN1046 (DuoBody®-PD-L1x4-1BB) reverses T-cell exhaustion in vitro

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GEN1046 (DuoBody®-PD-L1x4-1BB), an investigational, potential first-in-class immunomodulatory bispecific antibody, is designed to elicit an anti-tumor immune response via simultaneous and complementary PD-L1 blockade on tumor or immune cells and conditional 4-1BB stimulation on T cells and NK cells. Previously presented preclinical data suggest that GEN1046 reinforces DC/T-cell interactions, induces memory T-cell and NK-cell expansion, and enhances antigen-specific T-cell activation and effector functions in vitro. Given T-cell dysfunction may represent a potential resistance mechanism to checkpoint inhibitors (CPI), we used a multi-omics approach to determine whether GEN1046 could reverse T-cell exhaustion in vitro. Publicly available single-cell RNA sequencing (scRNAseq) datasets across multiple solid-tumor indications (including treatment-naive and anti-PD-1 and/or anti-CTLA-4 pretreated samples) were harmonized and analyzed for co-expression of PD-1 and 4-1BB on various immune-cell subsets based on their transcriptome signatures. We developed, optimized, and validated a mixed lymphocyte reaction (MLR) assay in which healthy donor T cells were exhausted by repeated stimulation with anti-CD3/CD28 beads before co-culturing with allogeneic LPS-matured dendritic cells. In the T-cell exhaustion MLR assay, T cells showed increased expression of inhibitory receptors (eg, TIM-3, LAG-3) and hyporesponsive proliferation and cytokine secretion upon restimulation with anti-CD3/CD28 beads, which was partially reversed by PD-1 blockade. GEN1046 restored IFNγ secretion, reinvigorating the exhausted T-cell response in vitro, with an effect roughly two-fold higher than that of PD-1 blockade. When combined with an anti-PD-1 antibody, cytokine secretion was further potentiated compared to each agent alone, suggesting potential synergy of the GEN1046 and anti-PD-1 antibody combination; additional molecular profiling from the T-cell exhaustion MLR assay will be presented. Using solid

tumor public scRNAseq datasets, we demonstrated co-expression of 4-1BB and PD-1 on exhausted CD8+ T cells in the tumor microenvironment. In addition, in patients who received PD-1-blocking agents, an increase in exhausted CD8+ T cells expressing PD-1 was observed. In summary, GEN1046 restored IFNγ secretion by exhausted T cells in vitro more potently than PD-1 blockade, and these effects could be further potentiated by combining GEN1046 with an anti-PD-1 antibody. These results support evaluation of GEN1046 in the post-CPI setting and the combination of tumor-targeted 4-1BB co-stimulation with PD-1 checkpoint blockade for the treatment of solid tumors. GEN1046 is being evaluated in an ongoing phase 2 clinical trial in patients with NSCLC whose disease has progressed on prior CPI therapy (NCT05117242).

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B-Cūr: A universal discovery platform enabling the selection of stable and rare antibodies from patients to discover novel therapeutic targets.

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Here, we describe a universal, proprietary platform that enables the discovery of novel therapeutic targets via a target agnostic approach. Monoclonal antibodies (mAbs) are crucial for therapeutics and diagnostics in numerous diseases. However, most currently used mAbs are of mouse origin and engage specific pre-selected targets. The use of human B cells to discover and generate mAbs is on the rise due to the emergence of new techniques to tap into the human antigen experienced B cell repertoire.
Primary B cells have a limited proliferative lifespan, however introduction of BCL6 and Bcl-xL prevents terminal differentiation and apoptosis. B-Cūr B cells are amenable to high-throughput processing through indefinite expansion capabilities, expression of the cognate B cell receptor on the cell surface and concomitant secretion of mAbs. In addition, B-Cūr B cells are more stable than EBV-transformed cells and the produced mAbs show excellent safety profiles and physicochemical properties. The B-Cūr platform shows >85% immortalization efficiency in isolated antigen-experienced B cells. Furthermore, the induction of activation-induced cytidine deaminase (AID), resulting in ongoing mutation in the B cell receptor/Ab sequence, allows for in vitro selection of affinity and stability variants with labeled antigens. This process yields information on critical and non-critical residues for binding and can generate surrogate mAbs for in-vivo studies. B-Cūr has been successfully applied to B cells from various animal species, including rabbits, mice, rats, pigs, non-human primates, and llamas. Transduced B cells from these species show the same phenotype as transduced human B cells. The B-Cūr platform forms the basis of the ZooMab® platform (MilliporeSigma exclusive license).
Recently, the EMA granted market approval to the D25 mAb (Beyfortus®, Sanofi/AstraZeneca), discovered using B-Cūr. This mAb, Nirsevimab, recognizes a new and unique epitope on the prefusion state of RSV-F protein, and is superior to the standard of care (palivizumab).
Kling Biotherapeutics has discovered several unique tumor targets and their corresponding human antibodies that are under preclinical development.,KBA1412, a CD9 targeting human antibody is currently being evaluated in a Phase 1b dose escalation and dose expansion study in patients with

advanced solid tumors (ClinicalTrials.gov: NCT05501821).
Kling Biotherapeutic's target-agnostic approach for mAb discovery enables the identification of novel disease targets from cured responders who have shown a highly successful immune response leading to virus clearance or tumor eradication. Our approach provides a significant competitive advantage compared to traditional target and antibody identification approaches.

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Novel Imidazoquinolines with improved pharmacokinetic properties

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Limited response to immune checkpoint therapy has been attributed to insufficient infiltration of immune cells in the tumor as well as a suppressive phenotype of innate immune cells in the tumor microenvironment (TME). One possible strategy to render such "cold" tumors responsive to immune therapy is directing the myeloid component of the immune system toward an antitumor response by utilizing ligands of Pattern Recognition Receptors and particularly Toll-like receptors (TLRs) as immune stimulants. However, the application of TLR-agonists has so far been limited to treatment of skin tumors due to poor pharmacokinetic properties of available TLR-agonists and, correspondingly, intolerable side effects related to organism-wide cytokine induction following systemic administration. We prepared a series of TLR7/8 agonists conjugated to a dibasic macrolide carrier to improve their pharmacokinetic properties and direct the compounds to and trap them in acidic tissues and cellular compartments. The first-generation-conjugate was hydrolysable and coupled Resiquimod to Azithromycin via the primary amine involved in receptor interaction. The conjugate retained activity in in vitro in blood stimulation assays and in a murine model of colon carcinoma. In vivo we observed reduced weight loss in groups treated with the conjugate versus free Resiquimod at equimolar doses. To determine whether this was due to altered distribution of the new compound or it acting as a pro-drug releasing Resiquimod over time, we prepared a second series of TLR7/8-agonists that coupled the agonist Imidazoquinoline "war head" to the macrolide core at a site not involved in receptor binding and in a non-hydrolysable way. We could identify linker-agonist-combinations that were still activating either hTLR7 or hTLR7/8 in a HEK reporter system. Cytokine release in a human blood stimulation assay reflected the differences in receptor affinity; compounds activating only hTLR7 primarily lead to secretion of Interferon α and hTLR7/8-active compounds like Resiquimod induced both IFNα and TNFα. Although Resiquimod is thought to exert its effects in mice solely by TLR7-activation the same differences in cytokine release pattern could be reproduced in mice. In addition to their narrower receptor specificity, the stable conjugates show altered tissue distribution and increased half-life when compared to Resiquimod and Imiquimod. When administered daily for 3 days in pharmacokinetic studies, lymphopenia was reduced compared to unconjugated Imidazoquinolines. Combined, these results indicate that pharmacokinetics impact the tolerability of TLR7/8 agonists and optimization thereof could increase their usefulness in a clinical context.

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Identification of immune cell populations associated with anti-PD-1 resistance in murine melanoma using CITE-seq (cellular indexing of transcriptomes and epitopes).

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Immune checkpoint inhibitors (ICIs) have revolutionised the treatment of metastatic cancer, but up to 40% of melanoma patients remain intrinsically resistant to this effective therapy. Therefore, understanding the effects of immunotherapy on the immune system remains a critical goal to identify mechanisms of resistance and new therapeutic targets.
Investigations to identify mechanisms of therapeutic resistance focus on the T-cell state during and after therapy and modulation of the immunosuppressive tumour microenvironment. It has been shown that anti-PD-1 resistance is associated with dysfunctional T-cells due to exhaustion, insufficient tumour immunogenicity and resistance to IFN-g signalling. Furthermore, the presence of regulatory T-cells (Tregs), myeloid-derived suppressor cells (MDSCs) and tumour-associated macrophages (TAMs) has been attributed to an immunosuppressive TME contributing to therapeutic resistance. However, as resistance to immunotherapy has not yet been resolved, it remains essential to identify and characterise the immune cell populations that either efficiently mediate the immunotherapeutic effect or identify immune cells responsible for resistance to therapy.
In this project, a murine melanoma model was used to perform immunophenotyping of intratumoral CD45+ immune cells after treatment with anti-PD-1 using the CITE (cellular indexing of transcriptomes and epitopes)-seq method. Briefly, mouse subcutaneous melanomas were treated with anti-PD-1 immunotherapy and harvested for subsequent dissociation into single-cell suspensions. The surface epitopes of immune cells were stained by using a library of >80 different oligo-tagged CITE-seq antibodies and an enrichment for viable CD45+ cells was performed by FACS sorting to remove dead cells and debris. Approximately 25,000 CD45+ cells were loaded into the 10X Genomics machine using the Chromium Next GEM Single Cell 3’ kit and the sequencing was performed on an Illumina NovaSeq6000 with 40,000 reads per cell. Afterwards, the data was pre-processed by de-multiplexing, genome alignment and quality control.
By combining the expression of canonical transcripts and the CITE-seq signal of known surface markers, we were able to identify 21 distinct intratumoral immune cell populations. The results show drastic effects in the mononuclear phagocyte compartment, where changes are most evident in the dendritic cell and macrophage populations. The T-cell compartment is also differentially affected by the anti-PD-1 treatment, with regulatory CD4+ T-cells and exhausted CD8+ T-cells showing the most pronounced changes.
In conclusion, this study demonstrates that the CITE-seq method successfully combines the epitope and transcriptome data of a single cell sequencing approach to improve the immunophenotyping of intratumoural immune cells. Furthermore, it contributes to the identification of new potential mechanisms to overcome immunotherapeutic resistance.

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splice2neo combines the effect of somatic mutations on splicing with RNA-seq support to predict tumor-specific splice junctions as neoantigen candidates

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Splicing is dysregulated in many tumors, and thereof resulting tumor-specific transcript isoforms can encode neoantigens. Detecting tumor-specific splicing is challenging because splice junctions identified in tumor transcriptomes may also appear in normal tissues. However, somatic mutations that are truly tumor-specific can lead to the loss or gain of canonical splicing sequence motifs. Here, we consider splice junctions that can be linked to somatic mutations as tumor-specific candidates and thereby serve as a source for neoantigens.
We developed the R-package splice2neo that integrates predicted splice effects from somatic mutations with splice junctions detected in tumor RNA-seq. Furthermore, splice2neo allows the exclusion of known canonical and healthy tissue splice junctions, annotation of resulting transcript and peptide sequences, and supports the targeted re-quantification of supporting RNA-seq reads.
We established a stringent detection rule on mutation effect scores and read support to predict tumor-specific splice junctions as targets in a discovery cohort of 85 melanoma samples. Applying the detection rule to a verification cohort of 27 melanoma samples, we identified 1.7 splice junction targets per tumor and estimated a false discovery rate of 0.04. For individual examples of exon-skipping events, we confirmed the expression in tumor-derived RNA by quantitative real-time PCR experiments. A subset of splice junctions encode neoepitope candidates with strong MHC I or MHC II binding. Compared to predicted neoepitopes derived from non-synonymous point mutations, the splicing-derived neoepitope candidates had a lower self-similarity to corresponding wild-type peptides.
Splice2neo facilitates the identification and analysis of tumor-specific splice junctions that are associated with somatic mutations and can lead to neoantigen candidates to expand the target repertoire for cancer immunotherapies. Splice2neo is available at https://github.com/TRON-Bioinformatics/splice2neo.

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Abstract has been withdrawn

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Abstract has been withdrawn

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Combination therapy with GEN1042 (DuoBody®-CD40x4-1BB): safety and preliminary efficacy in advanced solid tumors

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GEN1042 (DuoBody-CD40x4-1BB) is a novel, investigational, agonistic, bispecific antibody that combines targeting and conditional activation of CD40 and 4-1BB on immune cells. In the dose-escalation part of the ongoing phase 1/2 trial (NCT04083599), GEN1042 demonstrated biologic and early signs of clinical activity with a manageable safety profile in patients with advanced solid tumors. Preclinical data presented previously suggest that co-administration of a PD-1 inhibitor can amplify the magnitude of immune response that is generated by GEN1042 treatment. It is hypothesized that additional combination with chemotherapy may further enhance antitumor responses through increased PD-L1 expression and antigen release. We present results from the combination safety run-in (SRI) and expansion cohorts evaluating the safety and preliminary efficacy of GEN1042 + pembrolizumab (PEM) ± chemotherapy. CPI-naive patients with advanced, metastatic/unresectable/recurrent non-CNS tumors were treated with GEN1042 + PEM ± chemotherapy (nab-paclitaxel + gemcitabine or cis/carboplatin + 5-FU) until unacceptable toxicity or disease progression. The primary endpoints for the SRI and expansion cohorts were dose-limiting toxicity (DLT) and objective response rate (ORR), respectively. Secondary endpoints included antitumor activity (based on RECIST v1.1 criteria), adverse events (AEs), pharmacokinetics, and pharmacodynamic biomarkers. As of the data cutoff date (July 27, 2022), 20 patients (median age, 70.5 y), including 3 with melanoma, 5 with HNSCC, and 12 with non-small cell lung cancer, received GEN1042 + PEM, and 17 patients (median age, 64.0 y), including 5 with head and neck squamous cell carcinoma (HNSCC) and 12 with pancreatic ductal adenocarcinoma, received GEN1042 + PEM + chemotherapy. Treatment-related AEs occurring in at least 15% of patients (all grades; grade ≥3) were pruritus (20%; 0%), rash (15%; 5%), and pyrexia (15%; 0%) in patients receiving GEN1042 + PEM and transaminase elevation (23.5%; 5.9%), fatigue (23.5%; 0%), rash (17.6%; 5.9%), nausea (17.6%; 0%), and diarrhea (17.6%; 0%) in patients receiving GEN1042 + PEM + chemotherapy. No DLTs were observed in either treatment group. Immune-related AEs were manageable and as expected. Data demonstrate encouraging preliminary clinical activity in patients with HNSCC receiving GEN1042 + PEM + chemotherapy; updated results will be presented. In summary, GEN1042 + PEM ± chemotherapy demonstrated initial antitumor activity with a well tolerated safety profile in patients with previously untreated advanced/metastatic disease.

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Humanized PD-1 knock-in mice as a preclinical model for testing novel immunotherapies in combination with anti-human PD-1 therapeutics

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Checkpoint inhibitor treatment has become a common therapy for various cancer types. PD-1 is an inhibitory receptor expressed on activated T cells and dampens T cell activity when binding to its ligand PD-L1, which is expressed on tumor and myeloid cells. Therapeutic molecules blocking the PD-L1/PD-1 interaction have been proven to increase anti-tumoral immune response and prolong survival.
Several anti-PD-L1 and anti-PD-1 therapeutics are already approved and in clinical use. Fully immunocompetent syngeneic tumor mouse models are widely used in the development of novel immunotherapies. Limited cross-species reactivity of human specific antibodies created the need of the development of humanized mouse models. In these humanized mouse models novel antibody formats as well as combination therapies of new drugs can be tested with already clinically approved human checkpoint inhibitors.
The anti-tumor efficacies of pembrolizumab (Keytruda), a human specific anti-hPD-1 antibody, and the mouse specific anti-mPD-1 counterpart (clone RPMI1-14) were tested in C57BL/6 wildtype and humanized hPD-1 C57BL/6 mice engrafted with syngeneic MC38-CEA colorectal carcinoma cells. The MC38-CEA tumor cells were implanted into the mammary fat pad (subQperior™), which prevents tumor ulceration and leads to a more homogenous tumor growth compared to subcutaneous implantation. Survival of tumor-bearing human PD-1 C57BL/6 mice was significantly prolonged when treated with pembrolizumab, whereas no effect in C57BL/6 wildtype mice was observed, supporting the reported specificity of Keytruda towards human PD-1. In line with this finding, the application of the anti-mPD-1 antibody led to a prolonged survival of C57BL/6 mice with no survival benefit of hPD-1 C57BL/6 mice.
The validation of the humanized subQperior™ platform was further complemented by characterizing intra-tumoral immune populations present in tumor tissues at 7 days post-treatment by flow cytometry. Two staining panels were performed: a well-established and validated 17 marker staining allowing to quantify the frequency of all major immune cell populations and a staining panel addressing the activity of T and NK cells after *ex vivo* stimulation with PMA/Ionomycin. An increased activity of CD8+ T cells and NK cells was observed as major players in the response to treatment with these anti-PD-1 antibodies.
In summary, the humanized subQperior™ platform using hPD-1 C57BL/6 mice is a suitable tool to evaluate novel cancer therapies in combination with human specific anti-PD-1 therapeutics and tumor ulceration was completely prevented by subQperior implantation of the tumor cells.

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Recombinant CD64-directed immunotoxin exhibits cytotoxicity and may become part of a tool set for site-specific diagnosis and treatment of acute myeloid leukemia

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Cancer immunotherapy is a promising innovative and effective treatment for many forms of cancer. Among hematological malignancies, acute myeloid leukemia remains an unmet medical need as it is mainly treated with chemotherapy, which is associated with serious side effects. Therefore, antibody-based targeted therapy is preferred as it can target and specifically eliminate malignant cells. An immunotoxin is a chimeric molecule consisting of a targeting molecule and a toxic component that specifically kills the target cells. We have developed an immunotoxin called H22(scFv)-ETA', which consists of a humanised single-chain fragment antibody (scFv) targeting CD64 overexpressed on the surface of AML cells and a truncated version of Pseudomonas exotoxin A (ETA') that kills CD64-positive AML cells. CD64 is highly expressed on monocytic blast cells in AML patients and not on hematopoietic stem cells, making it a suitable target. H22(scFv)-ETA' was recombinantly expressed in E.coli BL21 (DE3), channeled into the periplasmic space, and purified by metal ion and affinity chromatography and size exclusion chromatography. The cytotoxic efficacy of H22(scFv)-ETA' was assessed by Annexin V bioassay and binding assays were assessed using flow cytometry. H22(scFv)-ETA' proved to be cytotoxic to AML cancer cells expressing CD64. H22(scFv)-ETA' showed toxicity against CD64-positive cell lines HL-60 and U937. The development of successful scale-up production of H22(scFv)-ETA' is critical for large-scale production to enable further preclinical/clinical studies. The current phase of this study is focused on optimising the productivity of H22(scFv)-ETA' and its large-scale production. In the future, we seek to combine this recombinant immunotoxin with a CD64-target companion diagnostic.

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PD-L1 interactome at the immunological synapse between cytotoxic lymphocytes and tumor cells

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Immune checkpoint therapy has revolutionized immunotherapy, but so far, many patients selected for the treatment fail to respond durably. The mechanism of the immune checkpoint PD-L1 is complex and the mode of action of anti-PD-L1/PD-1 therapy is not fully understood.
Our group identified a fraction of breast cancer cells that resist to cytotoxic lymphocyte-mediated killing by polarizing their actin cytoskeleton towards the immunological synapse, a process that we

have termed the "actin response". Remarkably, the actin response is responsible for the rapid relocation of important inhibitory ligands to the immunological synapse, including PD-L1. Accumulation of PD-L1 at the post-synaptic side of the immunological synapse could increase the strength of immune blockade delivered to cytotoxic lymphocytes and thus promote tumor immune evasion and contribute to immunotherapy failure. The molecular links between PD-L1 and the actin response were investigated using a proximity-dependent biotinylation assay coupled with mass spectrometry. Eight potential PD-L1 interaction partners were identified and currently being validated. The effects of depleting synaptic PD-L1 interaction partners on PD-L1 subcellular distribution in tumor cells with an actin response, as well as on tumor cell susceptibility to cytotoxic lymphocytes will be investigated. A better understanding of the role of PD-L1 polarization during the interaction of cytotoxic lymphocytes and target tumor cells and the characterization of the underlying molecular mechanisms could have important translational implications and help to design more effective immunotherapy therapeutic approaches.

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BAY 2965501: A highly selective DGK- ζ inhibitor for cancer immuno-therapy with first-in-class potential

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The second messenger diacylglycerol (DAG) plays a key role in T cell receptor downstream signaling and thereby in T-cell activation. Diacylglycerol kinase zeta (DGK-ζ) is a lipid kinase that can down-modulate T-cell activation by catalyzing the conversion of DAG to phosphatidic acid, thereby acting as a ligand independent, intracellular immune checkpoint. Inhibition of DGK- ζ offers the potential to enhance T cell priming against suboptimal tumor antigens and to overcome multiple immune-suppressive mechanisms in the tumor microenvironment.

Bayer AG in collaboration with the German Cancer Research Center (DKFZ) have developed BAY 2965501, a highly selective, orally available DGK-ζ inhibitor which shows a unique binding mode and good DMPK profile. The compound enhances the reactivity of human and mouse T-cells, both in the priming and the effector phase, and overcomes the inhibitory impact of suppressive factors, such as adenosine and prostaglandin E2. Oral dosing of BAY 2965501 shows T cell dependent efficacy in syngeneic, murine tumor models. Furthermore, this results in reactivation of exhausted T cells *in vivo.* Preclinical safety studies showed only mild findings, so that good clinical tolerability is expected. Collectively, the preclinical data support first in human (FiH) phase I testing of therapeutic potential.

A FiH trial with BAY 2965501 in patients with advanced solid tumors including NSCLC, gastric/GEJ AdCa, ccRCC, and melanoma patients, is currently enrolling patients *(*NCT05614102*).* This study will evaluate the safety, tolerability, maximum tolerated or administered dose, pharmacokinetics, pharmacodynamics, and tumor response profile of BAY 2965501.

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Unleashing natural IL18 activity using an anti-IL18BP blocker antibody induces potent immune stimulation and anti-tumor activity

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Conventional cytokines have limited anti-cancer efficacy mostly due to narrow therapeutic window and systemic adverse effects. IL18 is an inflammasome induced proinflammatory cytokine that enhances T and NK cell activity and stimulates IFNg production. The activity of IL18 is naturally blocked by a high affinity (~440fM) endogenous binding protein (IL18BP). IL18BP is induced in the tumor microenvironment (TME) in response to IFNg upregulation. We measured the protein expression of IL18 in human tumors. IL18 was highly expressed across 72 individual tumors (median 12.2ng/gr) and was clearly elevated compared to serum (median 0.32ng/ml). By assessing total and free IL-18 we showed that most of TME IL18 is bound by IL18BP (median 10.1ng/gr), and its levels are above the amount required for T cell activation in-vitro (1ng/ml), implying that releasing IL-18 in the TME could lead to T cell immune activation.

To unleash endogenous bound IL18 activity, COM503, a fully human, high affinity anti-IL18BP antibody (Ab), was generated. COM503 blocks the IL18BP-IL18 interaction and displaces pre-complexed IL-18 to enhance T cell activation (increased IFNg 197%, $p<0.001$; CD137 86%, $p<0.05$) in ex-vivo stimulated human CD8+ tumor infiltrating lymphocytes-tumor cells co-culture assay. Moreover, COM503 induced human NK cell function as evident by increased IFNg secretion (26-fold, $p<0.001$).

To assess the effect of IL18BP blockade in-vivo, a surrogate anti-IL18BP Ab was generated and administered systemically to mice. In an orthotopic E0771 breast cancer model, anti-IL18BP Ab monotherapy resulted in a significant tumor growth inhibition (91%, $p<0.0001$), and increased survival ($p<0.0001$). Anti-IL18BP Ab induced pronounced multi-parametric TME modulation including an increase in CD3+ T cells infiltration (108.5%, $p=0.015$), and specifically in infiltrating effector IFNg+GrB+ CD8+ T cells (258.5%, $p=0.02$) and IFNg+ TNFa+ NK cells (76.9%, $p=0.001$). Similarly, anti-tumor effects were shown in MC38ova model (58% TGI, $p<0.001$), with a robust TME-localized immune modulation including increased CD8+ infiltration (85%, $p=0.009$) and IFNg secretion (76%, $p=0.052$). In contrast, no increase in inflammatory cytokines and lymphocyte numbers or activation state was observed in serum and spleen.

In summary, we demonstrated that IL18 is upregulated in the TME and is mostly bound by IL18BP. COM503, a potential first-in-class, high-affinity anti-IL18BP Ab, induces human T and NK cell responses in-vitro. A mouse surrogate anti-IL18BP Ab induces potent anti-tumor responses and pronounced TME-constrained immune modulation, this in contrast to systemically administered therapeutic cytokines, which generate a non-localized inflammatory response. Taken together, blocking IL18BP is a promising novel approach to harness cytokine biology for the treatment of cancer.

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The immunopeptidome of paediatric high-grade osteosarcoma

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Osteosarcoma is the most frequent paediatric bone cancer, affecting more than 1100 children and adolescents per year in the EU. In contrast to advances in other paediatric cancers, osteosarcoma treatment has not seen progress in nearly 40 years and high-dose chemotherapy followed by radical surgery has remained the standard of care. Genetic heterogeneity of osteosarcoma paired with a lack of actionable driver mutations has hampered the development of targeted therapies. Clearly, there is an urgent and unmet need for novel, personalized therapeutic approaches. Among paediatric tumours, osteosarcoma stands out as a candidate for personalized immunotherapies, with a high mutational burden, T cell infiltration and signs of intra-tumoral clonal T cell expansion. Our pilot study aimed to uncover osteosarcoma-specific, clinically relevant, T cell epitopes presented on the surface of tumours in the context of human leukocyte antigen (HLA) molecules. We applied mass spectrometry (MS)-based HLA ligandomics, the only analytical method to directly identify the HLA peptidome presented on the surface of cells or tissue in an unbiased way. Our discovery cohort consisted of clinical samples from 11 primary, untreated and 5 post-chemotherapy high-grade osteosarcoma cases. Tumour samples were snap frozen, homogenized and peptide-HLA complexes were isolated by affinity chromatography. HLA peptides were isolated by C18 solid phase extraction and analysed by nanoUHPLC-coupled tandem MS. MS data was analysed by a hybrid approach combining de novo peptide sequencing and database searching (Peptide-PRISM). Peptide-PRISM enables comprehensive identification of conventional HLA peptides, derived from coding sequences (CDS), as well as cryptic HLA peptides from non-canonical translation events. A total of 34,600 unique peptides presented across 37 HLA class I alleles were identified, including 1,000 cryptic peptides. HLA ligands shared with healthy tissue were subtracted using an extended benign-tissue HLA ligandome database, yielding an osteosarcoma-enriched HLA ligandome of 1100 peptides. An additional layer of filtering, using transcriptomics data of osteosarcoma-enriched transcripts, defined a stringent osteosarcoma-specific core of 110 candidate epitopes, with shared epitopes across patients. Using HLA-matched, healthy-donor PBMCs in an in vitro culture system, we could prime T cell reactivities against CDS as well as cryptic peptides. Our data represents the first mapping of the bona-fide processed and presented HLA ligandome in a cohort of osteosarcoma patients and unveiled immunogenic, shared T cell epitopes with potential therapeutic application.

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Investigating membrane protein function in the anti-tumor immune response: a cross-presentation screen

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Myeloid-based cross-presentation of tumour antigens and cross-priming of cytotoxic lymphocytes (CTLs) are key processes that promote antigen-specific tumour cell killing. In recent years, macrophages have been shown to be fit to cross-present and activate CTLs, albeit to a lower degree than dendritic cells. However, tumour-associated macrophages (TAMs) appear not to cross-present effectively in the tumour microenvironment (TME). As a matter of fact, their presence in tumors is generally regarded as a marker of poor prognosis. Understanding cross-presentation by TAMs is crucial to enhance anti-tumour immunity by taking advantage of their privileged access to the TME and thus, to tumour-associated antigens (TAAs). In this study, we aim to identify novel macrophage cross-presentation modulators. To this end, we used our newly designed protein library, the “Membranome”. This library contains a tagged, secretory modified form of all 1820 annotated membrane proteins in UniProt. The Membranome library has been confirmed to be well expressed in HEK293T cells. We have recently successfully used it to uncover new binding partners of several immune cell types (for the latter, refer to the abstract of Sebastian Hoffmann). In the present study, we used the Membranome to perform an arrayed cross-presentation screen in primary blood-derived human macrophages. Briefly, Membranome-treated macrophages were fed a peptide containing 3 repeats of a 9 aa peptide present in NUF2 (pNUF2), a protein upregulated in numerous cancers. Subsequently, an NFAT-luciferase reporter cell line and a bispecific T cell engager molecule binding CD3 and the HLA-A2/pNUF2 complex were added. Efficiency of cross-presentation and T cell activation was correlated to luciferase activity. We identified numerous proteins including TNFA, TPSN and FURIN that led to up-regulation of luciferase activity, while CD3E had the opposite effect. These results were subsequently confirmed. We hypothesize that regulation of activity and availability of some of the top hits in our screen could be used to exploit the capacity of macrophages to cross-present, and hope to provide further insight for cross-presentation-based therapeutic strategies in the future.

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SARS-COV-2 antigens as a natural anti-cancer preventive immunization

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The coronavirus disease 2019 (COVID-19) pandemic has resulted in a global public health crisis and vaccines have played a crucial role in the protective strategy for COVID-19. It is know that SARS-CoV-2 vaccines as well as SARS CoV-2 infection induce T cells recognizing peptides derived from the whole range of viral proteins. We and others have previously shown the molecular mimicry between viral and Tumor Associated Antigens (TAAs) which might have a significant impact in controlling tumor growth and improving the clinical outcome in cancer. In particular, we have shown that TAAs may exhibit sequence homology as well as structural similarities with viral peptides and cross-reacting CD8 + T cells have been identified.
SARS-CoV-2 proteins of all variants were downloaded from UNIPROT and used to interrogate NetMHCpan V.4.1 tool. Viral epitopes with high affinity (<100nM) to the HLA-A*02:01 allele were predicted. Shared and variant-specific epitopes were identified. Their homology to TAAs from Cancer Antigenic Peptide Database was searched by Blast analysis. Several homologies in amino acidic sequence have been found between paired TAAs and SARS-CoV2 peptides, especially in the core positions of the peptides (P_4 and P_5). Predicted structural similarities confirmed the sequence homology and comparable patterns of contact with HLA and TCR α and β chains were observed. MHC class l-dextramer staining was performed and specific cross- reactive CD8+ T cell clones have been found for all the paired peptides in PBMCs of both infected and BNT162b2-vaccinated HLAA02:01 positive individuals.
Our results show that several SARS-COV-2 antigens are highly homologous to TAAs and cross-recognized by T cells in infected and vaccinated individuals. The implication would be that the World population may have undergone a natural anti-cancer preventive immunization able to elicit antiviral/anticancer memory CD8+ T cells. In the coming years, real-world evidences will provide the final proof for such immunological experimental evidence.

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Targetable T-cell epitopes in H3.3K27M altered diffuse midline gliomas

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Introduction Diffuse midline gliomas (DMGs) are malignant brain tumors of childhood with unfavorable prognosis. Due to their localization, they are difficult to resect and respond poorly to radiochemotherapy. Hallmarks of these tumors are H3K27M mutation or overexpression or the enhancer of zeste homolog inhibitory protein (EZHIP), both leading to epigenetic alterations, disturbed global histone and DNA methylation and imbalanced gene expression. In order to exploit immunotherapeutic options in DMG tumors, the aim of this project is to decipher the immunopeptidome of DMGs with a focus on the methylome-based oncogenesis. **Experimental Procedures** 25 native tumor tissues or primary cell lines will be analyzed to achieve >90% HLA allele coverage within the Caucasian population. HLA-I molecules were isolated from tumors using anti pan-HLA-I monoclonal antibodies, peptides were separated by acidic elution, purified solid phase extraction and analyzed by mass spectrometry (MS). Conventional HLA peptides from coding

sequence (CDS) as well as non-canonical (cryptic) HLA peptides from allegedly non-coding regions were identified using Peptide-PRISM. NetMHCpan binding predictions and non-existence in benignome databases have been used as further selection criteria. Immunogenicity of target candidates was evaluated by CD8+ in vitro priming assays. **Results** So far, a total of 40,380 HLA-I peptides have been identified in 15 patients (96,4% classic; 3,6% cryptic). 1,832 peptides were classified as tumor-specific (by our database), of these 27.6% were of cryptic origin and 72.4% derived from CDS. Interestingly, cryptic peptides were recurrently found in other DMG samples (in up to 4 out of 15 patients), but also in other tumor entities (certain peptides in up to 22 different tumors). This redundancy in other tumor entities was substantially higher than that observed in peptides from conventional proteins. With a first selection of 11 tumor-specific candidates we established further immunogenicity testing. Preliminary results show that de novo T-cell responses against these peptides can be generated from the naive T-cell repertoire of healthy donors. **Conclusions** We demonstrate that DMG tumors although being considered as immunologically cold present substantial numbers of cryptic and classic immunogenic HLA-I peptides. Cryptic peptides make up to 27% of all potential DMG specific T-cell epitopes and show a high rate of recurrency in different tumors. This special immunopeptidome might be related to the profound alterations of methylation patterns in these tumors. Current investigations aim to find a link between the global hypomethylation caused by the H3K27M-mutation and the immunopeptidome.

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AI-based tools for target identification foster the generation of novel TCR hits against solid tumor antigens

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T-cells are an essential component of adaptive immunity capable of recognizing pathogens and malignant cells. They use T cell receptors (TCRs) to screen antigens presented by HLA molecules on a cell surface. Upon recognition of a tumor-derived antigen - e.g. neoantigen encompassing a mutation site or peptide expressed at an abnormally high level, T cells might detect ongoing tumorigenesis and mount an anti-tumor immune response. This natural mechanism is utilized in the development of cancer vaccines and TCR-based immunotherapies for cancer. However, the key to successful therapy requires the identification of a proper target antigen.
Here we present the ARDentify platform - a set of highly accurate in silico prediction tools for the selection of clinically relevant tumor antigens. The platform allows for identifying population-wide or patient-specific targets and estimating the number of patients that can benefit from a given targeted therapy. It consists of ARDisplay presentation model that computes likelihood of antigen presentation in a complex with HLA class I or class II, with 2-fold higher accuracy compared to other approaches. Additionally, the ARDitox module allows for excluding candidates with high risk of off-target toxicity. The presented solution is designed to streamline the therapy development process by narrowing down the list of potential candidates, thereby minimizing both time and costs.

We applied the ARDentify platform to identify population-wide antigen candidates expressed in solid tumors using publicly available datasets. We derived neoantigens based on 25 TCGA cohorts (8197 patients), and tumor associated antigens derived from proteins of abnormal expression in tumor tissue (50 cohorts from 164 histological studies). We shortlisted the antigen candidates employing our ARDisplay and ARDitox modules and designed a panel of 34 peptide HLA I complexes (pHLAs). To identify TCRs recognizing the selected antigens, we employed oligo-barcoded multimers of the shortlisted pHLAs. Using the multimers we isolated antigen-binding cells from healthy donors CD8+ T cells and performed single-cell sequencing of TCRs and associated antigen barcodes. It allowed us to identify pHLA:TCR pairs in high-throughput manner and obtain TCR hits for all tested target pHLAs, yielding a total of 4134 full-length TCR sequences.
ARDisplay platform showed high success rate in experimental identification of TCRs for the selected antigen targets. We demonstrate that identification of TCRs targeting tumor-specific antigens among rare clonotypes of CD8+ T cells from healthy donors is feasible and allows generation of a large number of pHLA:TCR pairs that can be used for cancer immunotherapy development.

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SJ1 drives immune resistance in MITF^{low} melanomas

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A major focus in the field of immunotherapy is the identification of genes that mediate therapy resistance of cancer cells. In melanoma, one association to resistance is the expression of microphtalmia-associated transcription factor (MITF). A decrease of MITF is related to dedifferentiation, invasiveness, and resistance to immunotherapies. We hypothesize that MITF^{low} melanoma cells use so far unknown complementary pathways to evade immune responses. Our group established a high-throughput (HTP) RNAi screen to identify genes that regulate tumor cell rejection by cytotoxic T cells. For this project, HTP screens were conducted in a MITF^{low} and a MITF^{high} melanoma cell line that were both derived from the same immunotherapy non-responder patient. Together with previous screens in cell lines of various tumor entities, 220 genes that regulated tumor cell death were identified. Genes were subjected to bioinformatics analyses using published bulk and single cell RNAseq data sets of human melanomas and patient-derived melanoma cell lines. The analyses revealed that immune resistance genes were co-expressed in clusters with high inter-individual expression patterns. Several genes as well as clusters were additionally associated to the MITF^{low} melanoma phenotype across multiple data sets. One of those genes was SJ1 (masked name). SJ1 also showed a strong impact on tumor cell rejection by cytotoxic T cells in the HTP screen of the MITF^{low} melanoma cell line. We therefore performed extensive functional analysis to discover its mechanism of mediating immune resistance. Results showed that SJ1 can convey resistance not only in co-culture experiments with T cells but also that gene knockdown enhanced susceptibility of melanoma cells to treatment with supernatant of activated T cells, indicating an intrinsic resistance mechanism against apoptosis. Interestingly, although MITF^{low} melanoma cell lines showed primary

resistance to death receptor ligands such as TNFα and TRAIL, SJ1 silencing sensitized tumor cells to ligand mediated tumor cell death. Deeper analyses revealed that SJ1 stabilizes cell homeostasis. Knockdown of SJ1 induces phosphorylation of JNK that sensitizes the melanoma cells to death receptor ligand mediated apoptosis. Anti-apoptotic proteins such as BCL-2 are downregulated while death receptor 5 and caspase expression is increased. Accordingly, treatment with TRAIL results in strong induction of apoptosis in SJ1 silenced melanoma cells. In conclusion, our findings help to better understand complementary resistance mechanisms, even in the resistant MITF^{low} phenotype of melanoma, with SJ1 as a new potential target for immunotherapy.

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Combination of a bi-specific 4-1BB x PD-L1 immune cell activator and TAA x CD3 T cell engagers enhances anti-tumor efficacy

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Cancer patients benefit from targeted immunotherapy, but effective and durable treatment of solid tumors remains a challenge in the field. This is due to multiple mechanisms involved in suppression of anti-tumoral response, such as suppressive tumor microenvironment or immune depletion of tumors. Numab's proprietary MATCH^{TM} platform allows generating affinity tailored, stable, multi-specific compounds aiming to overcome these hurdles, while maintaining a favorable safety profile. The combination of 4-1BB (CD137, T-cell co-stimulator) activation and immune checkpoint inhibitor PD-1/PD-L1 antagonism has been shown to be highly active in preclinical models. Systemic application of first-generation anti-4-1BB antibodies however has resulted in dose limiting hepatic toxicities. We have generated a novel 4-1BB/PD-L1/HSA trispecific MATCH3 immunomodulatory drug candidate (NM21-1480), with optimized affinities, allowing overlapping 4-1BB activation and PD-L1 blockade. *In vitro* assays confirmed potent stimulation of T cell responses and tumor cell killing in dependency of PD-L1 expression. *In vivo* studies in mice and cynomolgus monkey revealed a potent anti-cancer immune response with beneficial safety profiles based on tumor-localized activation of 4-1BB and concomitantly blocking the interaction between PD-1 and PD-L1. NM21-1480 is currently clinically investigated in a Phase I/II trial.
CD3-based T cell engagers are highly potent therapeutic molecules which redirect T cell-mediated cytotoxic activity towards cells expressing selected tumor-associated antigens (TAA). We have designed affinity optimized T cell engagers targeting mesothelin (MSLN) or ROR-1 at a low affinity. *In vitro*, NM28-2746 (MSLN x CD3) and NM32-2668 (ROR-1 x CD3) induced T cell activation and tumor cell killing in a TAA density dependent manner, decreasing the risk for on-target/off-tumor adverse effects. *In vivo*, both molecules significantly reduced tumor growth in a model of MSLN+ human pancreatic adenocarcinoma and ROR-1+ mantle cell lymphoma, respectively.
Finally, we demonstrate that the combination of NM21-1480 with NM28-2746 or NM32-2668 has the potential to form a super-agonist synapse for enhanced T cell recruitment and activation. *In vivo* studies showed enhanced anti-tumor efficacy, recruitment of CD8 T cells to the tumor site and

immune memory formation.
Overall, our data not only suggests strong single agent efficacy of the tested drug candidates, but further suggest that combination of different immune-modulatory drugs can induce synergistic effects in immune cell recruitment and activation leading to improved tumor control.

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Identification of T cell targets by immunopeptidomics of small cell lung cancer

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Small cell lung cancer (SCLC) is the most aggressive subtype of lung cancer. SCLC is initially highly sensitive to chemo- and radiotherapy, however, it relapses in most cases without effective treatment options left. SCLC-specific or associated T cell targets are largely unknown. In this work, we aim to analyze naturally presented HLA-class I and -class II peptides by mass spectrometry (MS) in order to identify truly presented SCLC-associated antigens. Long term aim is to define patient shared or individual antigens, that could be targeted by novel immunotherapeutic approaches, e.g. peptide-based vaccination or cellular therapy.
SCLC tumor samples were obtained from two different sources: from fresh/frozen cryorecanalization or biopsies, and from patient-derived mouse xenograft models. Tumor lysis was followed by immunoprecipitation of HLA-I and -II peptide complexes with pan-HLA antibodies. After acidic elution and ultrafiltration, HLA bound peptides were identified by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). MS results were searched against the human proteome from Swiss-Prot database. To identify non-mutated tumor-exclusive ligands, a comparative analysis with a benign tissue reference database (publicly available HLA ligand atlas and in-house database) was performed. To search for mutated peptides, patient-specific mutated sequences derived from whole exome sequencing were included in the database for peptide sequence annotation.
So far, we have analyzed 17 SCLC tumor samples. The SCLC cohort covered 9 HLA-A, 16 HLA-B and 13 HLA-C allotypes, with respectively 8, 9 and 9 being shared by at least two samples. We optimized the peptide identification and selection strategy for xenograft models in order to improve overall peptide

yields and exclude mouse-derived MHC ligands. Altogether, we identified >35,000 HLA-I ligands and >25,000 HLA-II peptides. By comparative analysis of the peptides found on fresh/frozen SCLC tumor samples with a large reference database of benign tissues, we identified 3905 and 4693 non-mutated tumor-exclusive peptides for each HLA-I and HLA-II, respectively. More than 100 peptides were also present in samples from xenografts. Many of these tumor-exclusive ligands derived from gene products described to be associated with SCLC tumorigenesis, e.g. TP53, RB1, EP300, YAP1. Additionally, we identified HLA-I ligands that are shared with highest frequency between four different SCLC samples. So far mutated neoantigens could not be identified.
Further samples are already collected and will be analyzed to extend our knowledge on the SCLC-specific peptidome. Moreover, peptides are currently selected for T cell immunogenicity testing with autologous PBMC samples, PBMCs from HLA-matched SCLC patients, or PBMCs obtained from healthy donors. Identification of immunogenic SCLC-specific peptides will enable the development of personalized tumor vaccines.

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Identification and development of KBA1412, a fully human, first-in-class CD9 antibody for the treatment of cancer

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We investigated if functional anti-tumor antibodies with therapeutic utility can be isolated from cancer patients that have shown exceptional responses to treatment.
Memory B cells were isolated from the peripheral blood mononuclear cells (PBMCs) of a stage IV melanoma patient that was in long-term remission after transfer of *ex vivo* expanded autologous T cells. B cells were immortalized and expanded in a short-term culture using our proprietary protocol (B-Cūr, see poster Martijn Kedde). A target-agnostic screening for tumor-reactive B cell clones led to the identification of KBA1412 by virtue of its differential binding to melanoma cells as compared to healthy melanocytes. KBA1412 recognizes a non-mutated epitope on the cell surface glycoprotein CD9.
CD9 shows a wide cellular and tissue distribution and is implicated in a range of cellular functions, including motility, proliferation, and adhesion. Although CD9 is physiologically expressed on many different cell types, KBA1412 shows preferential binding to a wide range of human tumor types including malignant melanoma, colon-, gastric-, bladder-, lung-, ovarian-, and esophageal cancers. Binding of the antibody to CD9 expressed on cancer cells induces ADCC in a dose-dependent manner. Since CD9 is known to regulate leucocyte adhesion to endothelial cells and extravasation, we analyzed transmigration of T cells over endothelial cell layers and found that KBA1412 significantly enhanced the transmigration of T cells. *In vivo* antitumor activity of KBA1412 was tested in various

xenograft tumor models in mice carrying a human immune system (NSG-HIS). Effective tumor control was observed with KBA1412 as monotherapy and strong synergistic activity was observed in combination with a PD-1 checkpoint inhibitor. Strikingly, treatment of mice with KBA1412 resulted in profound infiltration of CD8 T cells and macrophages into tumors.
KBA1412 showed an excellent safety profile in non-human primate toxicology studies, no dose-limiting toxicities were observed with doses tested up to 10 mg/kg. Binding of KBA1412 to CD9 expressed on platelets resulted in a transient drop of peripheral platelet counts without any signs of bleeding or changes in coagulation parameters. Based on our data, KBA1412 is the first CD9 targeting antibody yet that could be qualified for clinical testing in man. We have initiated a clinical Phase 1 dose escalation and expansion trial to study safety, tolerability and preliminary efficacy of KBA1412 in patients with advanced solid tumors (ClinicalTrials.gov Identifier: NCT05501821).

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Bispecific T cell-engager targeting oncofetal chondroitin sulfate induces complete tumor regression and protective immune memory in mice

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The purpose of this study was to determine the efficacy of a bispecific T-cell engager targeting anti-CD3 and oncofetal chondroitin sulfate (ofCS) in mice with a functional immune system.
Developing cancer-targeting therapeutics has proven extremely difficult due to high heterogeneity between different cancers in terms of origin, type, and oncogenic mechanisms. This has led to a hunt for cancer specific targets shared between all cancer types. We have in the last years acquired extensive data demonstrating that a distinct chondroitin sulphate, ofCS, normally expressed only during fetal and placental development, is also present in high amounts on the surface of nearly all cancer cells. This carbohydrate structure plays a key role in the rapid growth, migration, and invasion of cancer cells, and thereby in the metastatic spread of the primary lesion. The recombinant malaria protein VAR2CSA (rVAR2) was used to identify ofCS, as it binds ofCS specifically and with high affinity without binding healthy chondroitin sulfate. By conjugating the immune activating fragment anti-CD3 to rVAR2, we have created a novel bispecific immune engager (V-aCD3), able to target a broad range of cancer cells. We have previously shown efficacy of a V-aCD3 bispecific construct in xenografted tumors in immunocompromised mice. However, this model does not bring the complex immune-mediated effects justice.
To show the full potential of V-aCD3, we have demonstrated its efficacy in syngeneic murine models in combination with immune checkpoint inhibitors. V-aCD3 shows an effect on its own which was enhanced when combined with immune checkpoint inhibitors, as this resulted in complete

elimination of solid tumors in both the immunologically "cold" B16-F10 and 4T1 models, and the "hot" CT26 model.
In addition, we have demonstrated that this effect is abscopal and creates a systemic increase in memory and activated T cells (both CD8+ and CD4+) without increasing regulatory T cells. Lastly, we have shown how this protective effect is long-lasting, as mice are either partially or fully protected against a rechallenge in the opposite flank.
Restoring or stimulating the body's own immune response against cancer holds the promise for effective tumor treatment with limited cancer recurrence. With the accumulated data presented, we propose that by utilizing the broadly expressed ofCS, novel and highly specific targeted cancer therapies applicable across several cancer types can be generated.

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Preclinical efficacy of an RNA-encoded T-cell engaging bispecific antibody targeting human Claudin 6

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Claudin 6 (CLDN6) is an oncofetal epithelial and endothelial tight junction protein that is expressed in several solid tumors but is absent from all healthy adult human tissues apart from the placenta. T-cell-engaging bispecific antibodies can recruit cytotoxic T-cells to tumor cells expressing antigens such as CLDN6; however, such antibodies have short serum half-lives in vivo, necessitating their continuous delivery via infusion pump to achieve sufficient therapeutic exposure in patients. We developed BNT142, nucleoside-modified mRNA encoding the bispecific antibody Ab-CD3x$(CLDN6)_2$ (referred to here as RiboMab02.1), formulated as lipid nanoparticles for intravenous administration and translation in the liver. Here, we present a preclinical characterization of BNT142's uptake and translation in mice and non-human primates, as well as RiboMab02.1's specificity, immunogenicity and anti-tumor activity. Our results show that RiboMab02.1 is accurately translated from BNT142 in vitro and in vivo, and that it exhibits dose- and target-dependent T-cell-mediated cytotoxicity of CLDN6-positive tumor cells. Weekly dosing of mice with BNT142 provided a sustained exposure to therapeutically relevant amounts of RiboMab02.1 in vivo and dose-dependent regression of CLDN6-positive OV-90 tumor xenografts in PBMC-humanized mouse models. Single injections of BNT142 in nonhuman primates afforded prolonged RiboMab02.1 exposure in vivo and were well tolerated. The data presented here suggests that BNT142-delivered RiboMab02.1 could be safe, effective anti-cancer drug, with a broad therapeutic window, and has helped initiate a Phase I/IIa dose escalation trial for patients with CLDN6-positive advanced tumors to evaluate the safety and pharmacokinetics of BNT142 (NCT05262530).

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KLRK1 and IL-7R expressing CD8+ T cells: a hidden target of IL-2 immunotherapy?

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Interleukin-2 (IL-2) is a cytokine with a dual function in the immune system. On the one hand, it is essential for $CD4^+$ regulatory T cell (Treg) homeostasis, but on the other hand, it supports the proliferation and activation of cytotoxic $CD8^+$ T lymphocytes. Because the IL-2 receptor of Tregs has a higher affinity than that of $CD8^+$ lymphocytes, it has been proposed that IL-2 competition may be a mechanism which Tregs employ to suppress $CD8^+$ T cells. Clinical trials of IL-2 immunotherapy of cancer show a strong correlation between IL-2 treatment, $CD8^+$ T lymphocyte proliferation, and tumor regression, indicating that higher availability of IL-2 releases $CD8^+$ T lymphocytes from Treg control. However, the effect of IL-2 on the phenotype and properties of $CD8^+$ T cells in the context of cancer/autoimmunity is not fully understood. In this study, we aimed to investigate the effects of IL-2 treatment or Treg depletion on the phenotype of $CD8^+$ T lymphocytes in several pathological conditions, such as autoimmunity, cancer, and chronic infection.
First, we have shown that Treg depletion led to the expansion of effector $CD8^+$ T cells and a higher incidence of lethal autoimmunity in the diabetes model. Interestingly, we observed a similar effect in Treg-replete mice treated with IL-2 immunocomplexes, despite Tregs beings expanded by IL-2 treatment. These results suggest that IL-2 supplementation overcomes Treg-mediated suppression of $CD8^+$ T cells.
Next, analyzing IL-2 immunocomplexes-treated and Treg-depleted mice, we identified a novel population of $CD8^+$ effector CD8+ T cells, which express natural killer cell receptor KLRK1, granzymes, IFITMs, but also IL-7R, which is associated with cell “stemness”. We called this cell population KILR ($KLRK1^+$ $IL\text{-}7R^+$) $CD8^+$ T cells. We hypothesized that KILR $CD8^+$ T cells are induced by IL-2, presumably as a consequence of a $CD8^+$ T cells being released from Treg control. Importantly, we have shown that IL-2 immunotherapy induces KILR $CD8^+$ T cells and improves survival in mouse models of cancer. Last but not least, we have identified KLRK1 and IL-7R expressing $CD8^+$ T cells in publicly available single-cell RNA sequencing datasets from studies on IL-2 immunotherapy or its modifications.
Our findings propose KILR $CD8^+$ T cells as an important target of IL-2 immunotherapy that has not been previously acknowledged. These findings contribute to our understanding of the effects of IL-2 on $CD8^+$ T cells, and the identification of new targets for cancer and autoimmune immunotherapy.

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Novel myeloid immune checkpoints identified by iOTarg, a function-based high-throughput discovery platform

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The tumor microenvironment (TME) is comprised of a complex mixture of cells from various origins that differentiate locally or are recruited to support tumor growth. Among the most abundant immune cells at all stages of tumor progression are tumor-associated macrophages (TAMs), which play an important role in maintaining immunosuppression in the TME and attenuate the function of effector immune cells to promote tumor survival. Therapeutic strategies that dampen the suppressive potential of TAMs in the TME and reprogram them towards a proinflammatory phenotype are increasingly seen as instrumental for effective immunotherapy. Nevertheless, the key mediators of TAM-induced immunosuppression remain largely unknown. To this end, we developed a functional, high-throughput genetic screening platform (iOTarg™) based on human primary monocyte-derived M2-like macrophages to systematically interrogate the genes responsible for the immune-suppressive phenotype of TAMs, by way of CRISPR knockout (KO). We investigated a proprietary library of 1400 expressed and druggable genes in two independent donor-derived M2-like macrophage cultures and achieved nearly complete KO over a range of control genes. Functional impact of individual gene KO on macrophage viability and phenotype, as well as on T cell (TC) activity upon cocultivation, was measured using multiparametric assays. The results were confirmed in a secondary screen using three additional donors and including an additional tumor lysis readout. The KO of the CSF1R gene, which is essential for macrophage maintenance, led to a significant loss of macrophage viability, whereas KO of established TAM markers, TREM2 and Clever-1, induced a change in macrophage phenotype. Furthermore, KO of immune-inhibitory receptor LILRB2 reduced the M2-like phenotype and restored TC activity. For a subset of genes, KO-induced inhibition of M2 activation of macrophages and subsequent increase in TC activation culminated in strong tumor cell killing, revealing an untapped repertoire of potential novel TAM-associated immune-checkpoint targets. Overall, this study introduces a novel target discovery platform that investigates the functional role of TAM-expressed genes in controlling various aspects of macrophage behavior such as cell viability, phenotype, TC activation/suppression, and even its impact on TC-mediated tumor cell lysis. As a result, we could confirm well-known targets, some of which are already in clinical testing, as well as identify additional novel targets that could lead to first-in-class, TME-modulating therapeutics in immuno-oncology.

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CD70-directed CAR natural killer cells require IL-15 stimulation for optimal elimination of CD70$^+$ cancer-associated fibroblasts and tumor cells in colorectal cancer and pancreatic ductal adenocarcinoma

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Numerous hematological and solid tumor types aberrantly express the immune checkpoint molecule CD70 to facilitate tumor progression. Interestingly, in colorectal cancer (CRC) we previously discovered expression of CD70 on a subset of cancer-associated fibroblasts (CAFs) in the tumor microenvironment instead of on the tumor itself. This population of CD70$^+$ CAFs was clearly involved in tumor progression and their presence was identified as an adverse independent prognostic factor

for CRC patients. Recently, we have also found CD70$^+$ CAFs in tumor resections of pancreatic ductal adenocarcinoma (PDAC) patients. Due to their tumor-promoting nature and the fact that CAFs in general construct a physical barrier around the tumor, we hypothesize that eliminating CD70$^+$ CAFs has the potential to improve treatment of CRC and PDAC patients. Hence, we aimed to generate an off-the-shelf cell therapy that specifically targets CD70$^+$ CAFs and tumor cells.
As proof-of-concept for chimeric antigen receptor (CAR) natural killer (NK) cells, we developed CD70-directed CAR NK cells by electroporating CAR-encoding messenger RNA into the NK-92 cell line. We analyzed specificity of the CAR construct using a CAR-neutralizing antibody and sensitivity of the CAR NK cytotoxic capacity by including several CD70$^+$ CRC and PDAC CAF cell lines and tumor cell lines with decreasing amount of CD70 expression. In vivo efficacy was evaluated in a subcutaneous CD70$^+$ xenograft mouse model. Stimulation with IL-15 was achieved by either exogenous addition or incorporation in the CAR construct. We measured NK cell activation by IFN-γ and TNF-α secretion. Validation of the CD70-targeting CAR NK cells showed consistent transfection efficacy with high CAR expression. Regardless of the fact that we could demonstrate competent antigen-specific killing of CD70$^+$ tumor cells, we saw only limited elimination when target cells displayed a low amount of CD70 on their cell surface. We unraveled in vitro that CD70-directed CAR NK cells are significantly more capable of eradicating CD70$^+$ target cells with both low and high CD70 expression when stimulated with IL-15. We found that this is presumably due to a substantially increased CAR expression and NK cell activation. In vivo, IL-15 stimulated CAR NK cells also increased tumor growth delay and prolonged survival compared to the unstimulated CAR NK cells. Of note, we saw that this IL-15 stimulation is actually necessary to obtain effective elimination of CAF cell lines over time.
Our data demonstrates on the one hand the value of CD70$^+$ CAFs as a novel target in cancer treatment and on the other hand identifies CD70-directed CAR NK cells armored with IL-15 as an ideal strategy to specifically target these CD70$^+$ CAFs in CRC and PDAC tumors.

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Collagen fragments produced in cancer mediate T cell suppression through Leukocyte-Associated Immunoglobulin-Like Receptor 1

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The tumor microenvironment (TME) is a complex structure comprised of tumor, immune and stromal cells, vasculature, and extracellular matrix (ECM). During tumor development, ECM homeostasis is dysregulated. Collagen remodeling by matrix metalloproteinases (MMPs) generates specific collagen fragments, that can be detected in the circulation of cancer patients and correlate with poor disease outcome. Leukocyte-Associated Immunoglobulin-like Receptor-1 (LAIR-1) is an inhibitory collagen receptor expressed on immune cells in the TME and in the circulation. We hypothesized that in addition to ECM collagen, collagen fragments produced in cancer can mediate T cell immunosuppression through LAIR-1. Our analyses of TCGA datasets show that cancer patients with high tumor mRNA expression of MMPs, collagen I and LAIR-1 have worse overall survival. We show

that *in vitro* generated MMP1 or MMP9 collagen I fragments bind to and trigger LAIR-1. Importantly, LAIR-1 triggering by collagen I fragments inhibits CD3 signaling and IFN-γ secretion in a T cell line. LAIR-2 is a soluble homologue of LAIR-1 with higher affinity for collagen and thereby acts as a decoy receptor. Fc fusion proteins of LAIR-2 have potential as cancer immunotherapeutic agents and are currently being tested in clinical trials. We demonstrate that collagen fragment-induced inhibition of T cell function could be reversed by LAIR-2 fusion proteins. Overall, we show that collagen fragments produced in cancer can mediate T cell suppression through LAIR-1, potentially contributing to systemic immune suppression. Blocking the interaction of LAIR-1 with collagen fragments could be an added benefit of LAIR-1-directed immunotherapy.

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The immune landscape during malignant transformation of the vulva and its prognostic potential

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Vulvar squamous cell carcinoma (VSCC) is an aggressive and highly mutilating disease. It arises through the malignant transformation of healthy vulvar tissue, via a precursor lesion, to invasive carcinoma, in an HPV, p53 or other mutation dependent manner. Despite the differences in etiology, no tailored treatment strategies are currently offered for these molecular subgroups. Current treatments consist of surgery or (chemo)radiation, with limited success and high morbidity. In-depth information on the immunological make up of VSCC is pivotal in order to assess if immunotherapy may form a potent alternative treatment.
To unravel the immunological changes that occur in the vulva throughout the stages of cancer development, a total of 162 different patient samples comprising healthy vulva, vulvar high-grade squamous intraepithelial lesions (vHSIL, precursor lesion) and VSCC were immunologically dissected. Multispectral immunofluorescence (15 markers, in situ high-resolution single-cell proteomics) was used to study both the myeloid and lymphoid immune cell composition. Transcriptomic differences were studied with Nanostring nCounter (1258 individual genes). The impact of the immune composition on survival was analyzed by Kaplan Meier curves.
As healthy vulva transforms into precancer and eventually cancer, the number of stroma infiltrating myeloid cells and epithelium infiltrating lymphoid cells increases. In contrast to the lymphoid cell infiltrate, the composition of the myeloid cell infiltrate strongly differed per molecular subgroup of VSCC. Two myeloid cell phenotypes were identified to be strong predictors for improved survival, independent of T cell infiltration, disease stage or molecular subgroup: epithelial monocytes and stromal dendritic cells. Infiltration with these myeloid cell types was not associated with T cell infiltration. Survival analyses revealed that a strong infiltration with either T cells or the identified myeloid cells was associated with drastic clinical benefit: 5-year survival >90% when either one is high, versus 40% when both are low (p-value<0.001). Transcriptomic analyses suggested that tumors with low numbers of these infiltrating myeloid cells expressed stem-like features.
In conclusion, a hot myeloid and/or lymphoid infiltrate predicts excellent survival in VSCC patients. These patients are likely to respond well to neoadjuvant immunotherapy. Based on these findings a clinical trial exploring the potential of neoadjuvant checkpoint blockade in VSCC will open this year. By incorporating patient specific tumor characteristics in the design of the treatment regimen, personalized treatment can be realized resulting in higher success rates and better quality of life.

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Spatial Architecture of Myeloid and T Cells Orchestrates Immune Evasion and Clinical Outcome in TRACERx

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The role tumour microenvironment (TME) in cancer is tightly linked to the cell phenotypes and their spatial context, which can be modulated by cancer cells in favour of immune escape and tumour promotion. An improved understanding of the TME during tumour evolution is, therefore, critical to defining optimal therapeutic strategies. Multiplexed imaging is a powerful tool for profiling cell phenotypes and spatial tissue organisation; however, data analysis often involves complex steps and substantial manual intervention.

To characterise the co-evolution of the tumour and its TME, we performed imaging mass cytometry of 151 tumour and 49 normal regions from 81 patients within TRACERx, a longitudinal, prospectively recruited study of early-stage non-small cell lung cancer (NSCLC). To address the need for reproducible, benchmarked, and interpretable workflows for multiplexed imaging analysis, we first developed TRACERx-PHLEX, a user-friendly computational pipeline for comprehensive analysis of the TME, validated and compared against standard tools. PHLEX encapsulates deep learning-enhanced cell segmentation (deep-imcyto), detection of spatially resolved protein expression and automated cell-type annotation (TYPEx), and interpretable spatial analysis (Spatial-PHLEX) as three independent but interoperable modules. Fully automated, open-source and portable pipeline, PHLEX outputs spatial catalogues of cell-type specific co-expression of therapeutic targets and produces clinically relevant spatial information.

We next applied PHLEX on imaging mass cytometry and integrated it with paired genomics data in TRACERx. We showed that tumours with high clonal neoantigen burden had high intra-tumour CD8 T cell infiltration and spatial hubs of CD8 T cells and M2-like macrophages. Immune cold tumours were distinguished by dense fibroblast arrangements. Tumour-Associated Neutrophils (TANs) defined a distinct TME that supports subclonal expansion, glycolysis and hypoxia, characterised by sparse T cell infiltration and modulated by cancer-neutrophil crosstalk through PI3K and RAS signalling. Finally, we demonstrated that TANs predict clinical outcome in NSCLC. Histopathology-derived TAN scoring revealed a high risk of relapse and metastasis in TAN-high cancers in independent discovery (n=44) and validation (n=331) TRACERx cohorts.

These analyses support a central role of the neutrophil-rich TME in driving metastasis and delineate optimal therapeutic strategies based on spatial heterogeneity of immune-checkpoint expression.

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Abstract has been withdrawn

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In Vivo Cell Fate Reprogramming Elicits Anti-tumor Immunity

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Immune checkpoint inhibitors (ICI) have revolutionized cancer treatment. However, only a small subset of patients responds to therapy. Responsiveness to ICI has been associated with the presence of conventional dendritic cells type 1 (cDC1), but tumor-derived factors often limit cDC1 accumulation and function in the tumor. We previously demonstrated that overexpression of transcription factors PU.1, IRF8 and BATF3 (PIB) in fibroblasts or cancer cells imposes a cDC1 fate *in vitro*. Reprogrammed cells acquire high expression of antigen presentation complexes (MHC-I/II), co-stimulatory molecules and cDC1 functions including cross-presentation and cytokine/chemokine secretion. As such, we hypothesise that replenishing cDC1 in tumors by *in vivo* reprogramming of cancer cells into tumor-antigen presenting cells (tumor-APCs) will drive antigen-specific immunity. Here, we evaluated anti-tumor responses elicited firstly by *in vitro* generated tumor-APC through intratumoral injection and secondly by reprogramming cells inside tumors implanted in mice. Injection of TLR3-stimulated tumor-APCs in low immunogenic B16-OVA tumors extended animal survival, reduced tumor growth, and promoted lymphoid infiltration. To assess efficacy elicited by *in vivo* reprogramming in the tumor, we implanted B16 cells 16h after PIB-transduction mixed with non-transduced cancer cells. All animals were tumor-free for 40 days while controls displayed median survival of 27 days demonstrating the capacity of reprogrammed tumor cells to elicit anti-tumor immunity *in vivo* within the suppressive microenvironment and in absence of exogenous TLR triggering. Tumor-free mice showed increased antigen-specific T-cells in peripheral blood and median survival increase of 5 days post B16 re-challenge. $BRAF^{V600E}$ tumors in cDC1-deficient $BATF3^{KO}$ animals showed reduced tumor growth after *in vivo* reprogramming confirming the direct effect of replenishing cDC1 in tumors. Indeed, by collecting tumors after 5 days of reprogramming, we confirmed acquisition of cDC1 phenotype (CD45+MHC-II+XCR1+) by tumor cells. Importantly, human glioblastoma (T98G) and lung (A549) cancer cells also underwent phenotypic reprogramming *in vivo* in immunodeficient animals. Finally, we combined *in vivo* reprogramming with either aPD-1 or aCTLA4 treatment in ICI-resistant or sensitive melanoma (B16, YUMM1.7, B2905, $BRAF^{V600E}$). Remarkably, in all four models blocking PD-1 or CTLA-4 signaling was translated into further reduction of tumor growth or complete regression and high levels of systemic tumor-antigen specific CD8+ and CD4+ T-cells in the blood.
Collectively, we demonstrated that cDC1-reprogramming *in vivo* elicits immunogenic reprogramming of tumor cells that is translated into durable anti-tumor immunity as monotherapy or in combination

with ICI. This study pioneers *in vivo* cell fate reprogramming for immunotherapy and paves the way for induction of a cDC1-fate *in situ* by delivering reprogramming factors directly to the tumor.

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Differential infection of dendritic cell subsets by oncolytic vesicular stomatitis virus variants

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Oncolytic viruses (OVs) can eradicate tumor cells and elicit antitumor immunity. VSV-GP, a chimeric vesicular stomatitis virus (VSV) with the glycoprotein (GP) of the lymphocytic choriomeningitis virus, is a promising new oncolytic and vaccine vector platform. However, the interaction of VSV-GP with host immune cells is not fully understood. Dendritic cells (DCs) as professional antigen presenting cells are essential for inducing efficient antitumor immunity. Thus, we aimed to investigate the interaction of wild type VSV, VSV-GP and the less cytopathic variant VSV-dM51-GP with different murine and human DCs subsets. Immature murine bone marrow-derived DCs (BMDCs) were equally infected and killed by VSV and VSV-GP. Human monocyte-derived DCs (moDCs) were more permissive to VSV. Interestingly, VSV-dM51-GP induced maturation instead of killing in both BMDCs and moDCs as well as a pronounced release of pro-inflammatory cytokines. Importantly, matured BMDCs and moDCs were no longer susceptible to VSV-GP infection. Mouse splenic conventional DC (cDC) subsets could be infected *ex vivo* by VSV and VSV-GP. Systemic *in vivo* infection of mice with VSV-GP and VSV-dM51-GP resulted in strong activation of cDCs despite low infection rates in spleen and tumor tissue. Human blood cDC1 were equally infected by VSV and VSV-GP, whereas cDC2 showed preferential infection with VSV. Our study demonstrated differential DC infection, activation, and cytokine production after the treatment with VSV and VSV-GP variants among species and subsets, which should be considered when investigating immunological mechanisms of oncolytic virotherapy in mouse models and human clinical trials.

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Type 1 DCs drive anti-tumoral immunity to endogenous immunogenic neoantigens in lung cancer

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Reduced density and functionality of type 1 dendritic cells (cDC1) in lung tumor tissues is a key obstacle to activate anti-tumoral immunity in non-small cell lung cancer (NSCLC). Here we generated

a model of hypermutated NSCLC (KP^{neo}) to study cross-presentation of endogenous *bona-fide* neoantigens (neoAgs) and regulation of lung anti-tumoral $CD8^+$ T cell responses by DC-therapy. We show that cDC1 controls and broaden the specificity of anti-tumoral $CD8^+$ T cells to neoAgs. Consistently, a high cDC1 signature has prognostic value in highly mutated human cancers. Treatment with FMS-like tyrosine kinase 3 ligand (Flt3L)-based therapy enhances $CD8^+$ T cells activation and it is sufficient to control growth of KP^{neo} tumors in lung tissues, whereas antibodies to PD-L1 are not beneficial. Mechanistically, DC-therapy induces expansion of $CD8^+$ T cells, pre-DCs and cDC1 in the lung, promotes cytotoxicity in T cells and reduces expression of tolerogenic/inhibitory transcripts in lung cDCs. We conclude that boosting DCs activity is critical to leverage neoantigen content for therapeutic advantage in hypermutated lung tumors that do not respond to checkpoint blockade.

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Combining the organoid technology with CRISPR to study immune responses to hematological premalignancies and neoplasia

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Studies on cancer immunology have benefited from technological advancements. In particular, human organoids created vast opportunities to study the immune system. Although animal models and cell culture techniques produced many important insights, they have not shed much light on what happens in the early stages of malignancies. In contrast, the organoid technology has the potential to fill this gap by providing a complex system resembling true-to-life interactions of the human immune system with malignant cells in tissues.
Here, we aim to explore the cancer immunosurveillance and immunoediting hypotheses using the CRISPR/Cas9 system at the very time point of malignant transformation with a defined mutational landscape and burden, thereby mimicking hematological premalignancies and neoplasia in human tonsil and spleen organoids. CRISPR/Cas9 ribonucleoproteins were delivered into primary T cells by electroporation, introducing genetic alterations such as knockout of the tumor suppressor P53 (P53-k/o). To benefit from the genetically and phenotypically robust organoid system, we co-cultured P53-k/o cells with autologous tonsil organoids. Using Luminex to analyze 80 cytokines we did not detect altered cytokine secretion in response to the pre-malignant transformation. In a trans-well assay we did not observe any migration of immune cells towards the altered T cells. Using a 3-dimensional organoid co-culture and live microscopy of P53-k/o or unaltered T cells with autologous tonsil cells, we did not observe mutation-dependent cytotoxicity or increased contact of non-altered immune cells with the genetically modified premalignant T cells.
Because the cellular make-up of the tonsils is dominated by B and T cells, the experimental system lacks cellular diversity. Spleen organoids comprise a considerably broader cellular repertoire, and therefore are better suited for these experiments. Furthermore, P53-k/o might be insufficient to provoke an immune reaction. To account for this, we used CRISPR to generate oncogenes by homology-directed repair and introduced JAK2, calreticulin, and KRAS mutations. Moreover, we

induced a chromosomal translocation resulting in the Philadelphia chromosome, the main characteristic of chronic myeloid leukemia. Currently, we are co-culturing spleen organoids with autologous manipulated T and B cells to assess immune responses to these oncogenes using Luminex, trans-well assays, and live microscopy. This will enable us to assess effects of different mutation types, mutational load, or ratio of malignant cells to immune cells.
Together, combining organoids with the CRISPR technology allows us to study how malignant transformation affects anti-cancer immune responses at the very time point of alteration. In combination, these technologies are a powerful tool with the potential to advance our knowledge of cancer immunology, thus providing valuable insights for the improvement of cancer immunotherapy and ultimately patient outcomes.

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Actin remodelling and small vesicle recruitment hinder effective anti-tumor immunity by shaping the tumor cell side of the immune synapse

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Natural killer (NK) cells are key players in the fight against cancer. They physically interact with prospective target cells through a highly specialized cell-to-cell junction termed the immunological synapse (IS), which is used for directional delivery of cytotoxic molecules to the cancer cells. Conversely, tumors have developed defense mechanisms to escape from cytotoxic lymphocyte-mediated killing. Recently, we reported the critical role of actin cytoskeleton in setting of these defense mechanisms. Our findings show that, upon NK cell attack, a massive accumulation of actin rapidly takes place at the IS of resistant target cells. We termed this process “actin response” (AR). Correlative light and electron microscopy analysis revealed the presence of small vesicles (90-100 nm in size) and multivesicular bodies (MVBs) in the synaptic region of cancer cells with an AR. MVBs are the precursors of small extracellular vesicles (EVs). Tumor EVs contain an array of inhibitory molecules that could potentially contribute to modulate the activity of cytotoxic lymphocytes in the context of an IS. Using confocal microscopy and imaging flow cytometry, EV markers (CD63, CD81 and CD9) were localized and quantified at the tumor cell side of the IS. Cancer cells with an AR exhibited an enrichment of EVs at the IS when compared to cancer cells without an AR. Moreover, we used single-cell time-lapse microscopy to observe spatiotemporal resolution analyses of subcellular events in breast cancer cells upon NK cell attack. Our results identified that AR at the IS triggered MVB mobilization to the synapse, facilitating EV release and conferring resistance to NK induced cytotoxicity. Further insights on the molecular mechanisms underpinning this synaptic resistance pathway, such as the identification of selective targeting of linker proteins between the actin cytoskeleton and small vesicles, represent a promising therapeutic strategy to improve the efficacy of anticancer immunotherapies. Nevertheless, additional research is needed in order to evaluate the translational potential of targeting the AR in a clinical setting.

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Characterization of tissue-resident regulatory T cells in healthy human tissue and tumors on single-cell level

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Regulatory T (Treg) cells are best known for their role in regulating immune responses and maintaining self-tolerance. In addition to these “classical” functions, Treg cells residing in tissues are also able to promote tissue homeostasis and regeneration. This has been demonstrated in murine tissue injury models of a wide array of organs, including the lungs, liver, kidney, skin, and muscle, in all of which tissue Treg cells proved essential for tissue regeneration. We recently identified the human analogue to murine tissue regeneration-promoting Treg cells using single-cell epigenetic landscapes, and a conserved, microbiota-independent tissue-repair Treg signature with a prevailing footprint of the transcription factor BATF was defined. We then used this signature and applied it to a single-cell epigenetic landscape of human healthy skin, fat and blood CD4 T cells. Given the tissue regenerative/ remodelling function of tissue Treg cells, we now set out to elucidate the role of this cell type in human tumor development and metastasis. To this end, we isolated tissue Treg cells from fresh tumor tissue of various tissue types and disease stages with the corresponding normal tissue adjacent to the tumor (NAT). We then isolated nuclei and performed single cell ATAC-sequencing to investigate the chromatin accessibility of tissue Treg cells in human tumor and NAT samples across disease states. This technique allows us to generate tissue-specific signatures to identify the homeostatic regeneration program in human organs, and calculate the changes associated with malignant transformation of those tissues. In addition, we aim to characterize effector molecules of human tissue Treg cells in the tumor and neighbouring tumor-free tissues to identify commonalities and differences in the physiologic wound healing program and the impact of this program on tumor growth *in-situ*. These findings may enable us to directly target tumor growth-promoting Treg cells while sparing the homeostatic regeneration potential of the immune system, further extending the immunotherapeutic options in precision-based medicine.

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AATF/Che-1 RNA polymerase II binding protein overexpression reduces the anti-tumor NK cell cytotoxicity through immune check-point modulation

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The regulation of AATF/Che-1 on tumorigenicity is mainly due to its central role demonstrated in the oncogenic pathways of solid tumors, where it controls proliferation and viability. Its over-expression in different tumor contexts included pediatric leukemia, is well known, but the effect exerted by tumors overexpressing Che-1 on the immune response has not yet been investigated. Here we show that Che-1 is able to modulate the expression of Nectin-1 ligand in B-Cell Precursor Acute Lymphoblastic Leukemia (BCP-ALL) cell lines at the transcriptional level leading to the impairment of killing activity of NK cells. Che-1-dependent Nectin-1 down-modulation induces a functional modification in NK ligands expression on blast cells able to modify the interaction with activating receptors on NK cells
Indeed, we found impaired NK cells degranulation activity and IFNγ and TNFα production in NK cells co-cultured with BCP-ALL cells if compared with co-culture performed with Che-1-depleted BCP-ALL cell lines. In addition, NK cells showed reduced ERK 1/2 phosphorylation after 24 hours of co-culture with Che-1-expressing cells as a functional proof of inhibited NK cell proliferation. This effect is partially rescued upon Che-1 down-modulation. To support these findings we took advantage of a Che-1 transgenic mouse model crossed with the MITO-luc mice able to monitor blast cells hyper-proliferation in B-cell compartments. Thus we observed that Che-1 overexpression induced B-cell proliferation in mice spleens where NK cells exhibited impaired activation and a preferential immature status if compared with Che-1.negative MITO-luc mice, when analyzed by flow cytometry. In conclusion, the critical equilibrium between NK cell ligand expression on tumor cells and the interaction with NK cell receptors is affected by Che-1 over-expression and partially restored by Che-1 interference supporting the necessity to inhibit Che-1 as a pivotal target with a dual function in tumorigenicity as in anti-tumor immunity.

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Advancing IO drug development by high content imaging of immune cell co-cultures with organoids from diverse cancer indications

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While immuno-oncology (IO) drugs have shown great results for certain cancer indications in the clinic, the potential of many IO drugs in different indications remain to be uncovered. With the development of advanced 3D cell culture models of patient derived organoids (PDOs), a better preservation of the original patient tumor in terms of genetics, transcriptomics and morphology in comparison to other 3D in vitro systems can be achieved, leading to increased prediction of clinical outcome. We have established a biobank of tumor and normal organoids (>600), from multiple different tissue types. These organoids can act as surrogates for individual patients, making them suitable for patient population studies. Here, we present an organoid screening platform with immune cells in co-culture to evaluate the response to IO drug candidates *in vitro*. Using our proprietary image-based analysis we can quantify effects of IO drug candidates by analyzing functional read-outs such as tumor killing and migration or infiltration of immune cells into the tumors. PDOs from different indications, including colorectal, lung, breast, and ovarian cancers, were cultured in protein hydrogels in a 384-well format. Allogenic PBMCs were isolated from healthy donors and activated with superantigens or CD3/CD28 beads. PDOs and PBMCs were then co-

cultured together in the presence of IO drugs, with 4 technical replicates per condition. Using 3D high content imaging (HCI) and an automated image analysis pipeline, the tumor objects and immune cells were segmented and provided data on PDO numbers and volumes as well as PBMC numbers, migration and infiltration. Analysis of this information showed that PDOs have different sensitivity to killing by immune cells, as measured by reduction in organoid count and volume. Moreover, we found model-specific effects of PBMC activation status and compound treatment in killing and immune cell infiltration. Our tumor organoid platform with immune cell co-culture enables responder/non-responder profiling of IO drug candidates across a broad range of patient genetics and cancer types in a higher-throughput *in vitro* 3D screen, which is more physiologically relevant. Combined with our HCI analysis, immune cell mediated tumor killing could be quantified and correlated with the immune cell migration and infiltration providing deeper understanding of the mechanism of action of IO drugs and identifying indications, or subsets of tumors that share specific genetic or morphological characteristics, that could benefit from it. In short, this system allows IO drug testing ranging from monoclonal antibodies to adoptive cell therapies in a variety of clinically relevant models, advancing the development of drugs and the identification of suitable patients and indications as well as potential biomarkers.

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The role of IL4I1 in myeloid cells and its impact on immune control of chronic lymphocytic leukemia

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A pan-tissue aryl hydrocarbon receptor (AHR) signature revealed that across 32 tumor entities, **interleukin-4-induced-1 (IL4I1)** associates more frequently with AHR activity than other AHR activators, including idoleamine 2,3-dioxygenase (IDO-1). As an L-amino acid-oxidase, IL4I1 catabolizes aromatic amino acids which leads to the generation of indole metabolites and kynurenic acid which contribute to AHR activation. IL4I1 is strongly upregulated in many cancer types, where it is mainly expressed by tumor-associated myeloid cells. We previously showed that IL4I1 promotes cancer cell motility and **suppresses adaptive immunity** by affecting the T effector cell compartment, thereby **enhancing the progression of chronic lymphocytic leukemia** (CLL) in mice. In cell culture systems including antigen-presenting cells (APCs), T cells and tumor cells, we showed that **Il4i1 expression in APCs limits T cell fitness**, as an increased T cell activation and tumor cell killing capacity was observed when Il4i1-deficient APCs were used. To better characterize IL4I1-expressing myeloid cells and decipher their immune suppressive mode-of-action in cancer, we performed single-cell RNA-sequencing of the tumor microenvironment of CLL-bearing mice in *Il4i1*$^{-/-}$ or wild-type bone marrow chimeras. Spleen samples as the main organ of tumor development in this model were depleted of CLL cells, and transcriptome and T-cell receptor sequencing was performed using the 10X Genomics platform. This allowed us to identify a novel, recently characterized dendritic cell state, **mature regulatory DC** (mregDC), as the main population expressing *Il4i1*. Flow cytometry-based

characterization of mregDC in longitudinal tumor samples showed an accumulation of this cell state in parallel with tumor development, as well as a loss of antigen-presenting capacity over time. Current efforts focus on exploring the single-cell RNA-sequencing data to decipher mregDC biology and molecular interactions of mregDC with other immune cell types present in the CLL mouse model, as well as identifying IL4I1-expressing cell populations in lymph node samples of CLL patients.

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Human 3D in vitro models for the assessment of Cancer Immunotherapy Mode of Action

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Cancer Immunotherapy (CIT) strategies have enabled significant breakthroughs in cancer treatment. However, their development remains extremely challenging, also due to intrinsic limitations of currently available preclinical experimental approaches. The next generation of CIT-drugs will require a more dynamic and content-rich analysis allowing for mid-throughput readouts, in the most physiological human tumor microenvironment (TME) possible.
In order to complement currently available 2D in vitro and in vivo preclinical experimental models, we utilized the microfluidics OrganoPlate 3-lane 40 system by MIMETAS, to establish two distinct, fully human, imaging approaches for the screening of next generation CIT-drug candidates.
The first system is a 3D in vitro imaging model which enables the dynamic visualization and characterization of interactions between cancer cells, immune cells, stromal cells, and extracellular matrix (ECM), recapitulating the human TME organization and immune cell dynamics. After carefully cross validating our platform against in vivo and 2D in vitro analyses, we now employ this new system to shed light on the Mode of Action (MoA) of immune cell engagers, such as Glofitamab or Cibisatamab.
The second system is a fully human mid-throughput 3D in vitro screening system for imaging and quantifying immune cell trafficking and infiltration to tumors in response to CIT-drugs. It combines endothelial tubules, peripheral immune cells, solid or liquid tumors, ECM and tumor-resident immune cells. This model can provide insights on the impact on immune cell infiltration of immunomodulation and T cell redirection CIT drug candidates.
As a next step towards more physiological 3D in vitro models, we aim at implementing Patient-derived Tumor Organoids (PDTOs) in order to allow for a more translatable assessment of CIT-drugs MoA.
In summary, these models allow for mid-throughput screening of drug candidates, evaluation of combination strategies and a mechanistic understanding of their MoA in a fully human 3D in vitro system and are a valuable addition to existing preclinical models with their ability to mimic physiological immune cell dynamics, such as immunological synapse formation and trafficking. Consequently, these platforms support next-generation CIT-drug development.

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Identification of synergistic drug combinations with MEK inhibitors for the treatment of pancreatic cancer

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Pancreatic ductal adenocarcinoma (PDAC) is refractory to cytostatic and immuno-oncology therapeutic strategies, leaving surgical resection of the tumor as the only treatment that can significantly prolong survival. We are therefore exploring optimized treatment strategies targeting cancer cells as well as infiltrating immune cells and the tumor microenvironment (TME) in an autochthonous mouse model for PDAC. Single focal tumors are induced through *in vivo* electroporation of gene constructs into the pancreas which encode mutated *Kras* and facilitate knock out the tumor suppressor genes *P53*, *Cdkn2a* and/or *Smad4* via CRISPR/Cas9. The resulting tumors recapitulate the human disease with respect to genetics, histology and TME composition. Furthermore, this model allows the generation of tumors reflecting different PDAC subtypes, as well as the resection of primary tumors in combination with the (neo-)adjuvant regimens.
Like the human disease, these tumors are refractory to chemotherapy, whereas targeted therapy by means of MEK inhibitors merely leads to temporary delay of tumor outgrowth. We therefore tested the combination of MEK inhibitor treatment with different, potentially complementary modalities. While addition of PD-1/PD-L1 blockade did not enhance anti-tumor efficacy, we found the combination with agonist anti-CD40 antibodies to be highly effective against these tumors. Prominent regressions are observed, and in combination with primary tumor resection long-term complete responses can be achieved even in a metastatic variant of the model. Apart from the direct cytostatic impact of MEKi on the *Kras*-transformed tumor cells, the mechanism of action of this synergistic drug combination involves enhancement of the anti-tumor T-cell response, suppression of CD4+ Tregs, and reduction of MDSCs and M2-type macrophages in favor of M1-type pro-inflammatory macrophages.
A second highly effective regimen as identified in our experiments involves the combination of MEK inhibitors with the multi-kinase inhibitor regorafenib. In contrast to the aforementioned regimen, the complementary action of these drugs involves modulation of the TME by regorafenib. While the clinical translation of the MEKi/CD40 Ab regimen is hampered by toxicity issues encountered with agonist anti-CD40 Ab, the synergy as observed in the MEKi/regorafenib regiment provides a tangible path towards clinical testing.

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DC-SIGN-related function in vaccination and tumor control in a transgenic human DC-SIGN mouse-model

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The human DC-SIGN receptor expressed on various antigen presenting cells (APCs) binds glycans on e.g. pathogens and tumors. Because of its function, the potential to target the DC-SIGN receptor for the induction of T-cell immunity is studied widely. In contrast, DC-SIGN expression is also found to be expressed on macrophages, and it's expression on M2 tumor associated macrophages (TAMs) mainly coincides with anti-inflammatory cytokines. So far, the function and efficacy of DC-SIGN *in-vivo* has been studied in mouse models in which DC-SIGN is expressed under the murine CD11c promotor (CD11c-DC-SIGN). However, this does not fully reflect the human's expression pattern. Therefore, a new transgenic mouse has been studied, which expresses the human DC-SIGN receptor under its native promotor region (hDC-SIGN).
We investigated the expression of the DC-SIGN receptor in hDC-SIGN on *ex-vivo* and *in-vivo* APCs. Thereafter, we studied the potential to target DC-SIGN expressing APCs in the skin draining LN upon s.c. vaccination in combination with the adjuvant AddaVax which attracts immune cells such as moDCs and cDCs to the site of vaccination. Separately we analyzed the potential of DC-SIGN expressing APCs in hDC-SIGN mice to control tumor growth, when tumors these tumors overexpressed DC-SIGN binding glycans.
We show that in hDC-SIGN mice DC-SIGN is expressed on BMDCs, moDCs, cDC2's, and various macrophages throughout tissues, thereby better resembling the human's expression. S.c. injection of a DC-SIGN MoAb conjugated vaccine targets to various subsets of APCs in the skin draining lymph node, which effect is boosted by the addition of the adjuvant AddaVax. Interestingly, tumors that expressed DC-SIGN binding glycans showed diminished tumor growth in hDC-SIGN animals, while in both WT and CD11c-DC-SIGN mice tumor growth was increased. This highlights that the expression of DC-SIGN in hDC-SIGN mice controls tumor growth, probably due to its presence on macrophages, as CD11c-DC-SIGN mice did not show tumor control.
Our results highlight that the hDC-SIGN mouse shows differential expression of DC-SIGN receptor, that comes close to the human setting, and allows us to explore DC-SIGN-ligand interactions related to its function and strategy for targeted vaccinations and endogenous tumor recognition.

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Unravelling tumor-intrinsic resistance mechanisms to T-cell mediated cytotoxicity in pancreatic cancers

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Most human Gastrointestinal Cancers (GI) including colon, rectal and pancreatic cancers are resistant to Immune checkpoint inhibitors (ICB). The lack of response has been largely attributed to their low tumor mutational burden, but recent articles have demonstrated that neoantigen-specific Tumor Infiltrating Lymphocytes (TIL) can be successfully expanded and isolated. This suggests that other factors, both tumor intrinsic and due to the suppressive tumor microenvironment, likely contribute to the lack of response of ICB.
In this study we aim to identify tumor-intrinsic resistant mechanisms to T-cell mediated killing in a patientderived pancreatic cell line, TC-4177, *in vitro*. This cancer cell line is not effectively recognized when co-cultured with autologous PBLs capable of recognizing the $MMP14_{p.R158C}$ neoantigen expressed by these cancer cells, nor by $MMP14_{p.R158C}$-specific TCR-engineered $CD8^+$ T cells, suggesting that the lack of recognition is not due to T cell dysfunction but, rather, tumor intrinsic.
We performed a Genome-wide CRIPSR/Cas9 coculture-based screen on this patient-derived cell line to identify genes modulating T-cell cytotoxicity. We identified genes known to play a key role in T-cell-mediated immunity such as those involved in the antigen presentation and IFNγ and TNFα pathways. sgRNAs targeting the antigen itself (MMP14) and the MMP14 HLA-restriction (HLA-B) also conferred resistance to T-cell killing, further supporting the robustness of the screen.
Importantly, most genes shown to limit T-cell killing were mainly involved in autophagy, vesicle trafficking and ubiquitin-dependent catabolic pathways. Novel genes within these pathways were independently validated in the TC-4177 cell line and some were validated in additional pancreatic cancer cell lines. Overall, these results open newavenuesto render pancreatic cancers susceptible to T- cell killing and, potentially, to immunotherapy.

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Patient derived lung cancer organoids to study tumor immune interactions

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Lung cancer remains the leading cause of cancer related death worldwide and is the second most frequent cancer diagnosed. Lung cancers can be broadly classified into two forms, small-cell lung carcinomas (SCLC) and non-small-cell carcinomas (NSCLC). SCLC is a high-grade neuroendocrine

carcinoma which accounts for approximately 15% of all lung cancers. Although immunotherapies have shown great success in the treatment of other cancers, marginal clinical improvement has been observed in SCLC. As a result, there is a need to develop better models to understand SCLC responses to immunotherapy. Current transgenic mouse and patient derived xenograft models have been shown to capture the heterogeneity and complexity of SCLC; however, these models lack the adaptability to study the patients' immune components and tumor microenvironment. Here, we have generated and characterized a cohort of 20 SCLC patient-derived tumor organoids and confirmed that they possess similar molecular characteristics and morphological architectures of the corresponding patient tumor tissue. From autologous cocultures using organoids and patient material, upregulation of HLA-I on tumor cells led to the identification of 4-1BB$^+$ and PD1$^+$ tumor reactive T lymphocytes and TCR clonotypes for further downstream analyses. We demonstrate that patient SCLC cancer organoids would be ideal for investigating the tumor microenvironment in SCLC, tumor and immune cell interactions as well as assessing patients' responses to therapy.

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Germline homozygosity and allelic imbalance of HLA-I are common in esophago-gastric adenocarcinoma and impair the repertoire of immunogenic peptides

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The individual HLA-I genotype is associated with cancer, autoimmune diseases and infections. We aim to elucidate the role of germline homozygosity or allelic imbalance of HLA-I loci in esophago-gastric adenocarcinoma (EGA) and resulting repertoires of immunogenic peptides. We show that germline homozygosity of HLA-I genes is significantly enriched in EGA patients (n=80) compared to an HLA-matched reference cohort (n=7605). HLA-genotypes and sequences of either (1) ten relevant tumor-associated antigens (TAAs) or (2) patient-specific mutation-associated neoantigens (MANAs) were used to predict good-affinity binders using an in-silico approach for MHC-binding (www.iedb.org). Whereas the overall mutational burden is similar, the repertoire of potentially immunogenic peptides derived from TAAs and MANAs was lower in homozygous patients. Promiscuity of peptides binding to different HLA-I molecules was low for most TAAs and MANAs and in-silico modelling of the homozygous to a heterozygous HLA-genotype revealed normalized peptide repertoires. Transcriptome sequencing showed imbalanced expression of HLA-I alleles in 75% of heterozygous patients. 33% of these showed complete loss of heterozygosity, whereas 66% had

altered expression of only one or two HLA-I molecules. In a FluoroSpot assay we determined that peptide-specific T cell responses against NY-ESO-1 are derived from multiple peptides, which are often mutually exclusive for one HLA-I allele. The high frequency of germline homozygosity in EGA patients suggests reduced cancer immunosurveillance leading to an increased cancer risk. Therapeutics targeting epigenetic silencing of HLA-I molecules are an attractive approach to improve outcome in this disease.

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Single-cell analysis of the bone marrow niche from lung tumor bearing mice reveals cell subset specific myelopoiesis perturbing cues

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High infiltration of immunosuppressive myeloid cells in lung tumors prevents high response rates to immunotherapy. Yet the main source of myeloid cells, the bone marrow (BM) niche, remains largely understudied due to its inaccessibility, low fraction of stromal and hematopoietic stem and progenitor cells and the heterogeneous and continuously differentiating character of hematopoiesis. Recent studies suggest that tumor promoting myelopoiesis perturbing cues (MPCs) vary with cancer stage, (sub)type and cellular source.
To unravel the identity and source of MPCs in lung cancer, we used single-cell RNA sequencing to map and compare whole BM from healthy and orthotopic Lewis lung carcinoma (LLC) bearing mice. Data were integrated using Seurat's anchor-based integration approach. Subsequent clustering was performed at 0.5 resolution, allowing the identification of 20 clusters, re-clustered into 6 main clusters comprising: B cells, NK/T cells, granulocytes, myeloid cells, megakaryocytes/erythrocytes and hematopoietic progenitors. Using the Cacoa package, we identified a significant increase in the abundance of granulocyte progenitors, granulocyte monocyte progenitors, hematopoietic stem progenitor cells, promonocytes, and megakaryocyte-erythrocyte progenitors in LLC bearing mice. Within the granulocyte cluster, we further characterized four neutrophil subpopulations of which the

Ly6G$^+$ fraction drastically decreased while the opposite held true for the Lcn2$^+$ subset. Likewise, we identified the highest magnitude expression shifts in the neutrophil and monocyte subsets, indicating that tumor progression drastically impacted their phenotype and function. Upon transcriptional comparison of healthy and LLC-bearing mice, 5 potential MPCs were defined: *Ifitm1*, *Wfdc17, Hist1h2ap, Lcn2* and *S100a* family. These have not been described before in the BM niche of solid cancer bearing subjects, demanding their further investigation and clinical validation. Therefore, we rolled out a clinical trial (NCT05251805) to evaluate the collection and content of costal bone marrow aspirates from lung cancer patients. Finally, we are optimizing an innovative 3D crosstalk assay to spatiotemporally study and validate candidate MPCs during the lung tumor-BMN interplay *ex vivo*. Briefly, lung tumor spheroids are co-cultured with murine Lin/CD45$^-$ murine or human CD34$^+$ cells on tailored porous polymer scaffolds that mimic fenestrated bone structure.
By construing lung tumor–BM crosstalk characterizing MPCs, we provide fundamental knowledge and understanding that paves the way for novel biomarker identification and development of groundbreaking lung cancer treatments.

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Improving immunotherapy by overcoming tumor endothelial cell anergy

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Ongoing angiogenesis renders the tumor endothelium unresponsive to inflammatory cytokines, resulting in lack of endothelial adhesion molecules, such as ICAM-1, and escape from immunity. This process is referred to as tumor endothelial cell anergy. Anti-angiogenic agents can overcome tumor endothelial cell anergy by making endothelial cells sensitive to inflammatory signals and by inducing adhesion molecule expression. We recently demonstrated that primary tumors of renal cell carcinoma (RCC) patients treated pre-surgically with VEGF pathway-targeted drugs contain a more extensive inflammatory infiltrate, when compared to non-pretreated patients [1]. Overcoming tumor endothelial cell anergy is therefore generally supporting intrinsic anti-tumor immunity, as well as immunotherapy strategies. Indeed, since 2018 eight FDA approvals are registered for combination therapy of immune checkpoint inhibitors with anti-angiogenic drugs, mainly VEGF targeting compounds.
It can be considered counterintuitive that improvement of leukocyte infiltration in tumors can be reached with drugs that limit the main gateway of leukocytes for entering the tumor parenchyma. Nevertheless, the above-mentioned pretreated primary RCC tissues show a mean vessel density drop of 40-60% after two cycles of treatment with sunitinib or bevacizumab, while leukocyte infiltration was enhanced by 2-3-fold in these tissues. These results allow the expectation that the pro-inflammatory activity of angiostatic agents is temporary. However, it is currently unknown whether inhibition of angiogenesis and stimulation of leukocyte infiltration coincide at similar drug concentrations, neither it is known how the distribution of the two processes is regulated in time. In principle, it is possible that a non-angiostatic dose of anti-angiogenic agents is able to improve

leukocyte infiltration and anti-tumor immunity, which would be then continuously applicable. This concept will be discussed in the context of immune checkpoint inhibition and other immunotherapy approaches for cancer, such as vaccination or CAR T cell therapy.

1. Nowak-Sliwinska P ***et al***: Proinflammatory activity of VEGF-targeted treatment through reversal of tumor endothelial cell anergy. ***Angiogenesis, In press,*** 2022.

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A tryptophan metabolite activates tumor immunogenicity to potentiate adoptive T cell-based cancer immunotherapy

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Tryptophan metabolism plays an important role in antitumor immunity, but how tryptophan metabolites directly affect tumor immunogenicity is not clear. Here we set up an *in vitro* co-culture assay to examine the impact of a number of tryptophan metabolites on tumor immunogenicity, and identified a tryptophan metabolite, indole-3-aldehyde (I3A), could markedly upregulate tumor immunogenicity and activate antitumor T cell response. I3A-treated EG7 tumor cells significantly induced T cell activation reflected by IL-2 and IFN-γ secretion in tumor-T cell co-culture system. Mechanistically, I3A treatment induced significant downregulation of c-Myc oncogene, which consequently upregulates a plethora of immunogenic markers including HMGB1, calreticulin and antigen processing machinery genes. Treatment of established EG7 lymphoma and B16-OVA melanoma tumors with I3A markedly inhibited tumor growth in a T cell-dependent manner. Furthermore, I3A combined with adoptive T cell (OT-I) therapy significantly enhanced therapeutic efficacy than individual treatment in B16-OVA tumor model. Overall, our results uncover a previously unknown link between tryptophan metabolism and tumor immunogenicity, and provide a potential combination therapeutic means for cancer treatment by combining I3A treatment with adoptive T cell therapy.

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Patterns of immune evasion in primary and metastatic colorectal cancer

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Background: Metastases are a major cause of cancer-related deaths. Metastases often do not respond as well to immunotherapy as their primary tumors, which may be due to additional mechanisms of immune escape in metastastic cells. In our study, we aim to elucidate differences in the tumor microenvironment of metastases compared to their corresponding primary tumor. Special attention will be paid to defects of the antigen presenting machinery, T-cell abundance and the presence of tertiary lymphoid structures.
Materials and Methods: The analyzed cohort includes a total of 68 patients, 28 matched pairs of samples from liver metastases and primary tumors (colorectal and esophageal/stomach), 15 samples from non-metastasized colorectal carcinomas and 15 from hepatocellular carcinomas. Peripheral blood mononuclear cells (PBMCs) and healthy mucosa or liver tissue were included as additional controls. Composition maturation and differentiation of lymphocyte subsets and expression of immune-regulatory molecules were assessed by flow cytometry. Furthermore, immunohistochemical (IHC) staining and digital pathology (CD3, CD8, CD20) was performed to calculate an immune-score and to quantify tertiary lymphoid structure (TLS) abundance. 3'RNA sequencing and IHC were performed to elucidate defects of the antigen-presenting machinery in metastasis and the corresponding primary tumor.
Results: We found several differences of the immunological infiltrate in the metastases compared with the corresponding primary tumor. Analysis of T-cell abundance in primary tumors and the corresponding metastasis revealed a lower T-cell infiltrate in metastasis. Preliminary FACS analyses show a significant decrease of $CD4^+$ T- fractions in metastasis infiltrating lymphocytes (MILs) compared to TILs. This reduction is not due to regulatory T cells. Furthermore, we found no differences in PD1 or PDL1 expression on T cells between TILs and MILs. We will present detailed analyses regarding expression of co-inhibitory molecules on lymphocyte subsets in the different compartment.
Conclusion: First results of our study demonstrate decreased immune cell abundance in the metastatic environment, and we aim to elucidate the underlying mechanisms of immune escape. Especially combination therapies aiming to upregulate antigen presentation within metastases appear promising for combined immunotherapy approaches.

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The role of IκB Kinase epsilon in malignant melanoma

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IκB Kinase epsilon (IKKε) is known as an important mediator of NFκB as well as the interferon type I signalling. Several studies showed that it plays a role in the pathophysiology of various cancer types like ovarian, pancreatic and breast cancer. Previous data of our group indicate that IKKε is also involved in the progression of malignant melanoma as well as in the tumour-associated pain in mice. Furthermore, immunofluorescence analyses of human primary melanoma revealed that IKKε is not only expressed in tumour cells but also in immune cells in the tumour microenvironment, in particular in tumour-infiltrating T-cells. So far, the exact role of IKKε in the immune response in melanoma is not finally explained. Therefore, we want to study in detail the function of IKKε in the

immune regulation during melanoma progression. In our project, we apply different mouse melanoma models using wild type and IKKε knock-out mice as well as cell culture with primary T-cell subsets and B16BL6 melanoma cells, respectively. IKKε inhibition is accomplished either pharmacologically with the drug Amlexanox or by a stable knock-down.
Previous data of our group showed dysregulated levels of γδ-T-cells in different tissues of IKKε knock-out mice, e.g. in dermis and epidermis, thus suggesting that this special T-cell-subpopulation might play a role in IKKε-mediated immune responses which has not been described before. So, in the first step, we performed in vitro experiments with primary γδ-T-cells and found that pharmacological IKKε inhibition results in altered cytokine secretion after stimulation with PMA/Ionomycin and IL1/IL23, respectively.
Cell proliferation assays indicate that an inhibition of IKKε has an impact on the proliferation of mouse melanoma cells (B16BL6). In our mouse model, IKKε knock-out mice show reduced tumour proliferation compared to wild type mice. Over a period of 21 days, tumours grew more slowly. At the end of the experiment, tumour weight and volume were significantly lower in IKKε knock-out mice. To study the immune response in the tumour model, we performed immunofluorescence staining with various immune cell markers e.g. for T-cells and macrophages as well as FACS analysis to calculate differences in immune cell subpopulations that are involved in the tumour microenvironment. The results showed that immune cell composition is altered by deletion of IKKε. Taken together, our current data indicate that IKKε has an impact on T-cell responses as well as on the tumour progression in melanoma models in mice. This might be at least partially due to an altered immune response in the tumour microenvironment which, however, must be proven in a number of further experiments.

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Establishment of a mixed tumor model to study the therapeutic potential of an immune-modulatory cargo-expressing oncolytic virus

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Oncolytic virus (OV) therapy is an emerging anti-cancer approach that uses viruses to eliminate cancer cells due to their impaired antiviral defenses making them selectively susceptible for infections and subsequent killing. Besides direct killing, the viral oncolytic activity releases tumor-associated antigens, which are taken up by antigen presenting cells and stimulate the immune responses that can eliminate remaining tumor cells. OVs can be armed with immunomodulatory cargos (IMCs) to further support anti-tumor immunity induced by OV therapies. The vesicular stomatitis virus (VSV), pseudotyped with the glycoprotein (GP) of the lymphocytic choriomeningitis virus (LCMV), called VSV-GP represents a potent OV. Despite defective intrinsic type I IFN pathways and good *in vitro* susceptibility of the most syngeneic mouse tumor models, the strong systemic type I IFN responses induced by VSV-GP therapies limit the susceptibility of tumors to VSV-GP infection *in vivo*. This in turn not only reduce oncolytic capacity but also limits the production of immune-modulatory cargos (IMCs) encoded by the virus making difficult to evaluate potential therapeutic efficacy.

To overcome this limitation, we established a tumor model by mixing transformed murine lung epithelial cells (TC-1) expressing HPV-derived oncoproteins (E6 and E7) and the VSV-GP permissive IFNalpha-receptor knock-out TC-1 (TC-1-IFNaRko) cells. Using this mixed tumor model, we investigated the efficacy as well as the CD8+ T cell responses after treatment with VSV-GP and VSV-GP-IMC. CD8+ T cell responses were assessed by multicolor flow cytometry.
Whereas oncolytic virotherapy of the original TC-1 did not result in improved tumor control, virus treatment showed a prolonged survival in the mixed tumor model. More importantly, we found an enhanced therapeutic efficacy using VSV-GP-IMC compared to VSV-GP treatment. This together with the phenotypic differences of activated CD8 T cells between VSV-GP and VSV-GP-IMC treatment supports an improved effect of IMC in the mixed tumor model.
Thus, the TC-1/TC-1-IFNaRko experimental mixed tumor mouse model might be suitable for studying IMCs in context of oncolytic virotherapies utilizing type I IFN sensitive viruses.

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PD-1 and TIGIT expression delineate distinct functional T cell subsets with prognostic relevance in pancreatic cancer

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Background: Immunotherapy has led to a fundamental shift in the treatment of several cancers. However, its efficacy in pancreatic ductal adenocarcinoma (PDAC) is limited. Understanding the expression of inhibitory immune checkpoint receptors (ICR) by intratumoral T cells may help to unravel their involvement in insufficient T cell-mediated antitumor immunity.
Experimental design**:** We analyzed T cells from blood (n = 143) and matched tumor samples (n = 106) of PDAC patients using multicolor flow cytometry. The expression of ICRs by $CD8^+$ T cells, conventional $CD4^+$ T cells (Tconv) and regulatory T cells (Treg) was analyzed. Further, T cell differentiation, tumor reactivity and cytokine production were investigated as a function of ICR expression. A comprehensive follow-up was used to determine the prognostic value.
Results: Intratumoral T cells highly expressed PD-1 and TIGIT. Both markers delineated distinct T cell subpopulations. $PD\text{-}1^+TIGIT^-$ T cells highly expressed proinflammatory cytokines (IFN-γ, TNF-α, IL-2, and IL-17a), and markers of tumor reactivity (CD39, CD103), whereas TIGIT expression was linked to an antiinflammatory and exhausted phenotype. The presence of intratumoral $PD\text{-}1^+TIGIT^-$Tconv was associated with improved clinical outcomes, while high ICR expression on blood T cells was a

significant hazard for overall survival.
Conclusions: Our results display the association between ICR expression and T cell functionality in PDAC. PD-1 and TIGIT characterized intratumoral T cells with highly divergent phenotypes linked to clinical outcomes, further underscoring the potential of TIGT as a target for immunotherapies in PDAC. ICR expression in the blood may be a valuable tool for the stratification of PDAC patients.

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Oncolytic viruses alter the biogenesis of tumor extracellular vesicles and increase their immunogenicity

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Oncolytic viruses preferentially infect tumor cells and induce their immunogenic death, leading to the activation of an anti-tumor immune response. Extracellular vesicles (EVs) are produced by all cell types and mediate intercellular communication. Tumor EVs modulate the activity of both neighboring and distant cells, and are commonly associated with phenomena such as metastasis, immunosuppression or drug resistance. Viral infections usually increase EV secretion, but little is known about the effect of oncolytic viruses on the biology of tumor EVs. In this study, we aimed at understanding how oncolytic viruses influence the secretion and functions of tumor EVs. We infected human melanoma and thoracic cancer cell lines with different oncolytic viruses (poxviruses, paramyxoviruses, rhabdoviruses). After isolation by differential ultracentrifugation, we characterized EVs in terms of concentration, morphology and protein content. We used a CRE-based recombination assay to study internalization and release of EV cargo from infected cells. Finally, we assessed the immunogenicity of tumor EVs in a coculture model with anti-tumor $CD8^+$ T cell clones. We show that EVs secreted by infected tumor cells are more abundant and are enriched in proteins encoded by interferon-stimulated genes, such as class I MHC. EVs mimicking those from infected cells transfer their content to recipient cells more readily than those from uninfected cells. Importantly, $CD8^+$ T cells incubated with EVs from infected cells display enhanced cytotoxic functions against tumor cells. Overall, our results suggest that part of the therapeutic activity of oncolytic viruses could be mediated by a modification of tumor EV protein content, which would have an influence on the anti-tumor immunity.

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Three-dimensional (3D) bioprinting: new models in cancer immunotherapy

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Traditional two-dimensional (2D) cell culture where cells spread and grow on a flat surface is still widely used in cancer biology research. Despite the fact that 2D culture is inexpensive and easy to setup, it faces many disadvantages. For example, it poorly reflects the physiological conditions of

tissues and provides far less predictive values in drug screening. Three-dimensional (3D) cell culture has potential to overcome these pitfalls. 3D bioprinting, one of 3D cell culture methods, is an emerging technology with various advantages, as 3D bioprints resemble tissue structure, microenvironment and function, thereby providing realistic measurements of drug efficacy and toxicity. In this study, we 3D bioprinted colorectal cancer cells in alginate-based hydrogel and showed that tumour cells form tumour-like structures that resemble pathomorphological features. Furthermore, we were able to observe migration of peripheral blood mononuclear cells (PBMCs) toward cancer cells, when printing PBMCs cells as a layer beside the tumour cell layer. Adding a blocking antibody for a chemokine, we could study modulatory effects in various established 3D bioprinting models.

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Can we predict which TIL products will react against NSCLC tumours based on the immuneinfiltrates?

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Background: Adoptive transfer of tumour infiltrating lymphocytes (TIL therapy) has shown high efficacy in a phase III trial for melanoma patients and in phase I trials for other solid cancers. However, not all patients respond well to TIL therapy. Good prediction tools would help to select which patients may benefit most. The tumour microenvironment is infiltrated by different types of lymphoid and myeloid cells, which communicate with each other. We, therefore, hypothesized that the presence of specific immune cell types may explain the variation in TIL products.
Methods: To define tumour-specific alterations of immune infiltrates, we characterized the myeloid and lymphoid cell populations present in fresh tumour lesions and healthy adjacent tissue from 26 early-stage and 20 late-stage NSCLC patients by flow cytometry. TIL products were generated according to the clinical expansion protocol and tumour reactivity was determined based on cytokine production upon co-culture with the autologous tumour digest. Flow cytometry data were analysed in an unbiased fashion using Cytotree, and Spearman's Rank Correlation was used to correlate immune infiltrates with the expansion rate and percentage of polyfunctional T cells.
Results: The majority of immune cells in early-stage and late-stage tumour lesions included lymphoid cells (Early-stage: T cells 49%, B cells 13%, NK(T) cells 3%; Late-stage: T cells 48%, B cells 10%, NK(T) 4%), which were increased compared to the healthy adjacent tissue (T cells 33.6%; B cells 2.6%; NK(T) cells 5.7%). This increase was due to monocytic and dendritic cell infiltrates, which were decreased in tumour tissue (Early-stage: 9%; Late-stage: 5.2%) compared to healthy adjacent tissue (18%). None of the immune infiltrates correlated with the expansion rate of TILs. However, we

observed that specific immune infiltrates correlated with anti-tumour response rates. Neutrophils in tumour lesions positively correlated with the percentage of CD137 expression on CD4 TIL products after co-culture with autologous tumour digest. Interestingly, B cells negatively correlated with the percentage of cytokines expressed by both CD4 and CD8 T cells from the TIL products.
Conclusion: We found that specific immune infiltrates in the tumour tissue are associated with the functionality of TIL products, which may help select TIL products with highly responsive T cells against NSCLC tumours.

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Single-cell profiling and validation of tumor-specific TCR repertoires to study immune escape in Small cell lung cancer (SCLC) patients

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Despite several advances made, lung cancer remains the leading cause of cancer-related death. Our goal is to identify neoantigen-specific T cell receptors (TCRs) and employ them in adoptive T cell immunotherapy to study immune escape mechanisms in Small cell lung cancer. For this purpose, we plan to analyze at single-cell-resolution transcriptome (scRNA seq) and paired TCR α/β chain profiles (scTCR seq) of tumor-infiltrating lymphocytes (TILs) obtained from biopsies of individual naïve patients. Deploying the advantage of scTCR seq data, we are able to obtain matching αß TCR chains, which would have been lacking with bulk TCR seq profiles. TIL expansion with co-culture of autologous tumor cell lines is envisioned to allow specific expansion and enrichment of tumor-reactive TCRs. Comparative TCR repertoire profiling will support the identification of these tumor-reactive T cells. Relevant TCRs cloned from potential tumor-reactive TILs will be reconstituted in reporter T cells and analyzed for recognition and killing of autologous cell lines using a Jurkat-NFAT assay. Further, corresponding neoantigens predicted from mutations will be identified using ELISpot. So far, we observe that TIL expansions from patient biopsies are affected by various factors such as IL-2 concentration, number of days the TILs are expanded and the initial infiltration of immune cells in the biopsies. The TIL expansion results in a high expression of exhaustion markers and hyper-expanded clones in especially CD4+ T cells. With the scTCR seq profiles, we are able to observe overall clonality of the TILs at various time points. We observe that the clonal homeostasis is similar to TILs expanded for shorter duration than those expanded for 21 days. With our data analysis pipeline we are also able to integrate, gene expression with TCR profiles and identify the phenotype of the cells with a TCR of interest. For our future direction, once confirmed for their neoantigen-specificity, relevant TCRs will be used to trace back the frequency, distribution and transcriptome profile of neoantigen-reactive T cells in patient samples, as well as to study the clonality and expression of recognized mutations in tumor samples and engrafted cell lines. In a closely connected follow-up PhD project, T cells transduced with tumor-reactive TCRs will be used for *in vitro* and *in vivo* assays to study the mechanisms underlying outgrowing immune escape variants.

These studies will help generate insights into T cell immune recognition and escape in lung cancer patients.

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Melanoma-derived IκBζ as a key modulator of the tumor microenvironment and therapy response

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IκBζ, encoded by *NFKBIZ*, is an inducibly expressed co-factor of NF-κB, which can either induce or repress a subset of NF-κB target genes, such as *IL1B*, *IL6*, *CXCL8,* or *CCL20*. Whereas the role of immune cell-derived IκBζ as a key regulator of pro-inflammatory responses is well established, its role in cancer has only been rarely investigated yet. For example, in activated B-cell-like subtype of diffuse large B-cell lymphoma (ABC-DLBCL), IκBζ is constitutively expressed thereby upregulating IL-6 and IL-10 expression levels, which in turn enhances tumor growth and survival. However, the role of IκBζ in solid tumors remains unknown though.
In an unbiased screen, we found that the IκBζ protein is constitutively expressed in a subset of melanoma cell lines and patient samples. Of note, IκBζ expression levels did not correlate with known driver mutations in melanoma. Instead, constitutive IκBζ expression depended on transcriptional and post-translation events. Thus, as IκBζ is known to regulate the expression of key cytokines and chemokines, if expressed, we hypothesize that tumor-derived, constitutive IκBζ expression shapes the tumor microenvironment (TME) by transcriptionally regulating the recruitment and activation of associated immune cells. To reveal the functional consequences of constitutive IκBζ expression in melanoma, we depleted IκBζ in IκBζ-expressing melanoma cell lines (LOX-IMVI and D4M-3A) or overexpressed IκBζ in an IκBζ non-expressing cell line (MV3). Subsequently, we performed transcriptome analyses, which showed that melanoma-derived IκBζ induced the expression of several immune cell-modulating chemokines (e.g. *CXCL1* or *CXCL8*) or pro-proliferative cytokines, such as *IL1B* or *IL6*. Altogether, these findings suggest, that IκBζ affects the recruitment and activation of tumor-associated myeloid cells and T cells, thus modulating the TME and tumor growth. To validate this hypothesis, we performed *in vivo* experiments by subcutaneously injecting murine Braf mutant, Pten loss D4M-3A melanoma cells into C57BL/6N mice. While control tumor cells quickly proliferated, IκBζ-knockout tumors started to grow but stopped growing at day 7 post-injection. This was possibly due to a decreased cytokine and chemokine expression, as well as a detectable upregulation of MHC class I expression on IκBζ-depleted tumors, thus leading to a modulation of the TME. In agreement, we detected increased numbers of tumor-infiltrating macrophages, neutrophils, and T cells in the TME of IκBζ-depleted tumor cells.
Together, these results imply that abrogation of IκBζ expression in melanoma might (re)-sensitize melanoma for immunotherapy by increasing the recruitment and activation of especially cytotoxic T cells. Concluding, our data suggest that melanoma-derived IκBζ represents an interesting prognostic marker for patients and a new target for future therapeutic approaches in melanoma.

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Targeting of the immune suppressive tumor milieu by pan-functional miRNAs

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Immunotherapy based treatment approaches have tremendously improved clinical outcome in cancer patients. However, immunotherapy is not effective in all patients, because tumor cells can evade the immune system via so-called escape mechanisms. Thus, elevated expression of immunosuppressive checkpoint molecules (**ICMs**) on the surface of cancer cells leads to functional inhibition of tumor-reactive T and NK cells. Furthermore, the tumor microenvironment (TME) itself represents an immunosuppressive habitat. Tumors are often heavily infiltrated by regulatory T cells and by tumor associated macrophages (**TAMs**) with anti-inflammatory (M2-like) capacity. Thus, current immunotherapy approaches should be aimed at neutralizing the immunosuppressive TME to (re-)activate efficient anti-tumor immune responses. Based on a human miRNA library screen, we identified numerous miRNAs capable of inhibiting ICM surface expression on SK-Mel-28 and MDA-MB-231 cells. In parallel, we determined miRNAs highly expressed in pro-inflammatory human macrophages. Importantly, we discovered one miRNA, which reduced the expression of two ICMs, NT5E and CD274 in various cancer cell lines. Moreover, the same miRNA was found highly upregulated in both human and murine proinflammatory macrophages, and its expression drove re-polarization of anti-inflammatory macrophages. In addition, this miRNA induced upregulation of tapasin expression in cancer cells, thereby potentially increasing antigen processing and presentation, which might facilitate detection and elimination of tumor cells by the cellular immune system. We are currently establishing cationic Lipid-Nanoparticles (LNPs) for miRNA encapsulation and subsequent tumor delivery. *In vitro*, these **LNP-miRNAs** could reduce ICM expression on human and murine tumor cell lines. Next, this LNP-miRNA will be applied to tumor bearing mice to inhibit ICM expression on tumor cells and induce functional TAM repolarization *in vivo*. This strategy might help to reverse the immune-suppressive TME and facilitate efficient tumor attack by activated immune cells thereby improving the success of future immunotherapy approaches.

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Overcoming immune checkpoint therapy resistance by G-quadruplex stabilization

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In the past decade, checkpoint blockade has revolutionized cancer therapy and improved long-term survival for many patients. However, even in well-responding tumor entities about 50% of treated patients do not respond or their tumors develop resistances. One key feature of non-responding tumors is being immunologically “cold”, meaning they only contain a small number of tumor-suppressing lymphocytes. Thus, enhancing the infiltration of tumor-suppressing immune cells is a

promising approach to improve response rates and treatment outcome of checkpoint blockade. In this study, we injected Pyridostatin (PDS), a small molecule that binds secondary DNA structures called G-quadruplexes, intratumorally into B16-OVA tumor-bearing mice. By stabilizing G-quadruplexes, PDS causes DNA damage, micronuclei formation, telomere destabilization, downregulation of oncogenes, upregulation of T cell-attracting chemokines, and apoptosis in cancer cells.
Following intratumoral injection of PDS, we observed significantly decreased tumor growth, prolonged survival and successful checkpoint blockade. This effect was facilitated by the upregulation of pro-inflammatory chemokines within the tumor microenvironment, leading to significantly increased infiltration of NK and cytotoxic T cells (CTLs). We also observed that infiltrating NK cells and CTLs of PDS-treated tumors produced significantly more IFN-γ and Granzyme B. Overall, our results suggest that G-quadruplex stabilization via PDS treatment harbors the potential to induce intratumoral inflammation and sensitize non-responding tumors for checkpoint blockade.

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The immunopeptidome landscape associated with T cell infiltration, inflammation and immune-editing in lung cancer

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A key barrier for improving efficacy of cancer immunotherapy remains patient stratification. Patients with tumors characterized with high infiltration of $CD3^+CD8^+$ T cells, typically show better response to immune checkpoint inhibitors than patients with non-infiltrated tumors. In contrary, for other advanced personalized therapies that are dependent on the tumor antigenic landscape, such as neoantigen cancer vaccines and adoptive transfer of neoantigen-enriched T cells, stratifying biomarkers are not well defined. Furthermore, it is still unknown if the repertoire of HLA-bound peptides presented in T cell infiltration or excluded tumors is substantially different.
To capture the complex interplay between the tumor antigenic landscape and anti-tumor immunity in lung cancer, we surveyed 61 tumor regions and adjacent non-malignant lung tissues from eight lung cancer patients and performed deep antigen discovery combining immunopeptidomics, genomics, bulk and spatial transcriptomics and explored the heterogenic expression and presentation of tumor (neo)antigens. The large repertoire of HLA-I and HLA-II identified peptides, allowed us to deeply study immunopeptidomes and the antigenic landscape of $CD3^+CD8^+$ T cell excluded and infiltrated tumors. As HLA-II complexes were not expressed directly on the malignant cells in the majority of cases, we associated diverse immune cell populations with the HLA-II immunopeptidome and identified a panel of source proteins whose presentation is associated with $CD3^+CD8^+$ T cell infiltration. Interestingly, $CD8^+$ and $CD4^+$ cells were represented in the HLA-II immunopeptidome predominantly in their activated states in $CD3^+CD8^+$ T cell infiltrated tumors, while the presentation of activated B cells and DCs was associated with overall high inflammation. Surprisingly, higher presentation efficiency of tumor-associated antigens was found in $CD3^+CD8^+$ T

cell excluded tumors. In addition, a significantly higher frequency of predicted neoantigen sequences within HLA-I presentation hotspots was detected in the excluded tumors, potentially due to the absence of immune-surveillance. We demonstrated that the probability to induce spontaneous $CD8^+$ T cell responses against mutations located in HLA presentation hotspot was about 3 fold higher compared with mutations located in non-hotspot regions. Accordingly, in our cohort, the relative immunogenicity of tumors was higher in $CD3^+$ $CD8^+$ T cell excluded tumors than in T cell infiltrated tumors. We concluded that accumulation of mutations in presentation hotspots reflects limited immune pressure and that even when low in number, neoantigens in $CD3^+CD8^+$ T cell excluded tumors have potentially a better chance to be presented to T cells. Therefore, we propose that the choice of combination therapies should be tailored to the patient tumor's mutanome and the immune-microenvironment.

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Functional role of tumor-specific B-cell responses in preclinical models of immune checkpoint therapy

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Immune checkpoint inhibition (CKI) demonstrated breakthrough therapeutic efficacy in a variety of cancer types. Enhancement or de-novo induction of tumor specific T cell responses is assumed as major mode of action, but other lymphatic subpopulations also contribute to the efficacy of CKI. We have shown that B cells mediate immune response in gastrointestinal cancer and recent data suggest an important role of B cells for susceptibility of cancer patients to CKI.
To advance our understanding of B cell specific mechanisms in the setting of CKI, we used three different mouse models. In the subcutaneous PancO2-OVA tumor model, mice received anti-PD-1 treatment at onset of tumor appearance, which resulted in a significant increase of OVA-specific B cells and secretion of OVA-specific antibodies. This was accompanied by an increase of activated $CD86^+$ B cells and increased numbers of T follicular helper cells. For optimal mimicry of the major types of gastric adenocarcinomas, we used two recently developed orthotopic transgenic mouse models, which express a tamoxifen-inducible Cre-recombinase in the stomach-specific Anxa10 locus. Additionally, the genomically stable (GS) cancer model carries alterations in pathways $Cdh1^{fl/fl}$; $Kras^{G12D/+}$ and $Smad4^{fl/fl}$ resulting in a slow progressing tumor growth as observed by magnetic resonance imaging (MRI). The chromosomally instable (CIN) model contains mutations of $Kras^{G12D/+}$; $Tp53^{R172H/+}$ and $Smad4^{fl/f}$ and showed a more rapid, intestinal tumor growth. In both models, dysplasia of stomach tissue occurred two weeks after tamoxifen induction, which is apparent by a thickening of the stomach wall in MRI. Metastases of liver, lymph nodes and lungs were observed at later stages. We thoroughly characterized those tumor models with regard to their immune phenotype and detected B-cell clusters in the tumor microenvironment indicating the presence of tertiary-lymphoid structures, which is usually not the case in mouse models of cancer. Anti-PD1

treatment was conducted at early (2 weeks) and midterm stages (8 weeks) of cancer development, but in contrast to the subcutaneous Panc02-OVA model did not result in a significant reduction of tumor growth. However, we observed increased percentages of memory B cells, germinal center B cells and activated $CD86^+$ B cells, thus underlining the role of B cells in CKI.

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Abstract has been withdrawn

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Enhancing Predictive Performance of pMHC class I Presentation Models through Multi-Allelic Data Integration Techniques and Comparative Analysis of Architectural Choices

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Personalized cancer immunotherapies have emerged as a promising approach for cancer treatment by leveraging the ability of the immune system to recognize and eliminate cancer cells. A critical challenge in this field is predicting the presentation of peptide-MHC complexes (pMHCs) to elicit an effective immune response. In recent years, several machine learning-based approaches, such as NetMHCPan4.1, or MHCFlurry2.0, have been developed to model pMHC class I presentation. While most studies compare different approaches as tools, i.e. pre-trained models trained on different data, in this study, we focus on a fair comparison of different model architectures re-trained on the same data. This allows us to determine the optimal architectural approach for pMHC presentation prediction. In this work, we also introduce a new Transformer-based architecture for MHC class I presentation prediction that is adapted to handle binding affinity data and both single- and multi-allelic eluted ligand data from mass-spectrometry experiments. We particularly investigate different strategies to integrate multi-allelic data, including the one-shot deconvolution with our best single-allelic-only model, the iterative deconvolution of the multi-allelic samples, or the modification of our architecture to learn the deconvolution through the training process using techniques from the multiple instance learning paradigm.The proposed model substantially outperforms NetMHCPan4.1, a widely used public baseline, on both the NetMHCPan4.1 evaluation set and a proprietary dataset of MS ligands data (4.6% and 18.5% on per-genotype mean PPV), without using any additional data beyond the original NetMHCPan4.1 training set. This extensive comparative analysis disambiguates the contribution of architectural choices from the gain provided by additional training data, and, ultimately, leads to improved machine learning predictors for pMHC presentation.

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IL-17A-producing CD8$^+$ T cells promote PDAC via induction of inflammatory cancer-associated fibroblasts

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Objective: Pancreatic ductal adenocarcinoma (PDAC) is characterized by a highly desmoplastic stroma composed of cancer-associated fibroblasts (CAF) and interspersed immune cells. Non-canonical CD8$^+$ T cell subpopulation producing IL-17A (Tc17) promote autoimmunity and have been identified in tumors. Here we evaluated the Tc17 role in PDAC.
Methods: Infiltration of Tc17 cells in PDAC tissue was correlated with patient overall survival and tumor stage. WT or *Il17ra*$^{-/-}$ quiescent pancreatic stellate cells (qPSC) were exposed to conditional media obtained from Tc17 cells (Tc17-CM); moreover, co-culture of Tc17-CM-induced inflammatory (i)CAF (Tc17-iCAF) with tumor cells was performed. IL-17A/F-, IL-17RA-, RAG1-deficient and *Foxn1*$^{nu/nu}$ mice were used to study the Tc17 role in PDAC mouse models.
Results: **I**ncreased abundance of Tc17 cells was associated highly significant with reduced survival and with advanced tumor stage in PDAC. Tc17-CM induced iCAF differentiation as assessed by the expression of iCAF-associated genes via synergism of IL-17A and TNF. Accordingly, IL-17RA controlled the responsiveness of qPSC to Tc17-CM. Pancreatic tumor cells co-cultured with Tc17-iCAF displayed enhanced proliferation and increased expression of genes implicated in proliferation, adhesion, metabolism and protection from apoptosis. Tc17-iCAF accelerated growth of mouse and human tumors in *Rag1*$^{-/-}$ and *Foxn1*$^{nu/nu}$ mice, respectively. Finally, IL-17RA-expressed by fibroblasts was required for Tc17-driven tumor growth *in vivo*.
Conclusions**:** Here, we identified Tc17 as a novel pro-tumorigenic CD8$^+$ T-cell subtype in PDAC, which accelerated tumor growth via IL-17RA-dependent stroma modification. We described a crosstalk between three cell types, Tc17, fibroblasts, and tumor cells, promoting PDAC progression, which results in poor prognosis for patients.

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Tumor cell-secreted chemokines shape the immunosuppressive myeloid compartment in PDAC models

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In recent years, immune therapies have revolutionized treatment opportunities for cancer patients. However, current immunotherapies often fail to improve outcomes for patients with solid tumors. The development of an immune suppressive tumor microenvironment (TME) is considered the main reason for ineffective immunotherapies. The TME that develops in patients with pancreatic

adenocarcinomas (PDAC) is highly complex and tumor infiltrating myeloid cells have been identified as important drivers of immunosuppression in PDACs. Besides tumor-associated macrophages (TAMs), other myeloid cells of monocytic or polymorphonuclear (PMN) origin are collectively termed myeloid-derived suppressor cells (MDSC). It is still not fully understood which tumoral factors trigger recruitment and enrichment of MDSC in the TME.
We systematically analyzed the migratory capacity of naïve or tumor-primed myeloid cells towards tumor-secreted factors. For this, we have characterized the chemokine secretion of over 80 PDAC tumor cell lines which were isolated from KPC ($Kras^{G12D}$ $Trp53^{fl/R172H}$ Ptf1a–Cre) - mouse model (kindly provided by Prof. D. Saur, TUM) by performing multiplex immunoassays. The chemotactic effects of the various secretomes on MDSC were studied by performing *ex vivo* chemotaxis assay with isolated MDSC from tumor-bearing and naïve mice. Multivariate regression models were used to determine the chemokines with highest impact on polymorphonuclear (PMN-) MDSC and monocytic (M-) MDSC migration. To study the effect of the PDAC heterogeneity on the myeloid compartment *in vivo*, we used a syngeneic orthotopic PDAC mouse model and characterized immune cell expansion and infiltration in the blood, spleen and tumors by flow cytometry.
We found great heterogeneity in the secreted chemokines in different PDAC cell line secretomes. Interestingly, cluster analysis revealed that distinct secretion profiles were correlated with a specific tumor cell morphology (epithelial vs mesenchymal) that resulted in either PMN-MDSC-dominant or M-MDSC/TAM-dominant tumor microenvironments. Our results suggest that tumor-intrinsic chemokine secretion is the dominant factor for shaping the myeloid cell compartment in the TME. Both, M-MDSC-dominant and PMN-MDSC-dominant TMEs seem to be functionally redundant in their ability to suppress antitumoral immune responses but may need individual therapeutic strategies to overcome immunosuppression.

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Dissecting the phenotype, clonality and function of tumor-reactive NK cells in response to immunotherapy

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Immunotherapies have accelerated the progress of cancer treatments with the continuous effort to improve clinical efficacy and reduce side effects. Combination therapies targeting different immune compartments are frequently utilized to increase tumour control, prevent relapse and prolong survival. Together with T cells, natural killer (NK) cells are cytotoxic effectors fighting against cancer cells owing to their cytotoxicity and cytokine production. They serve an important role in case of MHC-I downregulated tumors, which evade T-cell immunity. Our lab's recent work demonstrated the bridging role of NK cells with tissue-resident traits in the tumor to promote adaptive anti-tumor immunity in response to IL-12 therapy. Nevertheless, the phenotype, clonality and function of tumor-reactive NK cells in tumor-bearing mice are largely unknown. Using multi-color flow cytometry, we demonstrated a high degree of heterogeneity among NK cell populations in terms of receptor expression, maturation, as well as cytokine and chemokine profiles in different solid tumor models. We are establishing a mouse tumor model with the overexpression of an activating ligand (Rae-1g) in

order to study tumor-specific NKG2D+ NK cell populations. In parallel, a single-cell fate-mapping model will be setup to study the migration and clonality of NK cells in peripheral blood, tumor and lymph nodes of tumor bearing mice. Ultimately, single cell RNA sequencing and functional studies will be performed to dissect the transcriptional composition and anti-tumor activity of tumor-specific NK cells from tumor bearing mice with and without IL-12 therapy treatment. The understanding of NK cell heterogeneity, function and their differentiation process in the solid tumor context will pave ways for further studies to improve NK-targeted immunotherapies.

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Glioma-induced neuronal remodeling promotes regional immunosuppression

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Glioblastoma cells remodel pre-existing neuronal circuits and take advantage of them for their proliferation and invasion. Moreover, our prior study demonstrated that a subpopulation of synaptogenic malignant cells expresses Thrombospondin-1 (TSP1, encoded by the *Thbs1* gene), thereby maintaining intra-tumoral neuro-functional connectivity. However, the effects of neuronal connectivity on the local immune environment remain to be elucidated. Understanding the functional significance of immunosuppression within functionally connected intratumoral regions may uncover therapeutic vulnerabilities. Here, through single-cell RNA sequencing analyses of clinical glioblastoma samples, we discovered that remarkably downregulated immune signatures colocalized with the intra-tumoral areas with high-functional connectivity characterized by upregulated *THBS1*. Using an SB28 murine glioma cell line with endogenous *Thbs1* stably expressed, we experimentally investigated glioma-neuronal-immune crosstalk through spatial transcriptomics, bulk RNA sequencing, and flow cytometry using preclinical syngeneic models. Unbiased gene expression program analysis using spatial transcriptomics for in vivo tumor-harboring mouse brains demonstrated negative co-localizations of neuro-synaptic activities (represented by the “Neuronal System [Reactome]” signature) and proinflammatory signatures (represented by the “TNF-α-via-NFκB [Hallmark]” signature). To investigate the significance of TSP1/*Thbs1*, we generated a CRISPR *Thbs1*-knock-out (KO) cell line. Bulk RNA-sequencing demonstrated that *Thbs1*-WT in vivo tumors exhibited gene expression programming consistent with synapse-associated genes and synaptogenic factors, recapitulating enriched connectivity in primary patient samples. Flow cytometry of brain-infiltrating leukocytes revealed that macrophages isolated from *Thbs1*-KO tumors were more frequently polarized into the proinflammatory “M1-like” phenotype (median M1/M2 ratio = 0.6 [WT] vs 1.34 [KO], $p < 0.006$). SB28-*Thbs1*-KO syngeneic models demonstrated slower tumor growth and significantly longer survival compared to *Thbs1*-WT counterparts (19 days [WT] vs 25 days [KO], $p <$ 4.5E-5). The survival difference was abrogated in the B6-SCID immunodeficient mice, indicating the critical role of adaptive immunity in the survival advantage associated with TSP1 downregulation.

Altogether, our results identify previously unrecognized immunosuppression mechanisms induced by glioma-neuronal circuit remodeling. Future therapeutic strategies targeting this glioma-neuronal-immune crosstalk may open up new avenues for glioblastoma immunotherapy.

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Immunomodulation induced by Interleukin-1 inhibition activates T cells and dendritic cells in colorectal cancer

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Colorectal cancer ranks as the third most common and the second deadliest cancer worldwide. Although early stages can be cured, recurrence and drug resistance are frequent phenomena demonstrating the high demand for new therapeutic options, especially in advanced metastatic settings. Therapeutic outcome is influenced strongly by the tumor immune microenvironment. Depending on the tissue context, inflammation can promote anti-tumor immunity on the one hand, but also tumor growth on the other hand. Here, we explore the functional role of the pro-inflammatory cytokine Interleukin-1 (IL-1) and therapeutic implications of its inhibition for colorectal cancer therapy, with a focus on liver metastases.
To maximize translational value, we apply a fully human pre-clinical explant model system. Explants are patient-derived and therefore contain the unselected set of cells that recapitulate both cellular context and tumor microenvironment of a patient, while simultaneously reflecting patient heterogeneity. To examine the effects of IL-1 inhibition, explants are treated with the clinically approved IL-1 receptor antagonist Anakinra (r-metHuIL-1ra, Kineret, Swedish Orphan Biovitrum) and analyzed by immunohistochemistry and multiplex cytokine profiling.
Multiplex cytokine profiling revealed different cytokine modulation patterns between primary and metastatic sites in response to IL-1 inhibition. Interestingly, there was a mutual increase in IFN-gamma originating from T cells and dendritic cells. Immunohistochemical staining on liver metastases confirmed an expansion of both $CD3^+$ T cells and DC-$Lamp^+$ dendritic cells in tumor areas, which was absent in the adjacent liver. Further, IL-1 inhibition yielded remarkable results in a prospective case of an advanced colorectal cancer patient with several liver metastases. The patient had already received all standard of care treatments and then received Anakinra monotherapy and subsequent single application of one cycle FOLFIRI plus Bevacizumab. The latter being a treatment the patient had received previously, but without response. With Anakinra however, two liver lesions responded and one progressed.
Taken together, these preliminary results indicate that IL-1 inhibition is a promising, feasible and safe treatment approach for advanced metastatic colorectal cancer patients. Explant data demonstrate that inhibiting IL-1 modulates both the T cell axis and the dendritic cell axis with a specific effect on

tumor tissue. The cytokine profile points towards a more advantageous immune landscape with an enhanced anti-tumor capacity. This is confirmed by the responding lesions in a prospective advanced metastatic case.

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Abstract has been withdrawn

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Mapping the TIGIT Axis in Pancreatic Cancer: Insights for Immunotherapy Targets.

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Pancreatic ductal adenocarcinoma (PDAC) remains one of the deadliest cancers with a five-year survival rate of less than 10%. Its resistance to therapy is strongly influenced by the unique tumor microenvironment (TME). This TME is characterized by a dense desmoplastic stroma consisting of cancer-associated fibroblasts, immune cells, and extracellular matrix components. This creates not only a physical barrier that hinders drug delivery and immune cell infiltration, but also fosters an immune suppressive environment. The emerging field of immune checkpoint inhibitors resulted in sustained anti-tumor response in several cancer types. However, PDAC patients enrolled in these clinical trials demonstrated no clinical benefit. Therefore, new and effective targets for novel immunotherapy combinations need to be sought. Our preliminary data, along with literature, suggest that TIGIT represents an emerging therapeutic target for the treatment of PDAC. However, the TIGIT axis is complex, including multiple inhibitory (e.g. TIGIT) and activating (e.g. DNAM-1) receptors expressed on various subtypes of immune cells, as well as different ligands (e.g. CD112 and CD155) expressed on both tumor cells and immune cells. Unravelling this complex axis in PDAC could provide crucial evidence for the development of future immunotherapeutic strategies. Therefore, in this study we mapped the complete TIGIT axis in 25 PDAC patient samples, from which we obtained both peripheral blood and tumor samples using a multicolour flow cytometry panel. On these matched samples we examined the expression of various receptors from the axis, including TIGIT, TACTILE, and DNAM-1 on immune cells of interest, as well as their co-expression with PD-1 and PD-L1. Additionally, we investigated the expression of the relevant ligands, such as CD111, CD112, CD113, and CD155, on tumor tissue slides using immunohistochemistry. Our initial findings revealed a significant decrease in the number of natural killer cells within the tumor tissue compared to peripheral blood samples. In contrast, regulatory T cells (Tregs) were found to be significantly increased in the tumor tissue. Additionally, these Tregs demonstrated a significantly higher TIGIT expression compared to circulating Tregs. Interestingly, we also found that the cytotoxic cells present in the tumor expressed significantly less DNAM-1 compared to their peripheral blood counterparts, suggesting potential immune suppression within the TME. Eventually, these results together with

immunohistochemistry data are currently being correlated with the patients' clinicopathological data to provide the necessary evidence for a rationally designed combination immunotherapy. Overall, these results will provide further insight into the complexity of the TIGIT axis in PDAC and highlight potential targets for future immunotherapeutic strategies.

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An Atlas and Compass of Immune-Cancer-Microbiome interactions in colon cancer

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Cancer progression is influenced by complex interactions between cancer cells, the immune system, and the microbiome. The lack of datasets containing genomic, immunological, and microbiological information with extensive clinical follow-up has made the identification of prognostic and predictive biomarkers challenging. In this study, RNA sequencing was performed on fresh-frozen colon cancer specimens from 348 patients, whole-exome sequencing of 562 samples, bacterial 16S-rDNA sequencing of 492 samples, and T-cell receptor profiling of 114 tumor samples. The Immunologic Constant of Rejection (ICR), a gene signature reflecting Th1-oriented immune activation, was found to have prognostic implications in colon cancer outperforming conventional prognostic molecular classifications, such as the consensus molecular subtypes. We demonstrated that an active immune tumor microenvironment in colon cancer is positively associated with a more clonal T-cell repertoire. This observation suggests that specific T-cell clones are expanded in colon cancer with a Th1 polarized immune response. We also explored associations between immune traits and the tumor-associated microbiome, and found that specific intra-tumoral bacteria were associated with improved survival. The combination of this bacterial composition with gene expression profiles that reflect immune activation identified patients with exceptional survival. This curated dataset and cancer-immune-microbiome metrics provide a valuable resource for gaining a better understanding of colon cancer immunobiology and the implementation of tailored interventions.

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Humanized mouse models for the preclinical evaluation of novel cancer immunotherapy options.

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Background: The preclinical evaluation of many novel immune therapies requires the use mouse models with a functional human immune system. In previous studies we have demonstrated that either peripheral blood mononuclear cells (PBMCs) or subpopulations of PBMCs such as T- and NK-cells or hematopoietic stem cells (HSC) can be used to establish a humanized immune system in a immunodeficient mouse system with functional T-, B-, and NK cells. By transplanting either cell-line or patient-derived tumor xenografts into humanized mice, we successfully generated a fully human tumor-immune-cell model for several tumor entities. Finally, we validated the functionality of these models using either immune-checkpoint inhibitors, cell therapies, or immune cell engagers.
Methods**:** HSC-humanized mice were generated by i.v. injection of CD34+ stem cells into immunodeficient NOG mice. PBMCs or enriched T- or NK-cell populations from a curated set of blood donors were used to humanize mice by either single or multiple i.v. injections. CDX and PDX models from different entities (i.e. lymphoma, neuroblastoma, and breast cancer) were transplanted into these humanized mice which were used to evaluate novel immune therapy options. The presence of immune cells and their activation status was analyzed by flow cytometry in blood and tumor samples.
Results: The hematopoietic stem cells (HSCs) engrafted after transplantation and established a functional human immune system with proliferating immune cells. Up to 20% of the human immune cells in the blood were functional T cells, characterized by high PD-1 expression fourteen weeks after HSC inoculation. Selected CDX and PDX tumors successfully engrafted on humanized mice without significant differences in tumor growth compared to non-humanized mice. Checkpoint inhibitor treatments induced tumor growth delay in selected models. Flow cytometry analysis of xenograft tumors revealed an increased percentage of tumor-infiltrating T cells. In addition, we identified a set of CDX and PDX models with concomitant injection of PBMC, T- or NK-cell preparations for the evaluation of immune cell engagers and other immune therapeutics.
Conclusions: We have established human tumor-immune-cell models of different entities using CDX or PDX in combination with different donor derived immune cell subsets as effector cells. We demonstrated successful engraftment of HSCs in immunodeficient mouse strains generating mice with a functional human hematopoiesis. These models have been used for preclinical evaluation of novel checkpoint inhibitors and immune cell engagers. Our human tumor-immune-cell models allow preclinical, translational studies on tumor immune biology as well as evaluation of new therapies, drug combinations and biomarker identification and validation.

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Subtype-specific immune interactions in Small Cell Lung Cancer indicate distinct vulnerabilites to immunotherapeutic approaches

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Small cell lung cancer (SCLC) is known as a tumor harboring high mutational load but most patients respond poorly to combined treatment of chemotherapy and ICI. With an overall survival rate of ca. 5 % and 60 % of patients shown systemic metastases, new therapeutic approaches are desperately needed. Tumor antigen-targeting RNA-vaccination shows a strong therapeutic efficacy in tumors with high mutational burden and represents a potential therapy for SCLC. Recent clinical data shed light on the role of neuroendocrine (NE) and non-neuroendocrine (Non-NE) subtypes, which seem to influence response to chemo- and immunotherapeutic treatment. Yet, there are no studies conducted exploring RNA vaccination as a potential therapeutic option keeping SCLC-specific subtypes and the underlining high tumor heterogeneity in mind.
Using cell line derive from a genetically engineered mouse model for SCLC, we established a transplantable, multimetastatic small cell lung cancer model as a fast and reliable tool to decipher the immune interaction with SCLC in distinct differentiation stages *in vivo*. In order to phenotype the tumor microenvironment of distinct subtypes, we examined infiltrating immune cells using flow cytometry. Additionally, spatial effects on immune infiltration in different subtypes and organs were examined using immunofluorescence staining. By engrafting cell lines in immunocompetent vs. immunodeficient mice, we aimed to determine the level of immunoediting the cell lines undergo during engraftment. Our data suggests that tumors expressing high level of neuroendocrine markers, seem to be less strongly edited by the immune system. The tumors further show low immune infiltration of T cells and macrophages, yet high levels of macrophages are recruited in close proximity of the tumor. SCLC tumors with low expression of neuroendocrine markers show in contrast to high-NE tumors high immune infiltrates and seem to underlie immunoediting in a stronger manner. The differences in immune infiltration in both subtypes imply a stronger response to RNA-vaccination therapy of Non-NE subsets of small cell lung cancer.
In summary, we provide a model that allows to examine subtype-specific analyses of SCLC metastases. Using this model we were able to compare immune cell subsets in different SCLC-specific subtypes. In future, we aim to use the model to generate therapeutic options for the treatment SCLC *in vitro* and *in vivo*.

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An unsurmountable immunologic barrier? A detailed view on the interaction of hypermutated colon cancer with autologous T cells

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Recently, immunotherapies revolutionized cancer treatment, but the proportion of patients actually benefitting is still limited. Reasons for therapy failure are ambiguous, thus underlining the need for an improved understanding of the mutual relationship between autologous T and tumor cells.
For our study, we selected two hypermutated colon cancer cases with retained HLA expression. Patient T cells were obtained from peripheral blood or tumor infiltrate and the proportion of exhausted as well as regulatory T cells was determined. In co-cultures, the interaction of respective cell populations was examined. Here, the degranulation capacity of T cells was tested in the following settings: (1) peripheral vs. tumor-infiltrating T cells; (2) untreated coculture vs. treated coculture (immune checkpoint inhibitors (ICI), inhibitor of proteinase inhibitor 9 (PI-9i)) and (3) untreated T cells vs. T cells stimulated with tumor-specific peptides.
Tumor-infiltrating T cells were dominated by $CD4^+$ T cells as well as a higher amount of exhausted T cells, explaining the less effective tumor cell recognition compared to peripheral T cells. With the amount of regulatory T cells not exceeding 8% in the analyzed T cell populations, immune suppression by inhibitory T cells seems, however, to be negligible. Of note, the highest relative degranulation was reached by peripheral T cells, which did not have contact to tumor cells before the degranulation assay. This highlights the immunosuppressive effects on the T cells induced by the autologous tumor cells. This was not ascribable to IL-10 secretion, but decreased levels of pro-inflammatory cytokines were observed in co-cultures. Unexpectedly, additional treatment with ICI did not restore anti-tumor immune activity. Stimulation with tumor-specific peptides clearly heightened amounts of degranulating cells, but even then, ICI could not further improve tumor cell recognition.
In final kill assays, unstimulated T cells were unable to eliminate tumor cells (3%). Here, ICI and PI-9i could improve killing (32 and 33%). Peptide-stimulated T cells were effective killers (77%), with only weak effects of ICI and PI-9i addition (both 83%). Most surprising, combining both classes of immune modifiers canceled out the anti-tumor effects of the single agents in both unstimulated (0%) and peptide-stimulated T cells (66%).
In summary, these results strengthen the supremacy of peptide-stimulated compared to unstimulated T cells. They further imply that for personalized immunotherapies, peripheral T cells could not only substitute for but even be superior to tumor-infiltrating ones. The unexpectedly low effects of the applied inhibitors (ICI and PI-9i) underline the importance of further research on tumor immune evasion strategies to overcome immunologic barriers erected by a given tumor.

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Abstract has been withdrawn

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Single-cell omics analyses identify the TIM-3 ligand Galectin-9 as novel immunotherapy target for chronic lymphocytic leukemia

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Failure of response to immunotherapy including checkpoint inhibitors or CAR-T-cell therapy in chronic lymphocytic leukemia (CLL) has been linked to dysfunctional effector T cells. Using single-cell analyses, we performed an in-depth characterization of the T cell compartment in blood and tissue samples of CLL patients and mouse models to gain insights into the spectrum of phenotypes and transcriptional programs of T cells and the underlying mechanisms of their development.
By mass cytometry (CyTOF) using 35 antibodies, we characterized T cells in blood (n=8), bone marrow (n=3), and lymph nodes (n=21) of CLL patients, as well as reactive lymph nodes of non-tumor patients (n=13). Integrative analyses of all data sets allowed us to identify and quantify 15 clusters of $CD4^+$ and 14 clusters of $CD8^+$ T cells. First, our data showed that T cells in blood and bone marrow are similar, but clearly distinct from lymph node-derived cells. Second, we observed an accumulation of several regulatory T cell subsets, as well as exhausted and precursor exhausted T cells in the lymph node samples, which showed a positively correlated abundance. Third, an increased frequency of regulatory and exhausted T cells was detected in CLL in comparison to non-malignant lymph nodes.
We further performed single-cell RNA-sequencing generating transcriptome and T cell receptor (TCR) data of T cells from lymph nodes of CLL patients (n=5) and spleen samples of the Eμ-TCL1 mouse model of CLL (n=2). These data confirmed the presence of several exhausted T cell clusters in CLL lymph nodes and spleens of Eμ-TCL1 mice, and allowed for the identification and transcriptional characterization of terminally exhausted T cells and their precursor state. By integrating single-cell TCR data, a clonal expansion mainly of the precursor exhausted T cells was observed, suggesting their reactivity for CLL cells.
Finally, we used the single-cell transcriptome data for interactome analyses and identified both known and novel ligand-receptor-interactions between CLL and different T cell clusters with a suggested function in supporting CLL cell growth or suppressing T cell activity. Among the latter, we focused on Galectin-9 which is expressed by CLL cells in patients and mice, and known as ligand for the immunoregulatory receptor TIM-3. We treated mice that had developed CLL-like disease with Galectin-9 blocking antibodies and showed that this treatment slowed down disease development. Associated with that, cytotoxic T cells appeared less exhausted.
Altogether, our study provides a single-cell resolved characterization of the T cell compartment in CLL that helps to understand T cell exhaustion in cancer and shows the potential of targeting the TIM-3 ligand Galectin-9 as novel immunotherapy approach.

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Tumor – associated inflammation in patients with advanced and relapsing hypopharyngeal carcinoma

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Hypopharyngeal carcinomas account for 5-10% of all head & neck squamous cell carcinomas (HNSCC). They are often diagnosed at an advanced stage and they then present a 40% 5-year overall survival. Therapy with immune checkpoint blockade has emerged as a first-line-therapy in relapsing or metastatic HNSCC. Therefore, a more detailed study of the immune and inflammatory cellular infiltrates in the tumor microenvironment (TME) may provide cues for treatment and prognosis in this specific HNSCC patient subgroup.
In a retrospective analysis of HNSCC patients at our center over a course of three years 29 stage III and IV patients (age range: 53 - 84, four female) with hypopharyngeal SCC were found, including 23 patients with first-time diagnosis (three of whom developed a recurrent loco-regional recurrence within the aforementioned time window), three with loco-regional relapsing and three with distant metastatic disease. The histopathological samples were analysed using a universal applicable tool using an established hematoxylin/eosin- based scoring system focusing on chronic and active inflammation based on leukocytic infiltration in the TME. Severity of chronic inflammation was scored as absent (score = 0), mild (=1), moderate (=2) or severe (=3), depending on the accumulation of inflammatory cells (lymphocytes, plasma cells, macrophages) and the formation of lymph follicles. Active, acute inflammation was semi-quantitatively scored as absent (score = 0), mild (=1) or moderate to severe (=2), depending on the density of neutrophil granulocyte infiltration in the TME. The primary (and relapsing, respectively) tumour samples showed in 43% (vs. 17% in relapsing cancers) absent, in 10% (vs. 50%) mild and in 47% (vs. 33%) moderate to severe active inflammation; the grade of chronic inflammatory cellular infiltration was mild in 27% (vs. 83% in relapsing cancers) and moderate to severe in 73% (vs. 17%) of patients. Patients who eventually developed relapsing disease showed a moderate or severe pattern of chronic inflammation at first diagnosis.
Discrete patterns of tissue – associated inflammation were found in the TME of the primary tumors in hypopharyngeal SCC patients. These patterns, especially the quite prevalent moderate to severe neutrophilic infiltration, deserve further study in search of potential links to clinical prognosis and / or response to different treatment modalities.

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Abstract has been withdrawn

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Actin cytoskeleton remodeling at the tumor cell side of the immunological synapse mediates multiple resistance mechanisms against cytotoxic lymphocytes

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Cytotoxic lymphocytes are key anti-tumor immune effector cells. They physically interact with cancer cells through a highly specialized cell-to-cell interface, called the immunological synapse, which is essential to recognize and kill target cells. Recently, we established that the resistance of cancer cells to cytotoxic lymphocytes correlates with rapid and massive polymerization of actin filaments at the post-synaptic (cancer cell) side of the immunological synapse, a process we termed "actin response". Pharmacological and genetic ablation of the actin response is sufficient to restore cancer cell susceptibility to destruction by cytotoxic lymphocytes *in vitro* and a potent anti-tumor immune response *in vivo*. Mechanistically, the actin response protects cancer cells by at least two complementary processes. First, it promotes the recruitment and receptor-independent clustering of inhibitory molecules to the immunological synapse which, in turn, prevents the activation of cytotoxic lymphocytes (as indicated, for example, by defective polarization of the MTOC and lytic granules). Second, the actin response is associated with the formation of actin-rich protrusions that penetrate the synaptic cleft and exert mechanical forces against the pre-synaptic (cytotoxic lymphocyte) side of the immunological synapse. These forces result in morphologically abnormal synapses characterized by significant enlargement of the synaptic cleft. Remarkably, correlative light and electron microscopy analysis revealed that cancer cell-originating protrusions are highly decorated with inhibitory and immune checkpoint molecules, such as PD-L1, and thus propagate inhibitory signals to pre-synaptic side of the immunological synapse. The actin response is a conserved process across a wide range of cancer cell lines and primary cancer cells, and can be induced in response to natural killer cell and cytotoxic T cell synapsing in cell suspensions, spheroids, and human tumor xenograft models. In summary, we report a novel, highly conserved, immune evasion mechanism that exploits fast remodeling of the actin cytoskeleton of cancer cells to generate strong resistance against cytotoxic lymphocytes.

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Design and preclinical testing of an anti-CD41 CAR T cell for the treatment of acute megakaryoblastic leukemia

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Acute megakaryoblastic leukemia (AMkL) is a rare subtype of acute myeloid leukemia (AML) representing 5% of all reported cases, and frequently diagnosed in children with Down syndrome. Patients diagnosed with AMkL have low overall survival and have poor outcome to treatment, thus novel therapies such as CAR T cell therapy could represent an alternative in treating AMkL. In the last decades, significant progress has been made in the field of novel immunotherapeutic agents that can be used as treatments for hematological malignancies. Chimeric antigen receptor T cells (CAR T cell) are the T cells modified to recognize specific antigen.
Currently, five FDA-approved CAR-T cell therapies are used in the clinic. This is the case for the anti-CD19 CAR T cells tisagenlecleucel, brexucabtagene autoleucel, lisocabtagene maraleucel and axicabtagene ciloleucel, as well as the anti-BCMA idecabtagene vicleucel. Thus, the existing CAR T cells are mostly targeting CD19, which is a specific antigen for B cells, with impressive for B-cell acute lymphoblastic leukemia and B-cell non-Hodgkin lymphomas.
We investigated the effect of a new CAR T cell which targets CD41, a specific surface antigen for M7-AMkL, against an in vitro model for AMkL, DAMI Luc2 cell line.
The performed flow cytometry evaluation highlighted a percentage of 93.8% CAR T cells eGFP positive and a limited acute effect on lowering the target cell population. However, the interaction between effector and target (E:T) cells, at a low ratio, lowered the cell membrane integrity, and reduced the M7-AMkL cell population after 24h of co-culture, while the cytotoxic effect was not significant in groups with higher E:T ratio.
Our findings suggest that the anti-CD41 CAR T cells are efficient for a limited time spawn and the cytotoxic effect is visible in all experimental groups with low E:T ratio.

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Tumor microenvironment mimicking 3D models unveil the multifaceted effects of SMAC mimetics

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Small molecule IAP antagonists - SMAC mimetics (SM) - are being developed as an anticancer therapy. SM therapy was demonstrated not only to sensitize tumor cells to TNFα-mediated cell death but also to exert immunostimulatory properties. Their good safety and tolerability profile, plus promising preclinical data, warrants further investigation into its various effects within the tumor

microenvironment. Using *in vitro* models of human tumor cell and fibroblast spheroids co-cultured with primary immune cells, we investigated the effects of SM in immune cell activation. SM treatment induces maturation of human PBMC- and patient-derived dendritic cells (DC), and modulates cancer associated fibroblasts towards an immune interacting phenotype. Finally, SM-induced tumor necroptosis further enhances DC activation, leading also to higher T-cell activation and infiltration into the tumor spheroid. These results highlight the relevance of using heterotypic *in vitro* models to investigate the effects of targeted therapies on different components of the tumor microenvironment.

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Enhancing Neoantigen Expression in Small Cell Lung Cancer via Inhibition of the Nonsense-Mediated Decay Pathway

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Cancer immunotherapy has revolutionized the field of oncology and is one of the most promising forms of lung cancer treatment. Small cell lung cancer (SCLC) is a highly aggressive tumor type with poor prognosis and limited response rates to current immunotherapeutic approaches. Paradoxically, SCLC is characterized by a remarkably high tumor mutational burden, but not all mutations are equally immunogenic. Frameshift (fs) mutations could encode for strongly immunogenic neoantigens, but due to the fs-induced generation of premature termination codons, fs-mutant transcripts are often degraded via Nonsense-Mediated Decay (NMD). According to publicly available data, SCLC is the tumor type with the highest frequency of fs-mutations. Thus, we aimed at targeting the NMD pathway to enhance immunogenicity in SCLC.
To this end, we first employed whole exome sequencing to detect tumor-specific mutations in SCLC samples – including immortalized SCLC cell lines and clinical samples – and predicted candidate neoantigens based on the ability of these mutant peptides to interact with sample-specific HLA-I molecules. So far, we have profiled tumor specific mutations for more than 400 SCLC patients. Next, we validated the expression and presentation of neoantigens in control versus NMD inhibited samples via transcriptome sequencing, whole cell proteomics and HLA-I-immuno-peptidomics. Interestingly, the changes in neoantigen landscapes observed upon NMD inhibition extended beyond fs-mutations, indicating that this approach might be even more powerful than anticipated. Importantly, we estimated tumor-specific NMD activity based on RNA transcriptome sequencing data and NMD reporter assays in order to predict which patients could better profit from NMD inhibition treatments. Based on these predictions, tumor samples with a high NMD activity and a high number of frameshift mutations were selected for co-culture with HLA-I-matched PBMCs. Our results showed that inhibition of NMD in tumor cells indeed resulted in enhanced T cell mediated killing in vitro. Immunogenicity of NMD-regulated neoantigens will be further tested in vivo using tumor xenograft mouse models humanized with autologous or HLA-I-matched PBMCs. To this end, we have currently established more than a hundred SCLC patient-derived xenografts. Our strategy not only provides a

novel immunotherapy approach for the treatment of so far low immunogenic tumors, but also enables patient stratification for an informed personalized care. In the future, NMD inhibition could be combined with checkpoint blockade to enhance immunotherapy efficiency. Our approach could furthermore be used for the rational design of personalized fs-neoantigen-based vaccines.

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PBRM1 loss in clear cell renal cell carcinoma leads to a proangiogenic phenotype via increased CXCL5 secretion that can be selectively inhibited by CXCR2 inhibition.

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Immune checkpoint blockades (ICBs) and anti-angiogenic tyrosine kinase inhibitors (TKI) have substantially contributed to improving the outcomes of metastatic clear cell renal cell carcinoma (ccRCC). However, there is still a lack of biomarkers to distinguish patients who would benefit from the aforementioned therapies. Mutations of polybromo-1 (PBRM1) occur in about one third of ccRCC. It is well known, that PBRM1-mutated tumors are highly vascularized. However, anti-VEGF TKIs often result in only a temporary tumor response, and most ccRCC become ultimately resistant to inhibition of the VEGFR axis. This project aims to dissect the molecular mechanism driving the proangiogenic phenotype of PBRM1-mutated ccRCC. After establishment of CRISPR-Cas9 induced polyclonal PBRM1-knockouts in the ccRCC cell lines 786O and Caki1, we investigated the transcriptional effects of tumormicroenvironmental stimuli (inflammatory as well as morphogenic signals) on PBRM1-loss versus control ccRCC cells. In an unbiased transcriptomic approach, we identified an elevated secretion of CXCL5 in PBRM1-loss cells. In accordance to this, our TCGA in silico analysis revealed that PBRM1-mutant ccRCC exhibited enhanced CXCL1, CXCL2 and CXCL5 expression (all CXCR2 ligands). Exposing the cells to pro-inflammatory cytokines (TNFα, and IL-17A) enhanced CXCL5 expression. The association between PBRM1-deficiency and an enhanced tumor angiogenesis was recapitulated using the spheroid sprouting assay, a 3D in vitro angiogenic model: The supernatant of PBRM1-knockout versus control cell lines induced an enhanced sprouting. CXCL5 was identified as one of the drivers of the pro-angiogenic phenotype of PBRM1-mutated tumors. Of note, enhanced sprouting capacity of the supernatant of PBRM1-knockout ccRCC cell lines can be selectively inhibited by CXCR2 inhibition and CXLC5 blocking antibody. Our findings thus provide a preclinical rational to inhibit the CXCR2/CXCL5 axis in PBRM1-mutant ccRCC.

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Extracellular vimentin is a suppressive vascular immune checkpoint

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Anti-angiogenic cancer therapies possess immune-stimulatory properties by counteracting pro-angiogenic molecular mechanisms. Using single-cell sorting and RNA profiling, we identified specific markers of tumor endothelial cells that are amenable for therapeutic targeting. We demonstrated that tumor endothelial cells ubiquitously overexpress and secrete the intermediate filament protein vimentin. Through screening of a compound library, we elucidated that vimentin is externalized through type III unconventional secretion mechanisms. Extracellular vimentin mimics, as well as potentiates, VEGF actions, through interaction with and activation of VEGFR2. It weakens cell-cell and cell-matrix interactions, thereby facilitating invasive tumor growth.
Antagonizing extracellular vimentin, either by antibodies or by knockdown strategies, results in inhibition of angiogenesis *in vitro* and *in vivo*. Importantly, both passive and active immunotherapies against extracellular vimentin are shown to inhibit tumor vascularization and tumor growth. Importantly, native and radiolabelled anti-vimentin antibodies specifically home to the tumor microenvironment. Vaccination to evoke a humoral immune response was effective and safe in different preclinical and clinical studies in animals.
Extracellular vimentin creates an immune suppressive tumor milieu. Exposure of endothelial cells to extracellular vimentin results in increased PD-L1 expression and suppression of ICAM-1 expression, as well as impaired adhesion of T-cells to the endothelial monolayer. Therapeutic targeting of vimentin rescues this phenotype, and contributes to a pro-inflammatory condition in the tumor. Next to induction of the endothelial adhesion molecule ICAM-1, and suppression of PD-L1, we observed altered immune cell profiles in vimentin vaccinated mice. In particular, dendritic cells were more frequently observed in vimentin vaccinated mice whereas monocytic myeloid-derived suppressor cells were reduced. These data were corroborated by extensive RNAseq analysis and cytokine profiling of these tumors. Finally, combining vimentin vaccination with immune checkpoint inhibition therapy further enhanced tumor growth suppression. Moreover, this combination resulted in a reduction in regulatory T-cells and an increase in cytotoxic T-cells in the tumors as compared to vaccination against vimentin alone.
Our data show that extracellular vimentin is a vascular immune checkpoint molecule and that targeting of this bioavailable marker provides a double-edged sword in cancer therapy, simultaneously alleviating immune suppression and repressing tumor angiogenesis.

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Evaluating the therapeutic efficacy of targeting crucial metabolic pathways in glioblastoma in combination with anti-PD-1 in vivo

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Glioblastoma (GBM) patients are in dire need of an effective therapy. GBM rewires crucial metabolic programs for proliferation and survival, thereby creating a hostile tumor microenvironment that impairs tumor immunosurveillance and response to immunotherapy. Our study investigates the potential of targeting mitochondrial respiration and glutaminolysis with various drugs to reprogram the metabolic environment of GBM tumors, with the aim of sensitizing them to immune checkpoint blockade (ICB) *in vivo*.
We examined the therapeutic potential of metabolic inhibitors in the orthotopic GL261 GBM murine model. Specifically, we tested the efficacy of Metformin and IACS-01759, which inhibit oxidative phosphorylation, and JHU-083, which targets glutaminolysis, in GL261 tumor-bearing mice. Mice were randomized based on tumor volume determined via magnetic resonance imaging. Metformin was administered via drinking water, while IACS-01759 was given orally at either a low or high dose daily. JHU-083 was administered orally either daily at a lower concentration or every four days at a higher concentration. Concurrently, mice were injected intraperitoneally with anti-PD-1. Survival was used as the primary read-out to evaluate the therapeutic effect, with humane endpoints for brain tumors being considered.
The administration of metformin and JHU-083 did not yield significant improvements in survival outcomes. However, we observed a significant improvement in overall survival when a low concentration of IACS-01759 was combined with anti-PD-1 compared to both vehicle control and IACS-01759 monotherapies. These preclinical findings offer promising evidence that targeting mitochondrial respiration using IACS-01759 has the potential to increase the sensitivity of GBM tumors to ICB therapy. Further research is necessary to determine the optimal administration strategy for this approach.

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Decoding the immunogenomic determinants of immunotherapy and radiotherapy response in soft tissue sarcomas

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Myxofibrosarcoma (MFS) and undifferentiated soft tissue sarcoma (USTS) are two subtypes of genetically complex sarcomas with distinct morphological features. The mainstay of treatment is surgery, potentially in combination with neoadjuvant chemo- or radiotherapy (RT). In recent years, patients with USTS or MFS have experienced benefit from T cell immune checkpoint blockade that produced responses in about 25% of patients, mainly in USTSs. To improve our understanding of the immunobiology of these STSs, we aimed to characterize the immune microenvironment through transcriptomic and immunophenotypic profiling.
In our retrospective study, we included 29 patients with high grade tumors (13 MFSs and 16 USTSs). Immune cell phenotypes and spatial interactions were mapped with a 40-marker imaging mass cytometry panel on tissues including pre-treatment biopsies, treatment-naïve resections and post-radiation resection. In parallel, immune-related gene signatures were obtained for treatment-naïve samples that underwent RNA sequencing, including seven MFSs and 13 USTSs. Histological response to RT was assessed by a bone and soft tissue pathologist and clinical follow-up data was collected for survival analysis.
In comparison with other genetically complex sarcomas, both USTSs and MFSs presented an enrichment for hallmarks of ongoing inflammatory responses, as assessed through RNA sequencing analysis of immune-related genes. The immunologic constant of rejection signature in combination with the microenvironment cell population counter deconvolution algorithm revealed a subgroup of highly infiltrated STSs as well as a general enrichment for T cell-related signaling. The 33 immunophenotypes discovered with imaging mass cytometry provided important additional information regarding the actual levels of infiltration. In general, USTSs comprise higher densities of immune cells than MFSs, which might explain the reported differences in response to checkpoint blockade between both subtypes. Intriguingly, we observed that both subtypes respond differently to RT, specifically that USTSs appear more radiosensitive than MFSs. This was apparent by the general decrease of all cell phenotypes in the USTSs and the increase of certain myeloid cell populations in MFSs after RT. By comparing the treatment-naïve microenvironment of responders with non-responders, we found that various types of T cells, as well as B cells and dendritic cells were more abundant in patients who responded to RT before treatment. These findings suggest a link between pre-existing anti-tumor immunity and responsiveness to RT.
In conclusion, soft tissue sarcomas are generally considered immunogenic as assessed by both transcriptomic and immunophenotypic profiling. USTSs are more often immunogenic than MFSs and appear more sensitive to RT. Enrichment for pro-inflammatory hallmarks before treatment could potentially be used as a predictive biomarker for response to RT and possibly immunotherapy in STSs.

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Natural killer cell metabolism and cytotoxicity are impaired by the hypoxic tumor microenvironment

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Chimeric antigen receptor (CAR)–engineered T cells have revolutionized cell therapy for cancer, but due to manufacturing and clinical challenges as well as an extremely high financial burden, other effector cells are being investigated. Natural killer (NK) cells form a very promising alternative as CAR vehicles, owing to their specialized cytotoxicity against tumor cells, unique biological attributes, favorable safety profile and potential use as an off-the-shelf cellular therapy. However, within solid tumors, NK cells are often dysfunctional. Hypoxia is recognized a major hallmark of the tumor microenvironment, but its effects on NK cells remain to be fully understood.
Here, we investigated the effects of hypoxia (1% O2) for 48 hours on the redox balance, mitochondrial health and cytotoxicity of the human NK-92 cell line, an unlimited NK cell source with significant clinical potential. To visualize the morphology of mitochondria of NK cells, we employed both confocal and electron microscopy. Mitochondrial fragmentation was significantly elevated in hypoxic NK cells and the ultrastructure of the mitochondrial cristae organization was distorted. Flow cytometry dyes were used to measure the mitochondrial membrane potential and mitochondrial ROS. We observed that hypoxic NK cells featured a lower number of mitochondria, which also displayed a lower mitochondrial membrane potential and higher mitochondrial ROS, compared to cells cultured in normoxic conditions. Functionally, hypoxic NK cells, as well as hypoxic CD70 CAR-NK cells, were severely restricted in target cell killing, as assessed by co-culture experiments.
In conclusion, the hypoxic environment of solid tumors has a detrimental effect on NK cell cytotoxic potential and metabolism.

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Expression of neolacto-series glycosphingolipids by tumors impairs the anti-tumor function of innate and adaptive immune responses.

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The transcriptional signature of neolacto-series glycosphingolipid (nsGSL) expression highly associates with patient survival in a number of cancers, such as glioma. We recently identified that nsGSLs on such tumor cells negatively affect immune T cell activation *in vitro* (Jongsma et al., Immunity 2020). Here, we show that nsGSLs also affect innate anti-tumor responses. In addition, we provide data on mechanisms that may be involved in nsGSL-mediated immune escape
Since T cell activation was demonstrated *in vitro* to affect the anti-tumor response, we investigated

effects of nsGSLs on anti-tumor T cell responses *in vivo*. We demonstrate in a murine model that nsGSL impaired OT1 T cell activation and benefit tumor growth *in vitro* and *in vivo*. Additionally, investigating other arms of the anti-tumor immune defense system, we engineered tumor cells with various levels of nsGSL expression and show that killing of nsGSL-overexpressing tumor cells by neutrophils, NK cells and gd-T cells was significantly reduced.
Using a flow cytometry approach with barcoded cell lines we discovered that nsGSLs sterically shield several, but not all, immune cell surface receptors. In depth analyses of shielded receptor properties revealed that they have significantly shorter extracellular domains compared to non-shielded receptors, which may relate to the limited extracellular length of nsGSLs. Secondly, using genome editing and pharmacological inhibitors, we found that negatively charged sialic acids of nsGSLs likely interact with positively charged amino acids of shielded proteins. This interaction inhibited antibody binding to surface receptors, which was highly dependent on affinity between antibody and ligand. We specifically assessed this using a well-characterized antibody panel against CD147. Low-affinity interactions of the central immune receptors HLA class I and CD47 with their ligands LIR-1, KIR2DL2 (HLA class I), and SIRP-α (CD47) were largely impaired by nsGSLs.
Overall, our data strongly indicate that expression of nsGSLs by tumor cells prevents productive communication towards immune cells through charge-based shielding of short receptors from their low affinity ligands. Because the GSL synthesis pathway is safely targeted as therapy in lysosomal storage diseases, our data warrant investigations on the efficacy of GSL synthesis inhibition to treat patients with nsGSL-rich tumors.

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Enhanced mutanome analysis towards the isolation of neoepitope-reactive T cell receptors for personalized immunotherapy of pancreatic cancer

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Pancreatic ductal adenocarcinoma (PDAC) represents an unmet medical need, in that surgical resection of the primary tumor is to date the only treatment that provides a perspective for prolonged survival. Unfortunately, a majority of patients experience tumor recurrence within 1-2 years, while non-resectable and recurrent disease are refractory to chemotherapy, targeted therapy and immune checkpoint blockade. Even though PDAC is generally considered a poorly immunogenic, ‘cold’ tumor, multiple studies including our own have provided evidence for tumor-reactive T-cell immunity, as well as for the prolonged post-surgery survival of patients with evidence of T-cell responses in the resected tumor. In view of this, T-cell therapy could be considered as an adjuvant strategy to counter tumor recurrence in patients with primary resectable disease.

We explored the possibility of targeting PDAC by means of TCR gene therapy, using T-cell receptors (TCRs) that target tumor-specific, mutanome-encoded neo-epitopes. Reliable calling of somatic mutations in primary tumor samples from pancreatic ductal adenocarcinoma (PDAC) has proven difficult due to low tumor cell content. We therefore developed a work flow that combines the analysis of next generation sequencing (NGS) data from patient-derived xenograft (PDX) models and primary tumor samples to enable the enhanced detection of driver mutations as well as single nucleotide variants (SNVs) encoding potentially immunogenic T-cell epitopes. A customized hybrid reference genome was generated to achieve accurate mutation calling in PDX samples.
For several of the candidate epitopes identified in this manner, we successfully generated TCRs through immunization of HLA/human TCR locus-transgenic mice that express a human TCR repertoire. Human T-cells expressing these fully human TCRs detected synthetic peptide concentrations in the 10-100 picomolar range, and were capable of discriminating between the neo-epitopes and their wild type counterparts. Furthermore, these TCRs mediated T-cell activation by naturally processed antigen, in that target cells transfected with gene constructs encoding the mutant epitopes, but not the wild type controls, were recognized. Lastly, these TCRs mediated selective recognition of tumor cell lines in which the neoepitope encoding mutations were originally identified.
In summary, our study provides a proof of concept for the generation of fully human tumor-reactive TCRs targeting mutanome-encoded neo-epitopes as expressed in human PDAC tumors by means of HLA/human TCR locus-transgenic mice.

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Organization, function and gene expression of tertiary lymphoid structures in pancreatic cancer resembles lymphoid follicles in secondary lymphoid organs and their abundance is related to superior survival

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Tertiary lymphoid structures (TLS) have been described in close proximity to tumor areas in a variety of cancer types. Abundance of TLS is related to cancer-specific survival and susceptibility to immune checkpoint inhibition. TLS in the tumor microenvironment are assumed to represent hotspots for T cell and B-cell activation leading to tumor-specific humoral and cellular immune responses. We aim to identify shared and TLS/secondary lymphoid organ (SLO)-specific features related to their functional overlap. TLS were found in 95% of 163 analyzed primary pancreatic ductal adenocarcinoma (PDAC) patients with heterogeneous abundance of TLS between patients. Immunohistochemical analysis of spatial distribution revealed that TLS were mainly localized in a 2000 µm invasive tumor margin with few TLS also occurring intratumorally. Patients with a high

density of TLS inside and surrounding the tumor showed a higher overall survival and there was a correlation between TLS abundance and high infiltration of $CD8^+$ T cells into the tumor. Five-color Immunofluorescence revealed structural similarities in the architecture of TLS with SLO as the major cellular compartments defining lymph follicles could be found in both structures and showed a similar spatial distribution. Tissue extraction by laser microdissection and Nanostring-based RNA expression analysis was conducted for 12 patients to compare gene expression in TLS, PDAC, SLOs and normal pancreatic tissue. In addition to the structural similarities of TLS and SLOs, we found largely overlapping expression patterns in a variety of immune related gene clusters. However, we also detected differences between TLS and SLOs, especially in expression levels of T-cell and complement-associated genes. Most TLS were positively stained for Ki-67, Pax5, AID and IgG, proving proliferation, class switching and affinity maturation of B cells in tumor-surrounding TLS. In B cell receptor sequencing of microdissected TLS and SLO, we identified clonal expansion and overlapping expanded sequences. In summary, our analyses of organization, function and gene expression patterns revealed a high overlap between SLOs and TLS in PDAC. Our results indicate a role of TLS in cancer immunosurveillance of PDAC, which may be susceptible to therapeutic targeting in this highly aggressive and immunotherapy-resistant disease.

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A rapid-processing *ex vivo* pipeline to unravel the immune phenotype of obesity-related hepatocellular carcinoma reveals impairment of MAIT cells

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In recent years, immune-checkpoint-inhibitor (ICI) therapy revolutionized the foundations of hepatocellular carcinoma (HCC) treatment. Unfortunately, not all patients respond to ICI therapy and subgroup analyses of latest trials suggest patients with HCC due to non-alcoholic fatty liver disease (NAFLD) benefit considerably less than patients with HCC due to viral hepatitis. With the globally rising prevalence of NAFLD due to increasing obesity in the general population, understanding the tumor immune microenvironment of NAFLD-associated HCC is key to apprehend possible mechanisms for augmented ICI resistance. Therefore, we developed a rapid-processing *ex vivo* immune cell isolation pipeline for matched human HCC, surrounding liver and blood samples. Isolated immune cells are used for simultaneous single-cell RNA sequencing as well as full spectrum flow cytometry, providing an unbiased approach to decipher leucocyte heterogeneity on a transcriptomic and protein level. Our 27-color full spectrum flow cytometry panel for tissue specimen allows us to analyze all major immune cell subsets, with a focus on checkpoint expression patterns on T cells (including unconventional T cell subsets) and myeloid cells. Interim analysis of 57 patients (and control subjects) already revealed a NAFLD/HCC-associated reduction in Mucosa-associated invariant T (MAIT) cells in the peripheral blood with elevated immune checkpoint expression, along with decreased tissue infiltration, which correlates with the stage of fibrosis. Furthermore, the number of tumoral MAIT cells is significantly lower compared to surrounding liver tissue and subgroup-analysis indicates that this phenomenon is even more distinct in NAFLD-associated HCC, suggesting an impairment of this T cell subtype specific to this etiology. In conclusion, our pipeline

enables us to compare varied immune cell subsets from HCC patients with and without NAFLD. This will reveal new specific immune signatures to provide a more comprehensive picture of human NAFLD-associated HCC and provide insights on the mechanisms of NAFLD-associated ICI therapy resistance.

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Melanoma cell intrinsic LAG-3 expression correlates with metastasis stage and might indicate resistance to anti-PD-1 monotherapy

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The immune checkpoint lymphocyte activation gene 3 (LAG-3) is discussed to contribute to anti-programmed cell death protein 1 (PD-1) therapy resistance. Melanoma patients with a programmed cell death ligand 1 (PD-L1) expression of < 1 % benefit from combined treatment with the LAG-3 antibody relatlimab and the PD-1 antibody nivolumab. However, to date LAG-3 expression has mainly been shown for tumor infiltrating lymphocytes.

We immunohistochemically stained metastases from patients with advanced melanoma collected before or shortly after their first dose of immunotherapy (anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) or anti-PD-1 or anti-CTLA-4/anti-PD-1) for LAG-3 (n = 60) and its ligands α-synuclein (n = 29), LSECtin (n = 29), major histocompatibility complex class II (n = 28), fibrinogen-like protein 1 (n = 28), galectin-3 (n = 26), as well as Ki-67 (n = 25), PD-1 (n = 42), and PD-L1 (n = 33). All slides were scored via the H-score system (range 0-300) or via a percentage cutoff by two independent scorers and categorized into the groups "high/positive" or "low/negative". In case of disagreement, a third observer additionally evaluated the stainings.
We detected LAG-3 expression of different intensities for the melanoma cells of the metastases. When correlating the staining intensity (high/low) with clinical parameters, we observed that LAG-3 expression was associated with M1 stage (M1a/M1b/M1c/M1d, $p < 0.001$, n = 56). Moreover, metastases from stage IV patients that were pre-treated (non-adjuvant) with immune checkpoint inhibitors (ICI) more often revealed high LAG-3 expression compared to metastases from ICI-untreated patients ($p < 0.05$, n = 56). Concerning the clinical outcome, stage IV patients with high LAG-3 expression treated with anti-PD-1 monotherapy tended to progress faster, showing a lower progression free survival (median 3.3 months, 95 % CI [2.4; 4.2]) compared to patients with a low LAG-3 expression (median 22.3 months, 95 % CI [0.0; 48.5]; $p = 0.061$). However, this was not seen with combined ICI treatment. The correlation of LAG-3 staining intensities with the respective potential ligand stainings did not reveal a significant association between the tumor cell intrinsic

expression of LAG-3 and any of its ligands.
In conclusion, LAG-3 is commonly expressed in tumor cells of melanoma metastases and first results indicate a negative impact on clinical outcome of anti-PD-1 monotherapy.

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Incorporation of TRDV segments into TCR alpha chains

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In human tumor models we tried to identify and clone the TCR of tumor-reactive T cells enriched in mixed lymphocyte tumor-cell cultures (MLTC). In a particular MLTC, we identified a predominant Vbeta chain using the Beta Mark Vbeta Kit, but a corresponding alpha chain could not be found via RT-PCR using *TRAV*-specific forward primers. Therefore, we applied 5'RACE to amplify the alpha chain sequences. The 5'RACE product revealed an alpha chain that encompassed 89bp of the *TRDV1* 5'UTR, followed by the TRDV1 coding sequence joined in frame to *TRAJ24*. The ORF reaching from the *TRDV1* start codon to the *TRAC* segment was intact, suggesting a functional TCR. To analyze this MLTC population in greater depth we subsequently conducted 10X VDJ sequencing. CellRanger identified the beta chain known from the Beta Mark analysis, but no corresponding alpha chain in the filtered results. The corresponding TRDV-containing TCR alpha chain could, however, be detected in the "all_contig_annotations" files.
In a separate project, we performed TCR sequencing of tumor-infiltrating lymphocytes (TILs) in a murine tumor model. Also here, a predominant clonotype contained an alpha chain joining *Trdv2-2* coding sequence joined in frame to *Traj49.*Transfection of both TCR cDNAs resulted in cell surface localization of TCR and CD3 as validated by FACS. Tumor recognition of the human, TRDV1-containing TCR could be demonstrated by IFNg ELISpot assay whereas the murine TCR did not show tumor recognition.
TRDV-containing alpha chains have been reported in the literature for two HLA I-restricted TCRs against HIV peptides. In order to find out whether TDRV-containing TCRs were unique events or whether Vdelta segments are commonly incorporated into TCRalpha chains, we queried the NCBI Sequence Read Archive (SRA) for 10X VDJ data and analyzed 21 human and 23 murine datasets. We found that especially *TRDV1*, *Trdv1* and to some extent *Trdv2-2* are more commonly incorporated into TCRalpha chains than some *TRAV* genes, making the *TRDV* segments a relevant contribution to TCRalpha diversity.
For apparently solitary beta chains in TCR profiles derived from 10X single cell sequencing, we suggest to scrutinize the "all_contig_annotations" files as these may provide an accompanying, TRDV-containing alpha chain.

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Abstract has been withdrawn

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Interleukin-38 promotes colon carcinogenesis via inhibiting T cell-mediated intestinal inflammation

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Inflammatory bowel diseases, such as Crohn's disease and ulcerative colitis, predispose patients for developing colorectal cancer. It is well-established that anti-inflammatory treatment not only counteracts the development of malignant tumors at an early stage, but also promotes colorectal cancer cell apoptosis. Interleukin-38 (IL-38) is a newly discovered cytokine of IL-1 family which promotes the resolution of inflammation, but whose role remains uncertain in the setting of cancer. We studied the expression of IL-38 in the development of intestinal inflammation and colorectal cancer. We found higher levels of IL-38 in tumor tissues from colorectal cancer patients compared with control individuals. The expression of IL-38 was negatively correlated with the infiltration of T cells and mismatch repair gene mutations. Moreover, increased epithelial IL-38 expression was negatively correlated with the overall survival of patients with microsatellite stable tumors. IL-38 deficient mice and mice treated with an antibody neutralizing IL-38 developed more severe colitis but, surprisingly, less tumors in the azoxymethane/dextran sodium sulfate (AOM/DSS) model. Interestingly the IL-38 antibody was only effective when given during the colitis stage. In tissues from IL-38 deficient mice and mice treated with anti-IL-38 antibody, significantly higher levels of inflammatory cytokines were noted, which correlated with increased infiltration of immune cells, especially macrophages and T cells. These results showed that the inhibition of IL-38, either genetically or by an antibody-mediated blockade, enhanced anti-tumor immunity and halted colon carcinogenesis. Overall, IL-38 appears to be a promising target for prevention and treatment of colorectal cancer, with particular relevance for patients with microsatellite stable tumors that do not benefit from current immunotherapy.

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Charting the tumor-reactive T-cell repertoire in DNA damage repair (DDR) deficient pancreatic cancer towards development of personalized T-cell therapy

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Poster Session II

Tumor Biology and Interaction with the Immune System

The failure of immunotherapeutic strategies including immune checkpoint blockade (ICB) in pancreatic cancer is due to limited numbers of somatic mutations in combination with a highly immune-suppressive tumor microenvironment. Patients with DNA damage repair (DDR) deficient pancreatic cancer do show – albeit limited – responsiveness to ICB. In prior work, we have demonstrated significantly increased tumor-specific T-cell responses in these tumors.
We are therefore exploring optimized, personalized treatment strategies for this pancreatic cancer subtype in an autochthonous mouse model for pancreatic cancer in which single focal tumors are induced in the tail of the pancreas through *in vivo* electroporation of gene constructs encoding mutant *Kras* as well as a sgRNA/Cas9 combination targeting the tumor suppressor gene *Trp53*. This model closely resembles the human disease with respect to genetics and histology. Furthermore, it allows for primary tumor resection and therefore pre-clinical testing of (neo)adjuvant regimens. We extended this model towards DDR-deficient tumors by including targeting constructs to inactivate *BRCA1*, *BRCA2*, *PALB2*, *MLH1* or *MSH6*. The resulting tumors exhibit greatly increased numbers (up to 500) of exonic mutations as well as enhanced T-cell infiltration.
Based on single-cell sequencing data sets from the tumor-infiltrating T-cell repertoire, we isolated multiple tumor-reactive T-cell receptors (TCR) and classified these based on their reactivity against a tumor cell panel and immunopeptidome preparations. In this manner, we identified TCRs targeting recurrent antigens, as well as TCRs targeting private neo-antigens.
Our ongoing work aims at systematic comparison of these two arms of the anti-tumor T-cell response, both with respect to the role in the natural T-cell response in emerging tumors, and the therapeutic potential in the (neo)-adjuvant setting.