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ICLE 2022 – Oral Presentations

O001
Topic: *ASa03 Engineered T cell therapy for solid tumours*

BNT211: A PHASE I TRIAL EVALUATING SAFETY AND EFFICACY OF CLDN6 CAR-T CELLS AND CARVAC-MEDIATED IN VIVO EXPANSION IN PATIENTS WITH CLDN6-POSITIVE ADVANCED SOLID TUMORS

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BNT211 comprises a chimeric antigen receptor (CAR)-T cell product candidate targeting the tumor specific antigen claudin 6 (CLDN6) and a CAR-T cell-Amplifying RNA Vaccine (CARVac). CARVac mediates expansion of adoptively transferred CAR-T cells, resulting in improved persistence and functionality.

Methods: BNT211-01 (NCT04503278, sponsor BioNTech), a first-in-human, open label, multi-center trial involves a bifurcated 3+3 design with separate CLDN6 CAR-T cell dose escalations (single flat-dose) for monotherapy (Part 1) and combination with CARVac (Part 2). In Part 2, CARVac is applied every 3 weeks from Day 4 post-transplantation including a one-step intra-patient dose escalation. Patients with CLDN6-positive relapsed or refractory solid tumors without further standard treatment options and ECOG 0-1 are eligible for recruitment.

Results: As of 18th Nov 2021, 15 patients had been treated. Part 1, dose levels (DLs) 1 and 2 and Part 2, DL1 had been completed, while Part 2, DL2 was ongoing. Few adverse events related to the drug product and a single DLT mainly linked to the lymphodepletion regimen have been reported. Pronounced cytopenia occurred in testicular cancer patients previously treated with high-dose chemotherapy and autologous stem cell transplantation. For these patients a lymphodepletion-free cohort was recently opened. Manageable cytokine release syndrome (grade 1-2) without any signs of neurotoxicity has been observed in 7 patients. Transient, moderate elevations of IL-6 serum levels occurred in remaining patients. Notably, CARVac resulted in flu-like symptoms resolving within 24 h. Analysis of CAR-T cell frequency in peripheral blood revealed robust engraftment in all patients. Preliminary efficacy data for 10 evaluable patients 6 weeks post-infusion showed 4 partial responses, 1 progressive disease and 5 stable disease. Most responses were seen in testicular cancer patients; remaining patients had stable disease.

Conclusions: CLDN6 CAR-T cells ± CARVac show a favorable safety profile at doses tested and encouraging signs of clinical activity.

O002
Topic: *ASa03 Engineered T cell therapy for solid tumours*

A UNIQUE ENGINEERED T CELL ENABLES ANTIBODY-MEDIATED CYTOTOXICITY OVERCOME THE LIMITATIONS OF CAR T CELL THERAPY AGAINST SOLID TUMORS

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The pioneering design of chimeric antigen receptors has revolutionized treatments of refractory lymphomas and demonstrated the therapeutic potential in re-wiring the immune system. Nonetheless, rapid exhaustion, cytotoxicity issues, and the presence of a suppressive tumor microenvironment limit their efficacy in solid tumors. Recently, by attempting to potentiate antibody-driven immunity, we characterized a novel subset of tumor-infiltrating $CD4^+$ T cells that express the high-affinity Fcγ receptor for IgG (FcγRI). This population accumulates in inflamed tissues of various pathologies and secretes high levels of Th1 cytokines and IL-17. Ectopic expression of the native FcgRI in T cells enables antibody-mediated cytotoxicity of tumor cells coated with antibodies. In an attempt to improve T cell ADCC. we engineered a novel receptor, based on the FcgRI scaffold, which endows naive T cells superior cytotoxic abilities, along with high antigen specificity. Administration of transduced T cells to immunocompetent mice bearing established tumors induced long-lasting immunity and eradication of these tumors. The majority of transduced T cells infiltrating the tumors were $CD4^+$ T cells, and it was followed by massive accumulation of T cells from the host. However, in cases where the tumor antigen was lost, tumors had escaped antibody-mediated therapy. Whereas the killing rates of anti-HER2 CAR and of T cells equipped with modified FcgRI were comparable, CAR T cells were found to secrete unattenuated levels of IFNg and IL-2.

Subsequently, while CAR T cells directed against HER2 were activated by cells expressing physiological levels of HER2, cytotoxicity driven by HER2 antibody was restricted to cells expressing high antigen density. Overall, this work describes a novel construct to enable T-cell cytotoxicity driven by to tumor-binding antibodies. This composition enables to overcome CAR T cells' inherent limitations, including specificity issues and premature exhaustion, and provides, for the first time, a mean to affect solid cancers.

O003
Topic: *ASa03 Engineered T cell therapy for solid tumours*

ORTHOGONAL GENE ENGINEERING ENABLES CD8+ T CELLS TO CONTROL TUMORS THROUGH A NOVEL TOX-INDIFFERENT SYNTHETIC EFFECTOR STATE

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T-cell engineering offers unlimited opportunity to rationally reprogram TILs, and in a paracrine manner the tumor microenvironment (TME). Here, we sought to engineer T cells using an orthogonal combinatorial approach, i.e. introducing genes whose products could mediate perturbations to reprogram both T cells and the TME. On one hand, we targeted the PD-1/PD-L1 inhibitory pathway with a secreted PD-1 decoy. On the other hand, two cytokines were selected: (i) to support T-cell stemness and expansion, we used a human IL-2 variant (IL-2^{V}) that does not engage the high affinity IL-2Ra-chain and (ii) IL-33, an alarmin demonstrated to inflame tumors. Adoptive cell transfer of CD8 T cells gene-engineered to secrete this combination of molecules led to cell-autonomous T-cell expansion (i.e avoiding lymphodepletion and cytokine support), engraftment and tumor control in immunocompetent hosts through reprogramming of both transferred and endogenous $CD8^{+}$ cells. Tumor-infiltrating $CD8^{+}$ lymphocytes adopted a novel and potent PD-1^{+} $TCF1^{neg}$ effector state characterized by TOX suppression and specific expression of multiples effector molecules, most prominently granzyme-C. These novel effector cells also downregulated other important exhaustion-associated transcription factors like *Batf, Nfatc1* and *Bhle40* and do not express neither *Cx3cr1* nor *Klrg1,* both distinctive markers of short-lived effector cells (SLEC) and the recently described transitory effector-like exhausted cells. While the IL2-variant promoted CD8 T-cell stemness, persistence and was associated with downregulation of TOX, the combination with IL-33 was necessary to trigger the novel polyfunctional effector state. In addition, we demonstrated that expression of multiple inhibitory receptors in this novel effector state was functionally inconsequential. Thus, we provide evidence that orthogonal combinatorial T-cell engineering can successfully overcome homeostatic barriers to successful ACT in the host and enables both optimal reprogramming of adoptively transferred T cells and mobilization of endogenous $CD8^{+}$ T-cell immunity into new functional synthetic states and efficient tumor control.

O004
Topic: *ASa03 Engineered T cell therapy for solid tumours*

DEVELOPMENT AND EVALUATION OF NEXT GENERATION IL-15 COENGINEERED MURINE CAR-T CELLS

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The adoptive transfer of CAR-T cells has emerged as a robust immunotherapy against some hematological malignancies but limited clinical responses have been achieved against epithelial-derived solid tumors. Barriers include a paucity of broadly-expressed target antigens, limited CAR-T cell homing, and a range of immunosuppressive mechanisms that can be upregulated in the solid tumor microenvironment (TME). Tumor vasculature endothelial cell (TVEC)-expressed antigens represent promising CAR targets as they are broadly and stably expressed, and readily accessible to circulating T cells. However, only modest tumor control has been achieved by anti-TVEC second generation (2G) CAR-T cells as a monotherapy. Here, we first optimized methods for both retroviral transduction and murine CAR-T cell culture in the presence of IL-7/IL-15 (versus IL-2) to achieve high engineering efficiency and robust and preferential expansion of central memory T cells (T_{CM}). With the aim of improving TVEC-antigen targeted CAR-T cell persistence and tumor control, we built a bicistronic retroviral vector encoding both murine interleukin (IL-)15 and a CAR (i.e., a 4G or next generation CAR construct) redirected against the vasculature endothelial growth factor receptor-2 (VEGFR-2). Overall, as compared to 2G-CAR-T cells, the 4G-CAR-T cells demonstrated superior in vitro and in vivo phenotype including higher expression levels of Ki-67 and the anti-apoptotic protein Bcl-2, and lower levels of PD-1. Furthermore, they exhibited superior proliferation, as well as cytokine secretion in response to VEGFR-2^{+} targets in vitro. Finally, the 4G-CAR-T cells sustained expression of the mIL-15 transgene in vivo, and displayed enhanced persistence and significantly improved tumor control. Interrogation of the TME revealed reprogramming favorable for immunity, including a higher proportion of activated NK cells and fewer M2 macrophages. Taken together our work introduces robust tools for the development and evaluation of 4G-CAR-T cells in immunocompetent mice, an important step toward the acceleration of effective therapies reaching the clinic.

O005
Topic: *ASa03 Engineered T cell therapy for solid tumours*

AS SMALL AS IT CAN BE: SHORT PEPTIDE-DERIVED TARGET MOLECULES FOR REDIRECTION OF UNICAR T-CELLS AND IMAGING OF SSTR2-EXPRESSING CANCERS

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Chimeric antigen receptor (CAR) T-cells are undoubtedly a promising approach in cancer immunotherapy. Nevertheless, mild to severe toxicities are associated with this approach including on-target/off-tumor effects and cytokine release syndrome. Aiming for increased clinical safety, adaptor CAR technologies were developed which include the modular universal CAR (UniCAR) platform developed by our group. UniCAR T-cells are exclusively activated in the presence of a target module (TM), which establishes the cross-link between cancer cells and UniCAR T-cells. These TMs are highly versatile molecules that can be constructed not only by using antibody fragments but also e.g. peptides specifically targeting a receptor or molecule on the cells surface. Somatostatin receptor (SSTR) subtype 2 is highly expressed in a variety of malignancies and has therefore been studied as a marker and target for cancer diagnosis and treatment. Currently, SSTR2 agonists and antagonists, such as Tyr^3-octreotate (TATE) and BASS or JR11, respectively, are particularly well established and clinically implemented mostly used for diagnostic nuclear medicine. Given this and the proven flexibility and efficacy of the UniCAR system, we hereby aimed to develop small peptide-derived TMs targeting SSTR2 that can be used for both immunotherapeutic and diagnostic approaches. For that, the above-mentioned SSTR2 agonist and antagonists were chemically linked to the E5B9 peptide and equipped with the radiometal chelator NODAGA. These TMs were tested *in vitro* and *in vivo*, in which they have proven to specifically redirect UniCAR T-cells to human neuroendocrine and breast SSTR2-expressing cancer cells. Furthermore, the enrichment of these anti-SSTR2 peptide TMs at the tumor site was confirmed by positron emission tomography (PET) studies. We hereby designed novel small peptide-derived TMs that can be used for redirection of UniCAR T-cells to SSTR2-expressing cancer cells as well as for PET imaging, proving to be promising and innovative immunotheranostics tools to foster cancer treatment.

O006

Topic: *ASa03 Engineered T cell therapy for solid tumours*

FREQUENT CRISPR-CAS9 INDUCED ANEUPLOIDY IN PRIMARY HUMAN T CELLS

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Multiple ongoing clinical trials use site specific nucleases to disrupt T cell receptor (TCR) genes in order to allow for allogeneic T cell therapy. In particular, the first U.S. clinical trial using CRISPR-Cas9 entailed the targeted disruption of the TCR chains and programmed cell death protein 1 (PDCD1) in T cells of refractory cancer patients. Here, we used the same guide RNA sequences and applied single cell RNA sequencing to primary human T cells, 4 days after CRISPR-Cas9 transfection. Importantly, we found a high correlation between the chromosomal location of the targeted gene and the resulting aberration. In particular, cleaving the TCRα locus, near the chromosome 14 centromere, leads to functional aneuploidy, with up to 9% and 1.4% of the cell having a chromosome 14 loss or gain, respectively. Cleaving the TCRβ locus, in the middle of the chromosome 7 q-arm, leads to the truncations in 9.9% of the cells, while cleaving the PDCD1 gene, near the chromosome 2 q-arm telomere, expectedly has little effect on global gene expression. Aneuploidy was found among all T cell subsets and was associated with transcriptional signatures of reduced fitness but did not lead to complete cell cycle arrest. Loss of heterozygosity was further validated using fluorescent *in situ* hybridization and the temporal dynamics of cleavage and incomplete repair was monitored using digital droplet PCR. We conclude that aneuploidy and chromosomal truncations are underappreciated, yet frequent, outcomes of CRISPR-Cas9 cleavage in clinical protocols. Monitoring and minimizing these aberrant products is crucial for future applications of genome editing in T cell engineering and beyond.

O007

Topic: *ASa03 Engineered T cell therapy for solid tumours*

OVERCOMING T CELL DYSFUNCTION IN ACIDIC PH TO ENHANCE ADOPTIVE T CELL TRANSFER IMMUNOTHERAPY

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It is well-known that the high metabolic activity and insufficient perfusion of tumors leads to the acidification of the tumor microenvironment (TME). This low intracellular pH (pH_i) of tumor infiltrating T cells may result in inhibited T cell function and anti-tumor activity. In *in vitro* studies, we have found that pharmacological inhibition of the acid loader chloride/bicarbonate ($Cl^-/HCO3^-$) anion exchanger 2 (AE2), with 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) enhanced $CD4^+$ and $CD8^+$ T cell function upon TCR activation, especially under low pH culture conditions. *In vivo*, DIDS administration delayed B16OVA tumor growth in immunocompetent mice as monotherapy and in particular when combined with adoptive T cell (ACT) transfer of OVA-specific T cells. Notably, genetic AE2 silencing in OVA-specific T cells using CRISPR/Cas9 improved $CD4^+$ and $CD8^+$ T cell function upon TCR activation *in vitro* as well as their anti-tumor activity *in vivo*. Similarly, genetic modification of OVA-specific T cells to overexpresses Hvcn1, a highly selective H^+ outward current mediator that prevents cell acidification, significantly improved T cell function *in vitro*, even at low pH conditions. Additionally, the adoptive transfer of OVA-specific T cells overexpressing Hvcn1 exerted a better control of tumor growth in B16OVA tumor-bearing mice.

Thus, our results suggest that preventing intracellular acidification by regulating the expression of acidifier ion channels such as AE2 or alkalinizer channels like Hvcn1 in tumor-specific lymphocytes enhances the anti-tumor immune response by making them more resistant to the acidic TME.

O008

Topic: *ASa03 Engineered T cell therapy for solid tumours*

RASA2 CHECKPOINT ABLATION IN T CELLS BOOSTS ANTIGEN SENSITIVITY AND LONG-TERM FUNCTION

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Adoptive T cell therapies have been transformative in the treatment of a subset of hematological malignancies. However, many patients fail to respond or face relapse, with even more limited success in treating solid tumors. Targeted gene editing has the potential to enhance T cell therapeutic function and improve clinical responses. We recently have developed a platform for CRISPR KO screens in primary human T cells which nominated RASA2 as a therpuetic target for T cell engineering. Here, we identify RASA2 as a signaling checkpoint in human T cells that is downregulated upon acute TCR stimulation but increases gradually with chronic antigen exposure. RASA2 ablation enhanced sensitivity to antigen and also improved both T cell acute effector function and long-term persistence. Antigen titration showed that RASA2 ablation enhances MAP kinase signaling and CAR-T cell cytolytic activity in response to low antigen levels. Repeated tumor antigen stimulation revealed that RASA2-deficient TCR-T and CAR-T cells show a striking advantage in persistent cancer killing in vitro. Deletion of RASA2 in multiple preclinical models of TCR- and CAR-T cell therapies prolonged survival across animals xenografted with either liquid or solid tumors. Our findings from multiple genome-wide screens and preclinical studies highlight RASA2 as a promising new target to enhance both persistence and effector function in TCR-T and CAR-T cell therapies for cancer treatment.

O009

Topic: *ASa06 Rare, autoimmune and infectious diseases as cell therapy targets*

B CELL ENGINEERING FOR THE PREVENTION AND TREATMENT OF DISEASE

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Lysosomal storage diseases comprise over 70 inherited diseases characterized by deficiencies in specific lysosomal enzymes, activator proteins, or transmembrane proteins. In the case of enzyme deficiencies, a lack of enzymatic activity results in the substrates of the affected enzyme, typically lipids or carbohydrates, accumulating within cells. This leads patients to develop a range of symptoms that typically present during childhood and progress over time, culminating in severe manifestations including organ failure and neurodegeneration. While most lysosomal storage diseases are inherited as autosomal recessive traits, lysosomal storage diseases have a frequency of roughly 1 in 5,000 people. The most common treatment for lysosomal storage diseases is enzyme replacement therapy, which requires patients to undergo 1-2 hours of intravenous infusion of recombinant enzyme every 2 weeks for the duration of their lives. While enzyme replacement therapy is somewhat effective at mitigating symptoms, therapies allowing for more stable levels of enzyme and thus less frequent medical care would be transformative for these patients. Our lab has previously developed a B cell engineering technology by which CRISPR/Cas9 is used to generate B cells that both express and secrete a specific protein of interest, namely antibodies[1]. Here we describe our work using this existing platform to engineer B cells to act as a long-term and renewable source of enzymes deficient in lysosomal storage diseases. 1. Moffett, H. F. *et al*. B cells engineered to express pathogen-specific antibodies protect against infection. *Sci. Immunol*. **4**, (2019).

O010

Topic: *ASa07 New approaches in lymphocyte engineering*

GENE-TRANSFER METHODOLOGY AFFECTS CHIMERIC ANTIGEN RECEPTOR EXPRESSION AND REGULATION IN T-CELLS, AND DETERMINES ANTITUMOR POTENCY IN THE CONTEXT OF HIGH VS. LOW TARGET ANTIGEN DENSITY

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The development of CAR T-cell products with optimal safety and efficacy requires insights how distinct gene-transfer strategies affect CAR expression and ensuing T-cell function. We established a test platform comprising (i) T-cells, engineered with lentiviral (LV) and Sleeping Beauty (SB) transposon-based gene-transfer vs. CRISPR-Cas-mediated targeted CAR insertion into the TRAC locus, (ii) CAR constructs with distinct target and epitope specificity and graded affinity, and (iii) target cells with graded antigen densities. We analyzed CAR expression by droplet digital (dd)PCR, flow cytometry and dSTORM super-resolution microscopy. We found pronounced differences in absolute number, spatial distribution and dynamics of CAR expression during sequential stimulation campaigns. On the surface of LV/SB CAR T-cells, we detected a >2-fold higher CAR density compared to TRAC knock-in (KI) T-cells. Furthermore, targeted CAR insertion resulted in more consistent CAR expression compared to LV/SB-based gene-transfer in both CD4 and CD8 T-cells. In functional experiments, LV/SB CAR T-cells conferred stronger cytolytic activity compared to KI CAR T-cells in short-term lysis assays

against target cells with low antigen density (< 20 hours, multiple E:T ratios). With longer follow-up (> 20 hours) LV/SB CAR T-cells and KI CAR T-cells were equally effective. The cytolytic activity of LV/SB CAR T-cells and KI CAR T-cells against target cells with high antigen density was similar at all time points. Intriguingly, we observed a significantly lesser extent of activation-induced cell death (AICD) in KI CAR T-cells, suggesting lower CAR number and density provided protection from overstimulation. Extended analyses in vitro and pre-clinical in vivo models are ongoing. Taken together, these data show that non-targeted (LV/SB) and targeted (KI) CAR insertion result in distinct patterns of CAR expression and regulation that translate into distinct anti-tumor reactivity. Our data suggest the targeted CAR KI may be preferable for targeting high density antigens on tumor cells.

O011
Topic: *ASa07 New approaches in lymphocyte engineering*

CHARACTERIZATION OF A NOVEL RADIOHAPTEN CAPTURE SYSTEM IN CAR T CELLS FOR TRACKING IN VIVO AND IMPROVING CAR T EFFICACY

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Chimeric antigen receptor (CAR) T cells have become an integral therapeutic tool for the treatment of B-cell neoplasms. However, development of CAR T cell therapy outside of hematologic malignancies has been hampered, mainly due to lack of solid tumor efficacy, antigen escape mechanisms, and toxic side effects. As such, there is an unmet need to incorporate orthogonal methods of anti-tumor activity into CAR T cell therapy to enhance their efficacy. There is also a need for tracking CAR T cells within patients, as traditional pharmacokinetic methods are not suitable for these living drugs. Combining the technologies of CAR T engineering and radiohapten capture has recently emerged as a potential solution for non-invasive in vivo imaging of CAR T's and cellular delivery of therapeutic radioisotopes. We successfully engineered human 19BBz (anti-CD19) CAR T cells to express a humanized, high-affinity anti-benzyl-DOTA antibody (named THOR T cells) to enable in vivo monitoring of the CAR T cells with [^{86}Y]Y-DOTA-Bn PET. NSG mice bearing Raji tumors were intravenously (IV) injected with THOR T cells; we successfully visualized the transfused T-cells after [^{86}Y]Y-DOTA-Bn IV injection using PET/CT imaging. We demonstrate the pharmacokinetic utility of the THOR platform as they could be tracked using serial imaging at least as early as 7 days and as late as 42 days post T cell engraftment. Importantly, we show there is about 11-, 34- and 4-times more [^{86}Y]Y-DOTA-Bn uptake in the tumor site compared to blood, muscle and kidneys at 16 h post-tracer injection, respectively, demonstrating THOR T cells selectively uptake radioisotope at the tumor site and likely spare peripheral organs from therapeutic radiohaptens, such as [^{177}Lu]Lu-DOTA-Bn. In-progress studies will evaluate the capacity of THOR T cells to deliver cytotoxic [^{177}Lu]Lu-DOTA-Bn specifically and eliminate tumor cells.

O012
Topic: *ASa07 New approaches in lymphocyte engineering*

GENOME-SCALE SCREEN FOR SYNTHETIC DRIVERS OF T-CELL PROLIFERATION

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The engineering of patient T-cells for adoptive cell therapies has revolutionized the treatment of several cancer types. However, further improvements are needed to increase durability and response rate. While CRISPR-based loss-of-function screens have shown promise for high-throughput identification of genes that modulate T-cell response, these methods have been limited thus far to negative regulators of T-cell functions, and raise safety concerns due to the permanent nature of genome modification. Here we identify positive T-cell regulators via overexpression of ~12,000 barcoded human open reading frames (ORFs). Using this genome-scale ORF screen, we find modulator genes that may not normally be expressed by T-cells. The top-ranked genes increased primary human T-cell proliferation, activation, and secretion of key cytokines. In addition, we developed a single-cell genomics method for high-throughput quantification of the transcriptome and surface proteome in ORF-engineered T-cells. The top-ranked ORF, lymphotoxin beta receptor (LTBR), is typically expressed by myeloid cells but absent in lymphocytes. When expressed in T-cells, LTBR induced profound transcriptional and epigenomic remodeling, resulting in an increase in T-cell stemness and effector functions, as well as resistance to apoptosis and exhaustion in chronic stimulation settings. Using mutagenesis and epistasis approaches, we demonstrated that LTBR constitutive activates the canonical NFkB pathway via ligand short-circuiting and tonic signaling. Expression of several top-ranked genes, including LTBR, improved antigen-specific chimeric antigen receptor (CAR) T-cell responses in healthy donors and diffuse large B-cell lymphoma patients. Finally, the top-ranked genes discovered in $\alpha\beta$ T-cells also improved antigen-specific responses of $\gamma\delta$ T-cells, highlighting the potential for cancer-agnostic therapies. Our results provide several strategies for improving next generation T-cell therapies via induction of new synthetic cell programs.

O013
Topic: *ASa07 New approaches in lymphocyte engineering*

ADVANCED VIRAL VECTORS OVERCOME MAJOR HURDLE TOWARDS LYMPHOPREPLETE MOUSE MODELS FOR IN VIVO CAR THERAPY THROUGH RECEPTOR TARGETING

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The future of CAR therapy may be *in vivo*: Already used successfully in marketed gene therapy products, the direct (systemic) application of viral vectors for gene therapy is convenient, scalable and can result in profound improvements for patients. Harnessing the potential of *in vivo* delivery will facilitate the transition of CAR therapy from a last line treatment to a viable option for less severely diseased patients. *In vivo* CAR therapy requires transduction of lymphocytes, necessitating the development of representative lymphoreplete mouse models that help researchers devise immune evasion strategies. For these syngeneic models, viral vectors that efficiently transduce mouse lymphocytes are a crucial component, which is lacking so far, especially with regard to adeno-associated vectors (AAVs). We recently designed and characterized both lentiviral vectors (mCD8-LV) and AAVs targeted to murine CD8 by means of a designed ankyrin repeat protein (DARPin) selected through ribosome display. mCD8-LV achieved transduction efficiencies on murine splenocytes close to those of previously described LVs targeted to human CD8+ T cells. Moreover, they were highly selective for mouse CD8+ cells when added to whole blood from BALB/c mice. Added to cocultures of primary mouse T cells and CD19+ cells, mCD8-LV induced CAR-mediated killing of both endogenous and exogenous CD19+ cells. Building on recent advances in capsid design, we also generated DARPin-targeted AAVs (DART-AAVs) by inserting the DARPin into the GH2-GH3 loop of VP1. Remarkably, DART-AAVs not only achieved >99% gene transfer selectivity on primary cells, but exhibited gene transfer rates to primary mouse lymphocytes >20x higher than those of AAV2.These data suggest that receptor-particle interaction is the main determinant for mouse lymphocyte compatibility. Thus, modifying vectors with small binders simultaneously confers mouse-compatibility and high selectivity towards the therapy-relevant cell type.

O014

Topic: *ASa09 Other*

HUMAN T CELLS ENGINEERED WITH A LEUKEMIA LIPID-SPECIFIC TCR ENABLES DONOR-UNRESTRICTED RECOGNITION OF CD1C-EXPRESSING LEUKEMIA

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Acute leukemia relapsing after chemotherapy plus allogeneic hematopoietic stem cell transplantation can be treated with donor-derived T cells, but this is hampered by the need for donor/recipient MHC-matching and often results in graft-versus-host disease (GvHD), prompting the search for new donor-unrestricted strategies targeting malignant cells. Leukemia blasts express CD1c antigen-presenting molecules, which are recognized by T cell clones specific for the CD1c-restricted leukemia-associated methyl-lysophosphatidic acid (mLPA) lipid antigen. Because CD1c molecules are identical in all individuals and expressed only by mature leukocytes, and mLPA is highly enriched in malignant cells, we propose a donor-unrestricted adoptive cell therapy (ACT) strategy with T cells redirected against $CD1c^+$ acute leukemia. To assess the feasibility of ACT for acute leukemia with CD1c-restricted mLPA-specific T cells, we generated a library of lentiviral vectors encoding a panel of human mLPA-specific TCRs and we transduced Jurkat 76 cells and human primary T cells. We thus identified the lead mLPA-specific TCR (DN4.99) to target $CD1c^+$ acute leukemia as the one that could be efficiently transduced by LV into primary T lymphocytes from any donor with high level of expression and strong endogenous TCR repression and displayed the strongest reactivity against $CD1c^+$ leukemia cells and mLPA specificity. We generated mLPA-specific T cells that killed diverse CD1c-expressing leukemia blasts in vitro, but not normal circulating $CD1c^+$ monocytes, B cells and DCs. Moreover, the adoptive transfer of DN4.99 TCR-transduced T cells delayed the progression of 3 models of leukemia xenograft in NSG mice, an effect that is boosted by mLPA-cellular immunization with monocyte-derived DCs. Our results highlighted a strategy to redirect T cells against leukemia via transfer of a lipid-specific TCR that could be used across MHC barriers with reduced risk of graft-versus-host disease.

EP001
Topic: *ASa01 CAR T cell therapies in hematological malignancies*

ENGINEERING IMMUNOTHERAPY RESISTANT HEMATOPOIESIS TO TREAT HIGH-RISK ACUTE MYELOID LEUKEMIA

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Acute myeloid leukemia (AML) is the most common leukemia but has the lowest survival rate. While highly effective for other leukemia/lymphoma, adoptive immunotherapies for AML are hampered by absence of leukemia-restricted targets, the most suitable of which are often shared with healthy hematopoietic stem/progenitor cells (HSPC). This restricts applicability of anti-myeloid/HSPC therapies to a limited time window before allogeneic HSPC transplant (HSCT), thus limiting the chances for disease eradication. Here, we exploited base-editing technology to introduce new antigenic variants of FLT3 and/or CD123 receptors in healthy HSPC to generate ''stealth'' hematopoietic lineages that are resistant to immunoglobulin-based drugs/CAR-T-cells. Differently from other approaches aimed at abrogating non-essential lineage markers (e.g.CD33), targeting genes directly involved in malignant transformation and essential for leukemia survival will minimize the risks of relapse by antigen-loss/-downregulation. By coupling transposon-based library screenings with orthologous/not-conserved amino-acid substitutions, we identified one *FLT3*-specific gRNA that, when coupled with adenine base-editor (ABE8e-TadA-8e-V106W), generates an extracellular-domain variant that preserves physiologic ligand-binding and subsequent intracellular kinase activation but is not recognized by a therapeutic monoclonal-antibody (mAb) and allows survival to CAR-T-cell *in-vitro* killing. Similarly, we identified one genomic-region of *CD123* that, when targeted by adenine base-editors, confers resistance to 3 different mAbs that are currently in advanced phases of preclinical development. Using ABE8e-mRNA electroporation on human CD34+ HSPCs, we achieved up to 60% base transitions in *FLT3/CD123*, both alone and in multiplex. After transplant into NBSGW-mice, treated HSPC sustained long-term haematopoiesis while maintaining editing levels comparable to input cells (average $\sim$40%), thus indicating successful editing of the most-primitive HSC. Importantly, treatment with FLT3-CAR-T-cell partially spared human HSC (CD34 + 38-90+) and myeloid cells in the marrow of mice engrafted with edited HSPCs compared to controls. If successful, our strategy combined with conventional allogeneic-HSCT may enable more efficacious/less-toxic therapeutic options to AML and several other blood cancers.

EP002
Topic: *ASa02 CAR and TCR targets*

VEGF-A COMPETES WITH VEGFR-2-REDIRECTED CAR-T CELLS FOR TARGET BINDING AND IMPEDES FUNCTION

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The tumor vasculature represents an appealing target for CAR-T cell therapy. Indeed, antigens expressed by endothelial cells of tumor blood vessels are more stably and homogeneously expressed in comparison to those found on tumors cells, are broadly shared across cancer types, and are readily accessible to circulating CAR-T cells. In this study, we sought to explore limitations to the efficacy of second-generation (2G) murine CAR-T cells redirected against the vascular endothelial growth factor receptor-2 (VEGFR-2) via the well-characterized single-chain variable fragment DC101, with the aim to identify rational combination treatments allowing enhanced tumor control. We demonstrated that anti-VEGFR-2 CAR-T cells exhibited high activity levels in vitro but had negligible impact on the growth of subcutaneous B16 melanoma tumors. While we observed no downregulation of VEGFR-2 post CAR-T cell transfer, VEGF-A levels were significantly elevated. Despite that VEGF-A has been implicated in the deregulation of adhesion molecules, such as ICAM-1 and VCAM-1, we observed no such effects in our study. By computational modeling, we predicted an overlap in VEGFR-2 binding by VEGF-A and the DC101 scFv, and we subsequently showed that soluble VEGF-A (but not VEGF-C nor VEGF-D), physically competed with the CAR-T cells for binding their shared target, VEGFR-2. This competition resulted in impaired CAR-T cell adhesion and effector function in vitro that could be restored in the presence of anti-VEGF-A antibody. Moreover, we demonstrated that coadministration of anti-VEGF-A antibody enhanced CAR-T cell persistence and control of B16 tumors without causing toxicity, and was associated with reduced frequencies of PD-1^+ $Ki67^-$ and LAG-3^+ $Ki67^-$ CAR-T cells in the TME. To the best of our knowledge this is the first example of impaired CAR-T cell function due to competition by a soluble ligand. Together our work highlights a novel combinatorial strategy that can effectively ameliorate tumor vasculature targeted CAR-T cell therapy.

EP003
Topic: *ASa03 Engineered T cell therapy for solid tumours*

UNICAR CAR T CELL THERANOTICS FOR DIAGNOSTIC IMAGING AND THERAPY OF PROSTATE CANCER

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CAR T-cell therapy achieved unparalleled clinical success rates for treatment of patients with hematological malignancies. However, progress and clinical translation towards solid tumors is slow and hampered by many factors e.g. increased complexity, high heterogeneity and an immunosuppressive tumor microenvironment. Thus, CAR T-cell therapy alone might not result in durable antitumor responses. Combinatorial approaches are promising strategies to improve CAR T-cell efficacy in solid tumor treatment. In this regard, we here aim to combine conventional cancer theranostics with CAR T-cell immunotherapy in one single approach. By using the well-established UniCAR system, a novel, multifunctional tool termed PSCA-IgG4 target module (TM) was developed for dual prostate cancer theranostics. It comprises a human PSCA-specific binding domain, the hinge and Fc-domain of human IgG4 molecules as well as the UniCAR epitope E5B9. As shown by in vitro assays with PSCA-positive and PSCA-negative prostate cancer cells, the novel TM redirected UniCAR T cells for efficient tumor cell lysis in a strictly antigen- and TM-dependent manner. After radiolabeling with copper-64 or actinium-225, the novel PSCA-IgG4 TM was successfully applied for diagnostic imaging and targeted radioimmunotherapy. The ^{64}Cu-labeled PSCA-IgG4 TM showed maximal tumor accumulation with optimal tumor-to-background ratios after 1.5 days. Furthermore, targeted alpha-therapy with the ^{225}Ac-labeled TM significantly delayed the outgrowth of established tumors in mice. In summary, the here presented, novel PSCA-IgG4 TM is a promising candidate for dual theranostics of prostate cancer that may help to overcome present hurdles in solid tumor therapy. After radiolabeling it facilitates not only targeted alpha-therapy and diagnostic imaging of PSCA, but can be also repurposed as a TM for UniCAR T-cell immunotherapy.

EP004
Topic: *ASa03 Engineered T cell therapy for solid tumours*

EPITOPE SPREADING DRIVEN BY THE JOINT ACTION OF CAR T CELLS AND PHARMACOLOGICAL STING STIMULATION COUNTERACTS TARGET ANTIGEN ESCAPE

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Target antigen loss has emerged as a major cause of relapse after chimeric antigen receptor T (CART)-cell therapy. We reasoned that the combination of CART cells, with the consequent tumor debulking and release of antigens, together with an immunomodulatory agent, such as the stimulator of interferon gene ligand (STING-L) 2′3′-cyclic GMP-AMP (2′3′cGAMP), may facilitate the activation of an endogenous response to secondary tumor antigens able to counteract this tumor escape mechanism. Using two immune-competent solid tumor models we showed that CART-cell treatment led to the emergence of tumor cells that lose the targeted antigen, recreating the cancer immunoediting effect of CART-cell therapy. The combination of CART cells with the intratumoral delivery of 2′3′cGAMP showed a synergistic effect, being able to restrain the growth of STING-L-treated and untreated tumors. Interestingly, a secondary immune response against non-CAR-targeted antigens, as determined by MHC-I-tetramer staining, was fostered and the intensity of this epitope spreading effect correlated with the efficacy of the combination. This was consistent with the oligoclonal expansion of host T cells, as revealed by in-depth TCR repertoire analysis. Moreover, only in the combination group did the activation of endogenous T cells translate into a systemic antitumor response. Importantly, the epitope spreading and the antitumor effects of the combination therapy were fully dependent on host STING signaling and Batf3-dependent dendritic cells, and were partially dependent on perforin release by CAR T cells. This indicated that the CAR T-cell cytotoxic activity together with the immunostimulatory action of STING-L was necessary for the combination to be effective. Our data show that 2'3'cGAMP is a suitable adjuvant to combine with CART-cell therapy and to prevent tumor escape by target antigen loss.

EP005
Topic: *ASa03 Engineered T cell therapy for solid tumours*

TARGETING THE EXTRA DOMAIN A FROM FIBRONECTIN FOR CANCER THERAPY WITH CAR-T CELLS

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Tumor microenvironment (TME) of solid tumors creates remarkable barriers impairing the anti-tumor efficacy of CAR-T cells. Targeting key antigens of this TME with specific CARs might

overcome this limitation and improve their effectiveness. We have generated a CAR specific for the splice variant EDA of fibronectin, which is highly expressed in many types of tumors and not in healthy tissues. EDA CAR-T cells recognized and killed EDA-expressing tumor cell lines and rejected EDA-expressing tumors in immunocompetent mice. Notably, EDA CAR-T cells showed an antitumor effect in mice injected with EDA-negative tumor cells lines when the tumor stroma or the tumor endothelial cells express EDA. Thus, EDA CAR-T administration delayed tumor growth in immunocompetent 129Sv mice challenged with the EDA-negative teratocarcinoma cell line F9. Remarkably, the human version of EDA CAR, including the human 41BB and CD3z endodomains, exerted antitumor activity in NSG mice challenged with the human hepatocarcinoma cell line PLC, which highly expresses EDA in the tumor stroma and the endothelial vasculature. EDA CAR-T cells exhibited a tropism for EDA expressing cells and no toxicity was observed in tumor bearing or in healthy mice. These results suggest that targeting the tumor-specific fibronectin splice variant EDA with CAR T cells is feasible and offers a therapeutic option widely applicable to different types of cancer.

EP006

Topic: *ASa03 Engineered T cell therapy for solid tumours*

BOOSTING CAR T CELL EFFICACY BY MODULATION OF THE TUMOR MICROENVIRONMENT IN GLIOBLASTOMA

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Despite our studies that support protein p-xx as suitable TAAs in gliomas, and the significant overall survival observed in our pre-clinical models after treatment with p-xx-specific CAR T cells, tumor-bearing-mice eventually succumb to the disease. We hypothesized that a multifactorial *in situ* immunosuppressive response drives the expression of exhaustion markers such as PD −1 on infiltrating T cells, leading to tumor re-growth. It is suggested that the possibility of combining CAR T adoptive transfer with an adjuvant therapy that modulates the immunosuppressive tumor microenvironment (TME) may lead to a better outcome We analyzed TME composition after CAR T cell treatment by flow cytometry, this way we identified a prominent infiltrating cell population that may be exerting a suppressive action. Using a network-based prediction algorithm a potential drug candidate to target those cells was proposed. After a cycle of treatment with such drug, the target-cell infiltration in brain tumors was decreased by 4-fold. Finally, glioma-bearing mice that underwent two cycles of adjuvant therapy along with our usual CAR T regime experienced a significant extension of their median survival compared to the monotherapy modality. In conclusion, our study shed light in the possible role of infiltrating suppressive cell populations in CAR T cell therapy response in GBM. We showed that by specifically targeting such TME component it is possible to improve the activity of p-xx-specific CAR T cells. This indicates that combination of adoptive cell therapy with drugs targeting components of the TME may be a feasible strategy for the treatment of glioma patients.

EP007

Topic: *ASa06 Rare, autoimmune and infectious diseases as cell therapy targets*

TACROLIMUS-RESISTANT SARS-COV-2-SPECIFIC T-CELL PRODUCTS TO PREVENT AND TREAT SEVERE COVID-19 IN IMMUNOSUPPRESSED PATIENTS

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As solid organ transplant (SOT) recipients receive therapeutic immunosuppression that compromises their immune response to infections and vaccines, they have a high risk of developing severe COVID-19 and an increased risk of COVID-19-related death. The constant immunosuppression may result in reduction of efficiency of immunotherapy. Thus, a therapy is required that enables efficient viral clearance against SARS-CoV-2 whilst simultaneously maintaining immunosuppressive treatment in transplant patients to prevent transplant rejection. Here, we propose adoptive transfer of SARS-CoV-2-specific T-cells rendered resistant to the common immunosuppressant Tacrolimus to optimize performance in immunosuppressed patients. By using a GMP-compatible, vector-free CRISPR-Cas9-based, gene-editing approach, we knocked out the cell-intrinsic adaptor protein FKBP12, which is required for the immunosuppressive function of Tacrolimus, and generated Tacrolimus-resistant SARS-CoV-2-reactive T-cell products (TCPs) from the blood of SARS-CoV-2 convalescent donors. Functional and phenotypical characterization of these products in depth, including single cell CITE- and TCR sequencing analyses, showed that the gene modification did not impact the functional potency of the Tacrolimus-resistant SARS-CoV-2-specific TCPs compared to unmodified SARS-CoV-2-specific TCPs, but confirmed resistance to Tacrolimus and sensitivity to alternative immunosuppressive drugs from the same class (safety switch). Based on the promising results, we aim to clinically validate this approach in transplant recipients. Our strategy has the potential to prevent or ameliorate severe COVID-19 in the SOT setting whilst preventing allogeneic organ rejection. Our platform technology allows targeting of different SARS-CoV-2 variants and other viruses, thus multiplying its potential therapeutic use.

EP008

Topic: *ASa07 New approaches in lymphocyte engineering*

NANOPARTICLE-SENSITIZED PHOTOPORATION AS AN EMERGING NON-VIRAL ALTERNATIVE FOR T CELL ENGINEERING WITH NUCLEIC ACIDS

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Despite the clinical success and FDA approval of several CAR products, the manufacturing of T cell therapies presents unique challenges that need to be addressed to accelerate development and make such treatments more accessible to patients. At present, CAR T cells are typically generated by viral transduction or electroporation, which is associated with several drawbacks, including high manufacturing cost, safety issues, vector capacity constraints, and cytotoxicity. Among alternative physical transfection methods, laser-induced photoporation has recently emerged as a highly promising technology, combining high transfection efficiency and low cytotoxicity. Photoporation makes use of light-responsive nanoparticles (NPs), such as polydopamine (PDNPs) or iron oxide nanoparticles (IONPs) to induce transient membrane permeabilization by distinct photothermal effects, including direct heating or the generation of vapor nanobubbles (VNBs), allowing subsequent entry of external cargo molecules into the cells. We have recently demonstrated that polydopamine sensitized photoporation enables safe and efficient delivery of mRNA in expanded human T cells, yielding higher numbers of living transfected cells compared to state-of-the-art nucleofection [1]. In the present study, we extend our investigation to evaluate mRNA delivery in unstimulated human pan CD3+ cells, focusing on the investigation of cell phenotype, metabolic and proliferation rates, and activation status after treatment with photoporation or nucleofection. With this study we aim to provide new insights on the impact of the applied transfection method on the potency of engineered T cells. [1] Harizaj A, Wels M, Raes L, Stremersch S, Goetgeluk G, Brans T, … Braeckmans K. Photoporation with Biodegradable Polydopamine Nanosensitizers Enables Safe and Efficient Delivery of mRNA in Human T Cells. Adv Funct Mater 2021;31:2102472.

EP009
Topic: *ASa07 New approaches in lymphocyte engineering*

FUNCTIONAL CRISPR DISSECTION OF GENE NETWORKS CONTROLLING HUMAN REGULATORY T CELL IDENTITY

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Regulatory T cells (Treg) play a critical role in human immune homeostasis by suppressing inflammation and autoimmunity. Treg cells must maintain suppressive functions even in pro-inflammatory microenvironments, and this maintenance is in large part controlled by transcriptional regulation. The transcription factor (TF) FOXP3 is known to be crucial for establishment and maintenance of Treg cell identity. The complete set of critical transcription factors in human Treg cells and their downstream transcriptional targets remain unknown. Using both novel pooled as well as arrayed Cas9 ribonucleoprotein (RNP) screens in primary human Tregs under pro-inflammatory conditions we identified TFs that regulate expression of key Treg and effector T cell markers. We then deeply profiled a subset of these TFs by single cell RNA sequencing (scRNA-seq) of edited human Treg cells, revealing distinct gene modules that preserve Treg transcriptional identity. These modules highlighted key genes of Treg cell functions regulating cytokine secretion, transcriptional regulation and metabolism in Treg cells. We find that FOXP3 and PRDM1 individually regulate independent gene modules, while FOXO1 and IRF4 co-repress their own. We have also discovered that HIVEP2—which has not been previously implicated in Treg cell functions—participates with SATB1 in co-activating yet another gene module. Interestingly, this gene module is Treg cell-specific and is in this form not active in CD4 effector T cells. By identifying key genetic programs controlled by individual TFs that shape Treg or Teff cell identity, we gain knowledge that could be used to ultimately engineer Treg-based cell therapies.

EP010
Topic: *ASa07 New approaches in lymphocyte engineering*

ENGINEERING AVIDCARS FOR SMALL MOLECULE-DEPENDENT CAR T CELL REGULATION

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CAR T cells have shown impressive efficacy in the treatment of B cell malignancies. However, one drawback of this highly potent cellular therapy is its poor controllability after administration to the patient. To address this limitation, we engineered avidity-controlled CARs (AvidCARs) that can be regulated by administration of a small molecule drug. Briefly, the molecular mechanism of these ON-switch AvidCARs is based on low affinity antigen binding domains, which insufficiently activate the respective CARs in a monomeric state. However, upon small molecule-mediated CAR dimerization, the bivalent interaction with two antigen molecules on the target cell leads to avidity effects, thereby amplifying the low affinities of the individual antigen binding domains. We demonstrate both *in vitro* and *in vivo* that the activity of AvidCAR T cells is strongly dependent on small molecule-mediated CAR dimerization, thereby enabling the functional control of CAR T cells by administration of a homodimerizing small molecule drug.

EP011
Topic: *ASa09 Other*

A NOVEL TUMOR MODEL FOR THE STUDY OF CELLULAR IMMUNOTHERAPY USING HUMANIZED MICE

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Cancer treatment has changed fundamentally during the last several years due to the introduction of new immunotherapeutic modalities. With the use of genetically modified T cells expressing chimeric antigen receptors directed against the antigen CD19, initial complete remission rates of 50 - 90% in patients with relapsed or refractory hematological cancers such as acute lymphoblastic leukemia or diffuse large cell B-cell lymphoma can be achieved. This new treatment modality however is associated with life-threatening adverse events such as cytokine release syndrome and severe neurotoxicity. Also, translation of this potentially transformative approach to non-hematological, solid cancers has yet to be accomplished. Therefore, there is a need to accelerate research on generating translational models for such immunotherapies. Here we propose a novel immunocompetent tumor model using humanized mice engrafted with autologous Epstein-Barr virus transformed $CD19^+$ lymphoblastoid cell lines (LCLs). Upon subcutaneous injection of autologous LCLs, humanized mice reliably develop solid subcutaneous tumors, which are infiltrated by tumor infiltrating lymphocytes. The reconstituted immune system mediates a tumor specific immune response, as shown by loss of tumor control in non-reconstituted mice and tumor specific secretion of IFN-gamma by endogenous human T cells. The autologous tumor cells can be manipulated further, e.g. to tune immunogenicity. Thus, this humanized mouse model allows for the study of interactions of human tumor and endogenous human immune system as well as the interplay with adoptively transferred cells. The described model might in the future enable more integrated *in vivo* testing of genetically engineered immune cells for cancer therapy.

ICLE 2022–Poster Presentations

P001

Topic: *ASa01 CAR T cell therapies in hematological malignancies*

NOVEL STRATEGIES TO ENHANCE THE SAFETY OF CAR T-CELL IMMUNOTHERAPY: THE IMSAVAR PROJECT

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Adoptive immunotherapy with CAR-T-cells is a transformative treatment in hematology but can be associated with significant toxicity from e.g. cytokine release syndrome (CRS). Non-clinical testing to assess CRS is not standardized and there is a strong medical need to establish algorithms assessing the propensity of novel CAR-T-cell products inducing CRS and other toxicities. imSAVAR – immune safety avatar – is an EU Innovative Medicines Initiative project that tackles this challenge in a joint academia-industry consortium seeking to establish a platform for assessing the utility of innovative non-clinical models and endpoints for enhancing the safety assessment of e.g. CAR-T-cells. Step 1 comprises a systematic literature review on pathophysiology and clinical occurrence of CRS after CAR-T-cell therapy, followed by a survey amongst academia and industry stakeholders to identify gaps of non-clinical test systems used to assess CRS in step 2. Step 3 is a multi-stakeholder workshop to determine a roadmap for studies establishing a new set of non-clinical assays and endpoints that will be validated and help mitigate clinical safety concerns in Step 4. First, a conceptual map of CRS pathogenesis as an immune-related adverse outcome pathway (irAOP) comprising key molecular and cellular events was established and to each event existing and emerging non-clinical assays were allocated. Second, a survey was conducted with $n = 3$ CAR-T-cell products from within the consortium. Test systems available in imSAVAR include *in vitro* co-culture assays, genomic approaches, and organ-on-a-chip models. All data will be correlated with clinical data to identify optimal non-clinical assays for enhancing assessment and prediction of CRS. imSAVAR established an irAOP that will enable the development and validation of novel non-clinical assays that aim to enhance the characterization of CAR-T-cell-associated CRS during non-clinical development. This effort is ultimately anticipated to enhance the safety assessment of therapeutic CAR-T-products, thus potentially accelerating patient access to CAR-T products.

P002

Topic: *ASa01 CAR T cell therapies in hematological malignancies*

MECHANISMS OF RESISTANCE TO CHIMERIC ANTIGEN RECEPTOR T CELL THERAPY IN MULTIPLE MYELOMA

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Introduction: Dual antigen targeted chimeric antigen receptors (CARs) may overcome tumor heterogeneity and reduce antigen escape. CARs using A Proliferation Inducing Ligand

(APRIL) as binding domain, the natural high affinity ligand of B cell maturation antigen (BCMA) and Transmembrane Activator and CAML Interactor (TACI) on MM, have potent anti-tumor activity in pre-clinical models. However, a clinical trial was terminated early due to lack of responses. Because soluble APRIL induces growth signals in MM, we investigated mechanisms of resistance with different CAR configurations.

Methods: We generated retroviral vectors encoding monomeric (m) or trimeric (t) APRIL-CARs, each using CD28-CD3zeta (28z) or 41BB-CD3zeta (BBz) endo-domains, two different BCMA single chain variable fragment (scFv)-BCMA-CARs, or non-signaling controls (D). CARs were expressed in healthy donor T-cells for functional characterization.

Results: APRIL-CAR T-cells (m28z, mBBz, t28z, tBBz) efficiently killed MM cells in a co-culture stress-test with repetitive tumor challenges, and tBBz were more polyfunctional than mBBz CAR T-cells. As soluble APRIL promotes MM cell growth via BCMA activation, we assessed BCMA signaling (NFκB) and proliferation of MM cells co-cultured with APRIL- or scFv-DCARs. Both APRIL- and scFv-DCARs activated the NFκB pathway in MM1S or NCI-H929 cells, but this contact did not alter MM cell proliferation in vitro. Contact with scFv-CARs rapidly led to BCMA loss on target cells that was less pronounced with APRIL CARs. In two different mouse xenograft models (NCI-H929 and MM1S), anti-tumor function of tBBz-CAR T-cells was most potent, and we found no tumor promoting capacity of APRIL- or BCMA-D CAR T cells in vivo.

Conclusions: We have identified an APRIL-CAR with potent anti-tumor function and show that BCMA persists on target cells at higher levels than upon exposure to scFv-BCMA-CARs. Despite NFκB pathway activation upon APRIL- or scFv-CAR binding, neither APRIL- nor scFv-BCMA-CAR T cells promoted tumor progression in mice.

P003

Topic: *ASa01 CAR T cell therapies in hematological malignancies*

IMMUNE EFFECTOR CELLS TRANSDUCED WITH AN NKG2D CHIMERIC ANTIGEN RECEPTOR TO TREAT ACUTE MYELOID LEUKEMIA

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Acute Myeloid Leukemia (AML) is a hematological malignancy still incurable for almost all patients. Chimeric antigen receptor (CAR) therapy is showing promising results in other hematological disorders but remains challenging in AML since no specific antigens have been described yet. Using NKG2D as a CAR, a receptor with 8 ligands (MICA/B and ULBP-1-6) overexpressed in several tumors, could surmount AML targeting limitations. T cells are considered the gold standard immune effector cells for CAR therapy, but they show some toxicities that could be overcome using other cells such as activated and expanded natural killer cells (NKAE), that have natural anti-tumor properties and a short lifespan within the organism. In this project we perform a comparison between the AML anti-tumor activity of peripheral blood NKAE and T cells lentivirally transduced with an NKG2D-41BB-CD3z CAR. Our first *in vitro* results show modest but stable NKAE transduction levels for up to 13 days and robust T cell transduction for at least 15 days. Cytotoxicity of both NKAE and T cells was significantly higher when transduced with the CAR, achieving a quasi-total lysis of AML cells after 24 hours of coculture. Despite these encouraging results, we explored whether other co-stimulatory domains such as CD27z could provide higher cytotoxic properties so as to reduce effector:target ratios, thus decreasing toxic effects. Our preliminary results on T cells show they can also be efficiently transduced with the NKG2D-CD27z-CD3z CAR and exhibit significantly more anti-tumor effect than untransduced T cells. Interestingly, this cytotoxicity is also greater than the one shown by NKG2D-41BB-CD3z CAR transduced T cells. Further experiments using NKAE cells may be done to assess which of the constructs provides better cytotoxic profile against AML. *In vivo* experiments using immunodeficient NSG mice may be carried out to verify which CAR therapy results more efficient against AML.

P004

Topic: *ASa01 CAR T cell therapies in hematological malignancies*

LOWERING THE BINDING STRENGTH OF ANTI-CD19 CAR-T CELLS CAN REDUCE TOXICITY BUT AT THE COST OF IN VIVO ANTI-TUMOR EFFICACY

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Adoptive T cell transfer of high affinity chimeric antigen receptor (CAR-) T cells targeting CD19 has shown impressive clinical success. However, the development of potentially life-threatening side-effects still hinders broader clinical application. Recently, it was suggested that low affinity CAR-T cells might represent a strategy to maintain clinical efficacy while improving safety. However, a more systematic investigation of the role of CAR binding affinities is still missing. Therefore, we combined *in silico* predictions of single point mutations in the scFv-region and k_{off}-rate measurements to generate anti-CD19 CARs with a broad spectrum of reduced binding qualities and compared corresponding CAR-T cells *in vitro* and *in vivo* with commonly

used high affinity CAR-T cells. We were surprised that *in vitro* functionality profiles remained similar over a large range of CAR binding strengths. Only extremely weak binders showed reduced cytokine production and cytotoxicity. In strong contrast, already minor changes in CAR binding strength mediated significant differences *in vivo*. Lowering CAR/antigen binding correlated with a strong reduction of *in vivo* toxicities as determined in a humanized CRS mouse model. However, efficient tumor clearance was also dependent on the binding strength of the CAR. While high affinity CAR-T cells showed the best *in vivo* functionality, they also produced the strongest side effects. Impressively, cell number titrations demonstrated that for high affinity CAR-T cells, *in vivo* efficacy was maintained over a quite large range, while toxicity was decreasing with lower cell doses, allowing an optimum window with mild side effects, but without significant efficacy impairment. In summary, our data confirm that affinity reduction of CAR-target binding can be used to reduce CRS-toxicity. However, as it negatively affects tumor clearance, it might have limitations. Therefore, lowering the dose of high affinity CAR-T cells might be a more practical approach to minimize toxicities without losing *in vivo* efficacy.

P005

Topic: *ASa01 CAR T cell therapies in hematological malignancies*

NKG2D-CAR TCD45RA- CELLS BYPASS THE CANONIC TUMOR IMMUNOESCAPE MECHANISMS

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Background: The interactions between NKG2D receptor expressed on immune effector cells and its ligands (NKG2DL) expressed on leukemic cells are essential for anti-leukemia immune control. Leukemic blasts may develop different immune scape strategies like TGF-b release or NKG2DL shedding, which hinders anti-leukemia immune surveillance. NKG2D-CAR T cells have shown robust anti-leukemia effects *in vitro*. In a murine model of human T-ALL, mice treated with NKG2D-CAR T cells showed delayed tumor progression and prolonged survival, but the therapy failed to cure the animals. The aim of this study is to elucidate the resistance mechanisms behind these observations.

Methodology and Results: ELISA experiments revealed presence of sNKG2DL in the supernatant of leukemia cell lines. The sera of patients showed higher levels of sNKG2DL than those from healthy donors. FCM analysis showed impairment in NKG2D expression and enhanced proliferation of NKG2D-CAR T cells after incubation with supraphysiological concentrations of sNKG2DL, while lower concentrations had no effect. Europium-TDA assays showed NKG2D-CAR $T_{CD45RA-}$ cells cytotoxicity was not affected even at the higher concentrations used. Colony forming units (CFU) and side-population assays revealed NKG2D-CAR $T_{CD45RA-}$ cells were able to target leukemia initiating cells (LIC), as reduced number of colonies and percentage of side population were found after co-culture of leukemic cells with CAR T cells. Characterization of LIC after CAR T cell co-culture showed no alteration on NKG2DL expression; however, RNAseq analysis revealed upregulation of stemness and proliferation-related genes.

Conclusions: NKG2D-$CAR_{CD45RA-}$ T cells showed resistance to NKG2D downregulation upon exposure to sNKG2DL. The enrichment in stemness and proliferation markers in leukemic cells after treatment with NKG2D-$CAR_{CD45RA-}$ T cells, could indicate the inability of the therapy to target this compartment, and could explain treatment failure in the murine model. However further experiments need to be done to confirm these data.

P006

Topic: *ASa01 CAR T cell therapies in hematological malignancies*

CD33-DIRECTED IMMUNOTHERAPY WITH THIRD-GENERATION CHIMERIC ANTIGEN RECEPTOR T CELLS AND GEMTUZUMAB OZOGAMICIN IN INTACT AND CD33-EDITED ACUTE MYELOID LEUKEMIA AND HEMATOPOIETIC STEM AND PROGENITOR CELLS

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Immunotherapies, such as chimeric antigen receptor (CAR) modified T cells and antibody-drug conjugates (ADCs), have revolutionized the treatment of cancer, especially of lymphoid malignancies. The application of targeted immunotherapy to patients with acute myeloid leukemia (AML) has been limited in particular by the lack of a tumor-specific target antigen. Gemtuzumab ozogamicin (GO), an ADC targeting CD33, is the only approved immunotherapeutic agent in AML. In this study, we introduce a CD33-directed third-generation CAR T cell product (3G.CAR33-T) for the treatment of patients with AML. 3G.CAR33-T cells could be expanded up to the end-of-culture,

i.e. 17 days after transduction, and displayed significant cytokine secretion and robust cytotoxic activity when incubated with CD33-positive cells including cell lines, drug-resistant cells, primary blasts as well as normal hematopoietic stem and progenitor cells (HSPCs). When compared to second-generation CAR33-T cells, 3G.CAR33-T cells showed higher viability, increased proliferation and stronger cytotoxicity. Also, GO exerted strong anti-leukemia activity against CD33-positive AML cells. Upon genomic deletion of CD33 in HSPCs, 3G.CAR33-T cells and GO preferentially killed wildtype leukemia cells, while sparing CD33-deficient HSPCs. Our data provide evidence for the applicability of CD33-targeted immunotherapies in AML and its potential implementation in CD33 genome-edited stem cell transplantation approaches.

P007

Topic: *ASa01 CAR T cell therapies in hematological malignancies*

BI-SPECIFIC LOGIC GATED CAR T CELLS ELIMINATING CD19+ TUMORS WHILE PREVENTING NEUROTOXICITY VIA PERICYTE-INDUCED INHIBITION

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Chimeric antigen receptor (CAR) T cell therapy has revolutionized the management of selected hematological malignances. In fact 4 different anti-CD19 CAR T cell products to became the first FDA-approved genetically engineered immune cell therapeutics. However, these therapies come with significant toxicities, the main ones being cytokine-release syndrome and neurotoxicity. We recently reported a potential mechanism of CD19 CAR T cell-related neurotoxicity, identifying ''off-tumor'' expression of CD19 in brain pericytes using single-cell transcriptomics, as well as in vitro and in vivo mouse modeling (Parker/Migliorini Cell 2020). Pericytes are a key cell population responsible for the maintenance of blood-brain-barrier (BBB) integrity and their attack triggers BBB disruption and neurotoxicity. Our findings opened the opportunity to engineer a new generation of CAR T cells based on a ''logical'' CAR design that can only attack $CD19^+$ malignant B cells and spare healthy brain pericytes. To achieve this, we generated different candidates of bi-specific anti-CD19 activatory anti-pericyte marker inhibitory CAR constructs using four different inhibitory intracellular domains derived from T or NK cells. These inhibitory CAR T cells are specific for the canonical pericyte marker CSPG4 and were tested in vitro for inhibition of cytotoxicity, proliferation and proinflammatory cytokine production upon co-culture with the $CD19^+$ human B-ALL cell lines engineered to express CSPG4 as a proof of concept. We generated proof of concept evidence of killing and cytokine secretion inhibition upon bispecific anti inhibitory CSPG4/activatory CD19 CAR-Ts co-culture with CSPG4/CD19 double positive human B-ALL cell lines. In parallel, in an attempt to identify reliable pericyte cell surface markers, we analyzed human healthy and meningioma brain samples collected in our center after pericytes and endothelial cell sorting, using single-cell transcriptomics. The identified markers will be used to generate novel inhibitory CAR T cell products that will be tested for neurotoxicity prevention.

P008

Topic: *ASa01 CAR T cell therapies in hematological malignancies*

SITE-SPECIFIC IN VIVO T CELL ENGINEERING TO EXPRESS CHIMERIC ANTIGEN RECEPTORS

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T cells engineered to express chimeric antigen receptors (CAR-T cells) show great promise in treating hematological malignancies. However, the *ex vivo* culturing and engineering of autologous T cells is cumbersome, time consuming and expensive. Here, we propose to develop methods for the in vivo engineering of CAR-T cells, obviating pre-conditioning and reducing timelines and expenses without risking GVHD or graft rejection. We will use adeno associated viral vectors (AAV) coding for the CAR as well as for a nuclease targeting the CAR into the TCR alpha constant (TRAC) locus. Similar targeting schemes *ex vivo* were previously shown to reduce the variegated expression and premature T cell exhaustion associated with the commonly used integration techniques for CAR genes (retrovectors, lentivectors or transposons). Uniquely, our design allows co-encapsulation of the CAR gene together with the nuclease gene in a single AAV to facilitate efficient *in vivo* T cell targeting. Our AAV will encode ''ARCUS'', an engineered derivative of the I-CreI homing endonuclease, with the CAR gene flanked by homologous arms. In order to control cell specific transduction of human T cells, we used DARPins (designed ankyrin repeat proteins) which target the human CD8 receptor. Preliminary results include successful *in vitro* transduction of ARCUS-CAR AAV (MOI 500-750K) followed by CAR expression (2%-10%) under TRAC endogenous promoter. Furthermore, IFN-gamma secretion was elevated when CAR T cells (but not un-transduced) were co- incubated with target cells NALM6 $CD19^+$ compared to K562 $CD19^-$. We next aim to use this technique *in vivo*, to target human T cells in NSG mice that harbor leukemic B cells. Site specific *in vivo* engineering of CAR T cells may revolutionize the safety, efficacy and scalability of cellular immunotherapies for hematological malignancies and beyond.

P009

Topic: *ASa01 CAR T cell therapies in hematological malignancies*

HOME BREWN 3G.CD19.CAR T CELLS FOR RELAPSED/REFRACTORY CHRONIC LYMPHOCYTIC LEUKEMIA (CLL) – (HD-CAR-1)

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CD19-directed CART therapy is now part of the clinical armamentarium for treating patients with various B-cell lymphoma. Disease-inherent T-cell dysfunction might hamper CD19.CARTs in CLL . Here we show results obtained with 3rd generation CD19-directed CARTs in patients with high-risk r/r CLL. HD-CAR-1 is an IIT evaluating efficacy and safety of escalating doses of CD19-directed CARTs comprising CD28 and 4-1BB as costimulatory molecules in patients with advanced B-cell malignancies after FC lymphodepletion. Leukapheresis, manufacturing, administration, patient monitoring and follow-up were all conducted in-house. Patients with CLL were eligible if they had failed chemoimmunotherapy and at least one pathway inhibitor and/or alloHCT. 32 patients were enrolled. Seven patients with CLL had a median age of 62 years and up to ten prior treatment lines. All patients had failed at least one pathway inhibitor, and three alloHCT. TP53 abnormalities were present in five patients. Disease status at lymphodepletion was CR in three patients, PR in two patients, SD in one patient and PD in one patient. Despite heavy pretreatment, leukapheresis yielded sufficient T-cell numbers for manufacturing. Dose levels administered were I ($=0.1x10^7$ CARTs/m^2) in one patient, II ($=0.5x10^7$ CARTs/m^2) in one patient, and V ($=10x10^7$ CARTs/m^2) in five patients. Rapid CART expansion was observed in six patients. Peak levels ranged between 37,792 and 369,756 copy numbers/μg PBMC DNA and correlated with CART dose level. Toxicity was moderate with a single case of CRS >G2 and no severe neurotoxicity. However, prolonged G4 neutropenia occurred in one patient with ANC recovery on day +32. Responses were observed in all four patients available for response with MRD- CRs in both patients treated at dose level V. The 3rd generation CART HD-CAR-1 can be successfully generated from heavily pretreated patients with high-risk CLL and exerts a promising safety and efficacy profile.

P010

Topic: *ASa01 CAR T cell therapies in hematological malignancies*

A CHIMERIC ANTIGEN RECEPTOR (CAR)-BASED CELLULAR SAFEGUARD MECHANISM FOR ADOPTIVE T CELL THERAPY

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Adoptive immunotherapy based on genetically engineered T cells armed with chimeric antigen receptors (CARs) has shown tremendous clinical success in patients with B cell malignancies. However, their high potency also bears the risk of severe on/off-target toxicities. Therefore, it is necessary to develop strategies that improve the safety of CAR-T cell therapy. In this context, also the complete and highly selective removal of transferred cells in case of adverse events might become relevant, for example to revert long-term CAR-T cell related toxicities. Hence, our goal was to develop an efficient cellular safeguard mechanism that specifically eradicates therapeutically administered CAR-T cells in case of adverse events. This could be achieved by using a secondary CAR-T cell (*anti*-CAR CAR-T cell), which recognizes an additional tag implemented in the primary CAR construct. For this purpose, we generated CARs targeting a short peptide-sequence (StrepTag-II®) included in the extracellular domain of *anti*-CD19 CARs and tested these so-called *anti*-CAR CAR-T cells for their *in vitro* functionality with cytokine release and killing assays. Most importantly, we demonstrated highly efficient *in vivo* functionality when injecting *anti*-CD19 and *anti*-CAR CAR-T cells in immunocompromised and -competent mice. In addition, we could demonstrate that *anti*-CAR CAR-T cells are functional in clinically relevant pre-clinical animal models in which *anti*-CD19 CAR triggered B cell aplasia could be reliably reversed after the infusion of *anti*-CAR CAR-T cells. Remarkably, effective transfer of the *anti*-CAR cell product was possible in the absence of pre-conditioning, which should facilitate the applicability of the *anti*-CAR CAR-T cell approach. In conclusion, we developed a potent cellular safeguard system and showed that *anti*-CAR CAR-T cells can be used for selective *in vivo* depletion of engineered T cells early as well as late upon adoptive therapy with engineered lymphocytes.

P011

Topic: *ASa01 CAR T cell therapies in hematological malignancies*

A CONFORMATION-SPECIFIC ON-SWITCH FOR CONTROLLING CAR T CELLS WITH AN ORALLY AVAILABLE DRUG

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Adoptive transfer of CAR T cells resulted in groundbreaking success in the treatment of B cell malignancies. However, these impressive clinical results are often mitigated by severe side effects such as cytokine release syndrome and neurotoxicity arising from an uncontrolled activation of the CAR T cells after administration to the patient. Developing systems able to regulate CAR T cell activity is therefore of high importance for improving the safety of this therapy. A particularly promising concept is the conditional ''remote control'' of CAR function through the administration of small molecule drugs. Current strategies based on this model still have some drawbacks such as unfavorable pharmacokinetics or safety profile of the small molecule, or the usage of non-human potentially immunogenic protein components. In this study, we designed a novel molecular ON-switch system in which heterodimerization of proteins is tightly regulated by a clinically applicable small molecule. Two different protein scaffolds were engineered to bind the human retinol binding protein 4 (hRBP4) of the lipocalin family

only when the latter is loaded with a small molecule, A1120. X-ray crystallography confirmed that the engineered binders specifically recognize the new conformation of A1120-loaded hRBP4, providing us with a novel conformation-specific ON-switch. We show that the binders can interact with hRBP4 with high affinity only in the presence of A1120, and that the interaction is highly specific, as other known natural and synthetic ligands of hRBP4 do not trigger the switch. We further demonstrate that this molecular ON-switch can be used to regulate the activity of primary human CAR T cells, as addition of A1120 triggers tumor cells lysis and cytokine production. In summary, we present a novel conformation specific molecular ON-switch that can be used to regulate CAR T cell function with a clinically applicable small molecule.

P012

Topic: *ASi01 CAR T cell therapies in hematological malignancies*

MULTIPARAMETER CHARACTERIZATION OF CAR T CELLS

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Adoptive cell transfer of chimeric antigen receptor (CAR)-modified T cells has demonstrated great therapeutic success against certain malignancies. However, treatment efficacy varies with underlying mechanisms not fully understood or controlled to date. Emerging data suggest that the inefficient clinical outcome is related to different aspects, and intrinsic characteristics of the CAR T cells might be one of the important factors that determine clinical efficacy. During all phases of pre-clinical and clinical development it is fundamental to characterize CAR T cells using reliable tools and methods. In this work, we established a set of various *in vitro* assays for multiparameter characterization of CAR T cell products, which can be applied throughout all phases of drug product development. We performed in-depth characterization of healthy donor-derived CAR T cells by analyzing immunophenotype, cell fitness and effector functions. Specifically, we designed several flow cytometry panels and automated analysis for the extensive characterization of immunophenotypes of interest such as: proliferative capacity, differentiation, activation and exhaustion. Cell fitness status was determined by the rate at which cells undergo apoptosis following stress. Finally, effector functions was determined by the ability of the activated CAR T cells to secrete proinflammatory cytokines including IFN-g, TNF-a, GM-CSF and IL-2. With our established workflow, over 20 healthy donor-derived CAR T cells were generated and characterized. Thereby, we have explored donor-dependent variations and responses for most of the assessed parameters. Collectively, we established a workflow for multiparameter characterization of CAR T cells using automated and reproducible analysis with a high degree of standardization. This workflow provides comprehensive knowledge of phenotypic and functional characteristics of engineered CAR T cells, and can be widely applied during all phases of CAR T cell research and development.

P013

Topic: *ASi02 CAR and TCR targets*

CELL AVIDITY A KEY PARAMETER TO IMPROVE THE PREDICTABILITY OF T CELL FUNCTIONALITY FOR CELLULAR IMMUNOTHERAPIES

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T cell killing requires functional engagement between effector-target cell pairs and associated mechanical forces. Current strategies for selecting optimal T cell receptor (TCR), chimeric antigen receptor (CAR) T cells, and bi-specific (or cell engager) candidates focus on surface plasmon resonance (SPR) and fluorescent-activated cell sorting (FACS) technologies. These technologies provide useful information on affinities and kinetics of ligand-receptor interactions. However, several limitations persist including time-consuming processes, false-negative readouts of bulk populations and the inability to assess the strength of the collective, noncovalent cell-cell interactions involved in immunological synapse formation. Thus, affinity readouts have shown poor predictability of T cell functionality which could potentially lead to failed clinical trials and lost time. On the contrary, cell avidity (i.e. overall intercellular binding strength) provides a more complete and physiologically relevant live-cell parameter that reflects the true T cell-tumor cell interactions formed. Here we present data on TCR-,CAR-, and bi-specific based immunotherapeutic candidates obtained using the z-Movi® Cell Avidity Analyzer. This instrument uses acoustic forces to evaluate cell-cell interaction strengths, which strongly correlated to effective anti-tumor responses *in vitro* assays and *in vivo* mouse models. This demonstrates the benefits of understanding cell avidity to improve the prediction of T cell functionality and ultimately lead to better clinical outcomes.

P014

Topic: *ASa02 CAR and TCR targets*

STRATEGY TO INCREASE TUMOR SPECIFICITY OF EGFR-TARGETING THERAPIES

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The ideal target antigen is exclusively expressed on malignant cells. Unfortunately, most are not limited to cancer cells and lead to unintended on-target/off-tumor toxicity in healthy tissue (Bonifant et al., 2016). Therefore, the expression of the epidermal growth factor receptor (EGFR) on a wide variety of tissues represents a major limitation in the development of effective EGFR-targeted therapies. The hypothesis of this project is based on the biochemical properties of the extracellular module (ECM)

of EGFR, which undergoes a major conformational change upon ligand-binding, switching from a monomeric, tethered to a dimeric, extended structure (Kovacs et al., 2015). Based on this particular property of activated EGFR, we are engineering the hyper-thermostable protein-scaffold Sso7d for specifically interacting with ligand-bound EGFR by using yeast surface display (Angelini et al., 2015; Boder & Wittrup, 1997; Chao et al., 2006; Traxlmayr et al., 2012; Traxlmayr et al., 2016). Since cancer cells have evolved multiple mechanisms for EGFR-activation (Gazdar and Minna, 2008; Endres et al., 2013), we expect that such binders recognizing activated EGFR show much greater specificity for cancer cells compared with other binders that bind to all EGFR-molecules irrespective of their activation status. The most promising binders will be extensively studied regarding their biophysical properties (thermostability and aggregation) and binding to cancer cells with various EGFR expression levels.

P015
Topic: *ASa02 CAR and TCR targets*

GENERATING A NOVEL CAR-MACROPHAGE THERAPY FOR TREATMENT OF SOLID TUMORS

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Recent studies have highlighted the important role of the immune system in determining tumor faith. Along these lines, established immunotherapeutic strategy of engineered Chimeric Antigen Receptor T-cell (CAR-T) has proven successful in treating hematological tumors. Nevertheless, it has showed limited success in the realm of solid tumors. This stems from the harsh conditions at tumor microenvironment which limit T-cell infiltration and cytotoxicity. In contrast, myeloid cells massively infiltrate tumors and are well adapted to survive these conditions, albeit they usually adopt a tumor-promoting phenotype rather than cytotoxic one. Recently, I discovered that incubation of myeloid cells with IgM-coated tumor cells, induces secretion of lytic granules and massive tumor cell death. These findings open a new venue for designing novel immunotherapy by equipping monocytes with chimeric receptors that would target tumor antigen and consequently, signal through IgM receptor. However, I discovered that myeloid cells prevent expression of CAR by recognizing its extracellular, antibody-derived portion and induce ER stress mechanism. Overall, this work highlights the challenges in developing CAR-macrophage and provides a novel framework for utilizing the specific recognition capacity of the adaptive immune system and the ability of myeloid cells to survive at tumor sites.

P016
Topic: *ASa02 CAR and TCR targets*

HIGHLY EFFICIENT GENERATION OF TRANSGENICALLY AUGMENTED CAR NK CELLS OVEREXPRESSING CXCR4

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Natural killer (NK) cells are a noteworthy lymphocyte subset in cancer adoptive cell therapy. NK cells initiate innate immune responses against infections and malignancies with natural cytotoxicity which is independent of foreign antigen recognition. Based on these substantive features, genetically modifying NK cells is among the prime goals in immunotherapy but is currently difficult to achieve. Recently we reported a fully human CAR19 construct (huCAR19) with remarkable function in gene-modified T cells. Here we show efficient and stable gene delivery of huCAR19 to primary human NK cells using lentiviral vectors with transduction efficiencies comparable to those achieved with NK cell lines. These huCAR19 NK cells displayed specific and potent cytotoxic activity against target cells. To overcome insufficient movement towards malignant cell niches, we augmented huCAR19 NK cells with the CXCR4 gene, resulting in transgenically augmented CAR NK cells (TRACKs). Compared to conventional CAR NK cells, TRACKs exhibited a remarkably enhanced migration capacity, while retaining functional and cytolytic activities against target cells. Based on these promising findings, TRACKs may become a novel nominate for immunotherapeutic strategies in clinical applications.

P017
Topic: *ASa02 CAR and TCR targets*

ENGINEERING AVIDCARS FOR COMBINATORIAL ANTIGEN RECOGNITION

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The expression of chimeric antigen receptors (CARs) allows to efficiently direct T cells against tumor cells. Meanwhile, several CD19-specific CAR T cell products have already been approved for therapy of B cell malignancies. While on-target/off-tumor toxicity, i.e. the destruction of healthy cells that also express the target antigen, is tolerable in the therapy of B cell malignancies with CD19-specific CAR T cells, potential on-target/off-tumor toxicity poses a major risk in the treatment of virtually all other tumors. Therefore, to improve the tumor specificity of CARs, we have developed avidity-dependent CARs (AvidCARs) with AND-gate function. Most current CARs contain high-affinity binding domains and can thus trigger a strong signal by monovalent interaction with the target antigen. We hypothesized that CARs with sufficiently reduced affinity of their binding domains can efficiently trigger signaling only upon bivalent interaction with target antigens, but not upon

monovalent interaction. We further postulated that by integrating two different antigen binding domains, it would be possible to generate CARs that efficiently activate the T cells only when the target cells simultaneously express an antigen A *and* B (i.e. CARs with AND-gate function). This means that in these AND-gate AvidCARs, the bivalent interaction with two different target antigens leads to avidity effects that enhance the low affinities of the individual antigen binding domains and in this way prolong the interaction time of the CARs with the target antigens. Using the model antigens EGFR and HER2, we show both *in vitro* and *in vivo* that it is possible with such AND-gate AvidCARs to specifically and efficiently eliminate target cells expressing EGFR and HER2, while sparing cells expressing either EGFR or HER2 only.

P018
Topic: *ASa02 CAR and TCR targets*

PRODUCTION OF MONOCLONAL ANTIBODIES FOR THE T-CELL ENGINEERING AGAINST METASTATIC COLORECTAL CANCER

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Chimeric Antigen Receptor (CAR) T cell therapy represents an important strategy to fight hematological malignancies, including B-cell acute lymphoblastic leukemia and multiple myeloma, but unfortunately in solid tumors the results are still less promising. The main problem is the absence of specific tumor antigens in these malignancies. Here we aim to identify new surface targets through the generation and the screening of new monoclonal antibodies (mAbs) specifically targeting metastatic Colorectal Cancer Stem Cells (CRC-SCs) that have been described to play important roles in tumor progression. We immunized BALB/c mice with primary metastatic CRC-SCs and generated a random hybridoma library by performing 36 fusions that were directly cloned (25.000 wells). We chose this approach to identify antigens that couldn't be easily disclosed with complementary methods such as Next Generation Sequencing (NGS) (e.g., antigens with post-translational modifications). Thus, we selected hybridomas producing tumor specific mAbs by high-throughput flow cytometric (FACS) screening. We performed several steps of mAbs screening: the first by multiplexing labeled CRC-SCs and peripheral blood leukocytes (PBLs) and selecting only the 991 clones binding selectively tumor cells. The hybridomas were subcloned and validated again. This allowed us to verify the capability of the hybridoma to produce mAbs recognizing tumor cells during passing of time. Unfortunately, many hybridomas were instable and thus, the final number of suitable clones was reduced to 203. We purified mAbs with IgG isotype by using the ÄKTA pure chromatography system and performed immunohistochemistry (IHC). So far, we have identified 2 mAbs binding specifically CRC and not to normal mucosa. The next aims are the confirmation of the IHC data of these 2 mAbs followed by antigen identification and the characterization of its biologic function. Then, we will use the sequence-information of the mAbs to design novel CAR constructs targeting the human metastatic CRC.

P019
Topic: *ASa02 CAR and TCR targets*

IDENTIFICATION OF HIGHLY FUNCTIONAL AND CYTOTOXIC SARS-COV-2-SPECIFIC CD8+ T CELLS BY ORTHOTOPIC TCR REPLACEMENT ENGINEERING

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T cell immunity plays a pivotal role in the control of SARS-CoV-2 infections. Despite major efforts in quantifying SARS-CoV-2-specific T cell responses, the quality of such responses, in particular of $CD8^+$ T cells, has been marginally investigated so far, leaving the importance of $CD8^+$ T cells during COVID-19 and for protective immunity unclear. To assess $CD8^+$ T cell functionality, the identification and characterization of SARS-CoV-2-specific T cell receptors (TCRs) is indispensable. To first identify SARS-CoV-2-specific epitopes, we evaluated immunogenicity of candidate epitopes on convalescent donor material as functional and protective responses are supposed to be found. After a short *in-vitro* expansion and restimulation of SARS-CoV-2-specific $CD8^+$ T cells, we were able to detect response rates ranging from 33 – 100 %. Importantly, SARS-CoV-2-specific $CD8^+$ T cells were detected even one year after infection. For two HLA-restricted epitopes we subsequently identified TCRs by performing single-cell RNA sequencing. By re-expressing SARS-CoV-2 TCRs via CRISPR/Cas9-mediated orthotopic replacement (OTR), we confirmed functional avidity as well as cytotoxicity towards virus-infected cells. By combining experimental data with gene signatures of recent activation, we further defined a ''reactivity signature'' and a ''functionality signature'' to differentiate TCRs with high and low functionality. To test these gene signatures as a means of predicting TCR functionality in silico, we identified TCRs against nine additional immunodominant SARS-CoV-2 epitopes restricted to five different HLA class I molecules and could confirm TCR functionality. Finally, we showed that the SARS-CoV-2 TCR repertoire is highly polyclonal. In summary, our data demonstrate by single cell TCR identification in combination with OTR engineering that $CD8^+$ T cell responses upon mild COVID-19 infection are polyclonal, long-lasting, and highly functional. We furthermore demonstrated for several TCRs highly effective cytotoxicity towards virus-infected cells, which might open options for therapeutic use with adoptive T cell therapy in COVID-19 patients.

P020
Topic: *ASa02 CAR and TCR targets*

NOVEL ANTIBODY TO REDIRECT T CELLS FOR CANCER IMMUNOTHERAPY IN COLORECTAL CANCER (CRC)

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Adoptive immunotherapy with tumor-reactive chimeric antigen receptor (CAR) T cells, is an innovative therapeutic concept encouraging efficacy for the treatment of cancer patients. Concerning the treatment of CRC with CAR T therapy, one of the main difficulties is the lack of specific targets. In our study, we aimed to identify novel tumor-specific antigens on the surface of CRC stem cells (SCs) to specifically redirect CAR T cells to fight the tumor. We chose this model, because CRC-SCs play a vital role in CRC tumor initiation and progression. We immunized mice with CRC-SCs and screened for hybridomas that produced monoclonal antibodies (mAbs) binding to the surface of primary CRC-SCs but not to peripheral blood leukocytes by high-throughput flow cytometric screening (FACS). The specificity of the mAbs was further tested by IHC analyses on frozen and on formalin-fixed paraffin-embedded CRC tissue sections and on tissue micro arrays. Finally, we performed surface FACS analyses on freshly dissociated tumors and compared the binding pattern of the mAbs with the binding to normal mucosa of patients. Additionally, we performed FACS analyses with several commercial cell lines and CSCs obtained from different solid tumor types. All these experiments suggested that the selected mAbs selectively bind CRC and spare normal tissue. Using the sequence information of one tumor-specific mAb, we designed novel CAR constructs targeting the human CRC-SCs. As transfer method, we employed a lentiviral vector for the expression of the CAR molecule and a fluorescent reporter gene to allow the tracking of transgenic T cells. CAR T cells, established with the second-generation CAR construct, substantially killed CRC-SCs while sparing negative cell lines in vitro. In the next steps we are going to test these CAR constructs in vivo. In conclusion the isolated mAb could represents a powerful new tool to redirect T cells against CRC.

P021

Topic: *ASi02 CAR and TCR targets*

ULTRAHIGH-CONTENT IMAGING HELPS TO IDENTIFY CAR TARGET CANDIDATES AGAINST PANCREATIC ADENOCARCINOMA

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Introduction: CAR T cells have become a new pillar of cancer therapy. One major issue in CAR therapy remains the restricted availability of safe tagets. We show, how the newly developed tool MACSima can be integrated in a workflow to evaluate targets on tumor cells and healthy tissue expression and to support functional studies. The MACSima platform operates by iterative fluorescence staining, imaging, and signal erasure, enabling the operator to identify and compare the expression of dozens of targets on the very same tissue section.

Methods: We screened around 400 surface antigens on 17 patient derived xenografts (PDX) using flow cytometry. The novel MACSima platform was used to verify the expression of target candidates on human pancreatic tumors. 32 CAR constructs were designed specific for suitable targets and evaluated in vitro. Promising constructs were challenged in vivo. MACSima helped to investigate differences between treatment groups and escape mechanisms. Finally, a healthy tissue multiarray was analyzed, to confirm low expression of the chosen targets.

Results: We identified a set of 50 surface antigens on the PDXs in the initial flow screening. The MACSima platform helped to reveal, that CLA, CD66c, CD318 and TSPAN8 were targets with the highest tumor specificity and safety profile. While for CD66c, CD318 and TSPAN8 efficient CAR constructs were determined, no CAR T cells specific for CLA could be generated, due to self-antigenicity. For in vivo MACSima was used to show that low efficacies are not caused by target downregulation. MACSima unravelled also the restricted healthy tissue expression of CD66c, CD318 and TSPAN8, making them interesting targets for CAR treatment.

Conclusions: Here we introduce a novel comprehensive workflow for target identification in an immunotherapy setting. The novel MACSima platform proofed to be a useful tool throughout the whole procedure, resulting in the discovery of novel CAR targets.

P022

Topic: *ASa02 CAR and TCR targets*

DISCOVERING AND ASSESSING TRANSCRIPTOMIC SIGNATURES OF ANTIGEN-DEPENDENT ACTIVATION IN NEOANTIGEN-SPECIFIC TCRS ON A SINGLE-CELL LEVEL

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Introduction: Immunotherapy has become a pillar of anti-tumour therapy based on the natural immunogenicity of malignant cells. Targeting neoantigens that arise from tumour specific mutations can lead to durable regression in various types of cancer. It has also been shown that enhanced immunotherapy is affected by immune-cell subsets. Therefore assessing determinants of effective neoantigen-specific T-cell responses will be important to improve therapeutic approaches. Within a previous project, tumour infiltrating lymphocytes and PBMCs from melanoma patient Mel15 were used to identify mutated peptide ligands by mass spectrometry and in silico prediction. Using these ligands we discovered seven novel neoantigen specific T-cell receptors (TCRs) targeting three neoantigens (KIF2C^{P13L}, SYTL4^{S363F}, NCAPG2^{P333L}). All TCRs showed differences in their binding avidities against the mutated peptide-human leukocyte antigen (HLA) complex whilst lacking any reactivity against the wildtype counterpart.

Methods: To improve identification of neoantigen-reactive TCRs and assess transcriptomic signatures of antigen-specific T-cell activation we performed single cell RNA and TCR repertoire/VDJ sequencing from this patient. We developed a method to enrich PBMCs for neoantigen-reactive T cells using $CD137^+$-based selection. Autologous PBMCs of patient Mel15 were stimulated with neoantigens $KIF2C^{P13L}$ and $SYTL4^{S363F}$ and then enriched. After expansion enriched cells were restimulated with respective mutated peptides. We then sequenced an unenriched, unstimulated and an enriched, restimulated condition for comparison.

Results: Using this protocol we detected an enrichment of all previously identified neoantigen-reactive TCRs. After analysis of clonotype enrichment in both samples we selected 4 candidates and successfully identified 2 new TCRs with specificity to $KIF2C^{P13L}$. Comparing the transcriptome of our TCRs showed distinct differences in defined TCR groups.

Conclusion: Concluding, this approach offers itself to assess transcriptomic signatures of neoantigen-specific T-cell activation and can provide deeper insights into defined antigen-dependent activation patterns. Furthermore this protocol can be used as a sensitive tool for the identification of neoantigen-specific TCRs.

P023

Topic: *ASa02 CAR and TCR targets*

HARNESSING CD70-DIRECTED CAR NATURAL KILLER CELLS WITH IL-15 IS NECESSARY TO ERADICATE SOLID TUMOUR CELLS AND CANCER-ASSOCIATED FIBROBLASTS

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The immune checkpoint molecule CD70, ligand for CD27, is tightly regulated during physiology where it is only transiently upregulated upon antigen-activation on different immune cell subsets, while being absent on non-lymphoid tissue. However, numerous types of haematological and solid tumours hijack CD70 expression to facilitate tumour progression and immune evasion. We were the first to unveil that aberrant CD70 expression on cancer-associated fibroblasts (CAFs) in metastatic colorectal cancer provides the CAFs with tumour-promoting properties. Due to its limited expression on healthy tissues and the growing evidence of its tumour-enhancing effects, CD70 is an attractive target for cancer immunotherapy. We have generated off-the-shelf chimeric antigen receptor (CAR) natural killer (NK) cells, based on the CD27 receptor, capable of eradicating $CD70^+$ tumour cells and CAFs. We used the human NK-92 cell line as inexhaustible, clinically approved, and cost-effective source of NK cells. Messenger RNA electroporation was used as safe transfection method that limits off-target effects by inducing transient CAR expression. Validation of the developed CD70-directed CAR NK-92 cells showed consistent transfection efficiency, with high CAR expression (>80%) and competent antigen-specific killing of the $CD70^+$ NK-resistant Burkitt lymphoma Raji cell line in a co-culture setting. Blocking of the CAR's antigen-recognition domain with a neutralising antibody further demonstrated CAR-mediated killing. Of interest, incorporation of interleukin (IL)-15, a highly potent immunostimulatory cytokine, into the CAR construct resulted in protein co-expression, which substantially increased the cytotoxic capacity of the CAR NK-92 cells. In fact, co-stimulation with IL-15 appeared necessary to eliminate more robust $CD70^+$pancreatic and colorectal cancer solid tumour cell lines and CAF cell lines. Currently, we are setting up mouse experiments to evaluate the anti-tumour efficacy of the CD70-specific CAR NK-92 cells in vivo. Eventually, these results might pave the way towards the clinical application of our allogeneic off-the-shelf CAR NK cell therapy.

P024

Topic: *ASa03 Engineered T cell therapy for solid tumours*

SHED L1CAM IMPAIRS L1CAM-DIRECTED CAR T CELLS AGAINST NEUROBLASTOMA

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Target antigen expression by tumor cells and the hostile immunosuppressive microenvironment play crucial roles for CAR-T cell therapy success against solid tumors. L1CAM is overexpressed in neuroblastoma, and the cancer-specific glycosylation of the CE7 epitope makes it a suitable target antigen. Constitutive cleavage of the L1CAM extracellular domain by the ADAM metalloprotease sheds the CE7 epitope-containing L1CAM ectodomain into blood and ascites. The RGD motif in soluble L1CAM can also bind integrins in tissues. We hypothesized that soluble L1CAM in the blood or tissue matrix could neutralize CAR-T cells before they reach the tumor site or exhaust CAR-T cells more quickly through excessive CE7 CAR domain binding. Soluble L1CAM levels were elevated in serum from patients with neuroblastoma and mice harboring neuroblastoma xenografts, confirming *in vivo* L1CAM shedding. L1CAM-coated plates activated L1CAM CAR-T cells *in vitro*, but soluble recombinant L1CAM concentrations comparable with patient samples did not. CAR-T cells exposed *in vitro* to tumor cells lacking L1CAM expression, but pre-exposed to soluble L1CAM concentrations found in patients also upregulated IFNG release. L1CAM was flow cytometrically confirmed on the cell surface, presumably bound via integrins. Interestingly, CAR-T cells harboring the long spacer reacted faster to the L1CAM-bound tumor cells than CAR-T cells harboring the short spacer and showed stronger upregulation of the activation marker, CD137. Pre-exposing L1CAM CAR-T cells to L1CAM-bound tumor cells diminished *in vitro* cytotoxic potential against neuroblastoma cells. We provide proof-of-concept for the presence of soluble L1CAM in patients with neuroblastoma, and evidence that bound L1CAM on non-target cells can activate L1CAM CAR-T cells. Our findings suggest that the cleaved L1CAM ectodomain sequestered in extracellular matrix or bound to integrins on non-target cells is better able to neutralize L1CAM CAR-T cells than soluble L1CAM, and that CAR constructs harboring the long spacer are more susceptible to this neutralization.

P025
Topic: *ASa03 Engineered T cell therapy for solid tumours*

TCR-QR: IDENTIFYING TUMOUR-REACTIVE TCRS FOR PERSONALIZED T CELL THERAPIES

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Personalized immunotherapies such as adoptive cell transfer (ACT), from expanded tumor-infiltrating lymphocytes (TILs) have shown promising results across different tumor entities. Nonetheless, while it mediates complete regression in a fraction of some patients, many patients lack response to TIL therapy. One potential reason is that expanded TIL products include both tumor-reactive and bystander T cells, which thus may be leading to a lack of therapeutic efficacy and potency. Increasing the number of tumor-reactive TILs would substantially advance the efficacy of such personalized therapies. However, it is highly challenging to identify tumor-reactive TILs - and even more so in the limited timeframe needed for most personalized immunotherapies. Here, we use multi-dimensional data from single-cell transcriptome, epigenome, immune repertoire and functional reactivity assays to identify and eventually predict tumor-reactive TCRs. By introducing selected TCRs from TILs into our previously developed TCR testing platform (TnT, Vazquez et al. 2021 under revision at Cell), we identified enrichment of a subset of TCRs that showed expression of phenotypic markers that partly overlap with established markers of exhaustion and tumor reactivity such as CD39, CXCL13 and CD137. Combination with single cell chromatin accessibility sequencing (scATACseq) allowed us to project motif accessibility for TILs of interest and showed elevated motif activity of TCF7 and NFKB1. Future experiments will increase numbers of patient as well as tested TCRs per patient. In conclusion, TCR-QR consists of an integrated pipeline that exploits state-of-the-art methods in single-cell genomics, computational analysis, genome engineering and functional screening. This allows for new marker identification and patient sample integration on the multiOMIC level.

P026
Topic: *ASa03 Engineered T cell therapy for solid tumours*

NOVEL STRATEGIES FOR CAR T-CELL IMMUNOTHERAPY AGAINST GLIOBLASTOMA BASED ON MULTI-TARGETING AND MODULATION OF THE TUMOR MICROENVIRONMENT

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Glioblastoma (GBM) is an extremely aggressive primary brain tumor, with an overall survival of about a year after diagnosis. Conventional therapies have not resulted in major improvements in the survival outcomes of patients with GBM, so increasing efforts are being devoted to develop new therapeutic strategies, particularly in the flourishing field of CAR T-cell immunotherapy. However, CAR T-cell therapy in GBM, and in solid tumors in general, faces several challenges including the high level of heterogeneity that can lead to antigen escape, and the immunosuppressive tumor microenvironment (TME). To overcome the risk of antigen escape we propose the use of dual CAR T-cells that simultaneously recognize a novel but ''conventional'' tumor associated antigen (TAA) expressed on the cell surface, and an ''unconventional'' target antigen expressed in the tumor extracellular matrix (ECM). TAAs in the ECM are generally more accessible, abundant and stable than those located on the cell surface and provide an advantage in terms of antigen escape. We have demonstrated the specificity and functional activity of the mono-specific CAR T-cells *in vitro*, and their therapeutic efficiency *in vivo*, in murine and human GBM models, and we are currently assessing different modalities for the dual antigen targeting. In parallel, to overcome the obstacle of the TME, we are working on a combination therapy strategy for the dual CAR T-cells with an immunomodulatory agent that can revert the immunosuppressive milieu and re-invigorate T-cell effector mechanisms. In order to ensure a localized and safe expression of the agent in the tumor site, we are using a lipid-nanoparticle (LNP) mRNA based tool for *in vivo* targeted expression with promising preliminary results. Although GBM is a challenging candidate for CAR T-cell immunotherapy, we consider that the combination of dual CAR T-cells with nanomedicines that can remodel the TME represents a promising therapeutic approach.

P027
Topic: *ASa03 Engineered T cell therapy for solid tumours*

PROSTATE-CONFINED RADIOTHERAPY SUPPORTS TCR-REDIRECTED T CELLS TUMOR SEEDING INSTRUCTING LONG-LASTING PROTECTIVE IMMUNITY AGAINST THE PRIMARY TUMOR AND LIVER METASTASIS.

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Background: Tumor-confined hypo-fractionated radiotherapy (RT) exerts tumor cell intrinsic effects and also immunomodulatory activities. We had previously reported that tumor-specific TCR redirected T cells alone, lacked significant therapeutic activity against advanced prostate adenocarcinoma. We postulated that local RT might synergize with adoptive T cell therapy (ACT), and investigated combined delivery.

Methods: Prostate-confined RT was delivered to TRAMP (Transgenic Adenocarcinoma of the Mouse Prostate) mice by a

X-RAD SmART irradiator. Twenty-four hours after last radiation dose, mice received autologous T cells transduced with the SV40IV/Tag reactive TCR. Magnetic resonance imaging, Real-Time PCR, FACS and histological analyses were used to define immunological correlate of clinical responses.

Results: We report that RT and ACT concurred to acute tumor debulking when used in combination. Mechanistically, RT promoted tumor vessel permeability and a type-I interferon response, comprising the expression of T cell recruiting chemokines. This best explained selective enrichment of tumor-specific PD-1+ IFN-g and Granzyme B + T cells by 10 days of transfer in the prostate, draining lymph nodes, spleen and blood of RT+ACT mice, compared to ACT only. ACT-derived T cells also persisted in greater numbers to 4 weeks, when tumor debulking was complete. Stronger T cell immunity in RT+ACT mice further correlated with increased survival rates, and protection from liver metastasis.

Conclusions: Data support the synergy of RT and TCR engineered T cells against advanced primary prostate adenocarcinoma and also metastatic lesions outside the irradiation field.

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Topic: *ASa03 Engineered T cell therapy for solid tumours*

A HIGH-THROUGHPUT WORKFLOW FOR THE FUNCTIONAL SCREENING AND CHARACTERIZATION OF CLINICALLY RELEVANT MHC CLASS I-RESTRICTED T CELL RECEPTORS FOR ADOPTIVE T CELL THERAPY

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Clinical success of adoptive cell therapy (ACT) with T cell receptor (TCR)-engineered T cells is still limited by the restricted number of available TCRs. Besides some difficulties to access relevant tumor sites, the spatiotemporal distribution of tumor-specific T cells in patients is still unknown. On the contrary, peripheral blood of healthy donors is easily accessible and the huge diversity of the antigen unexperienced TCR repertoire should contain T cells for theoretically any (foreign) antigen specificity. However, naïve antigen-specific T cell populations are of extremely low frequency and contain a substantial fraction of T cells with low functional TCRs. To overcome these intrinsic limitations, we started from T cell apheresis and established a cell sorting-based technology to quickly isolate rare antigen-specific $CD8^+$ naïve T cells from large-size sources according to peptide-Major Histocompatibility Complex (pMHC) class I multimer staining. Secondly, we developed a flow cytometry-based functional screening to estimate the structural avidity, which correlates with functionality, of each individual TCR composing an antigen-specific population. When combined, epitope-specific TCRs can be isolated and concurrently ranked according to predicted avidity/functionality. As proof-of-principle, we targeted a clinically relevant neoepitope derived from a frameshift mutation in the tumor suppressor RNF43, for which 21 TCRs were successfully isolated. Remarkably, functional TCRs were predicted with a sensitivity of 60-70%. In parallel, we further improved the throughput of *in vitro* TCR characterization, as it remains a highly cost and time-consuming step. By exploiting TCR-engineered Jurkat reporter systems and cutting-edge measurements of T cell:target cell synapse interactions (so called ''cellular avidity'') as a relatively fast measurable readout correlating with T cell functionality, we succeeded in the simultaneous investigation of dozens of TCRs in a relative short time. In summary, we have established a novel workflow to rapidly move from donor material to the selection of candidate TCRs for TCR therapy.

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Topic: *ASa03 Engineered T cell therapy for solid tumours*

OPTIMIZATION OF CHIMERIC ANTIGEN RECEPTOR (CAR) T CELL TO IMPROVE SOLID TUMORS TREATMENT

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To develop novel treatment strategies against colorectal cancer (CRC), we generated novel chimeric antigen receptor (CAR) constructs based to the sequence information of novel CRC targeting monoclonal antibodies. The aim of this part of the project was the optimization of the lentiviral gene-transfer. A fundamental step in the preparation of CAR T cells is the production of functional lentiviral particles. By comparing several systems, we found that 3rd-generation lentiviral transfer constructs in combination with 2nd generation packaging plasmids represent an optimal system to improve transduction efficiency while retaining the significant biosafety features. Using this system, we tested the transduction efficiency of viral constructs in which we changed several parameters such as the size of the transgene, the promoters and orientation of scFv. We observed that the vectors with CMV promoter had a relatively low transduction efficiency in human T-cells of around 15%. To improve the efficiency, we shortened the transgene by removing the WPRE sequence. Using this modification, the titres were not increasing while the expression of the marker EGFP was reduced. Therefore, we changed the strategy to improve transduction efficiency and tested different internal promoters driving the CAR expression. We compared three different promoters in human T cell: CMV, EIF1a and hPGK promoters. We observed that lentiviral vectors with hPGK promoter gave consistently the best transduction efficacy of primary T cells yielding up to 50% of GFP^+ cells. Moreover, an important structural component of the CAR is the scFv. Considering this, we optimized our CAR constructs with a new orientation of scFv that resulted in a better in vitro anti-tumor activity of the CAR T cells. In the next steps only the CAR constructs with the highest cytotoxicity will be selected for in vivo validation using mouse models.

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Topic: *ASa03 Engineered T cell therapy for solid tumours*

CHIMERIC ANTIGEN RECEPTOR-T CELLS DERIVED EXTRACELLULAR VESICLES AS A POTENTIAL CANCER THERAPY

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Chimeric antigen receptor (CAR)-T cells are genetically engineered T cells, directed against a tumor-associated antigen. While CAR-T cell therapy shows great promise in hematological malignancies, its implication for solid tumors is far more difficult. This difficulty rise from the hostile tumor microenvironment and lack of tumor penetration. Extracellular vesicles (EVs) are cell-derived membranous vesicles varies in size and point of origin. Taking advantage of their small size on the one hand and the content similarity to the parental cells on the other, EVs may escape the immunosuppressive microenvironment of the solid tumor, penetrate it and act as a surrogate CAR T cell. In order to mimic the CAR-T cell activity by the EVs, the parental CAR-T cells need to possess activated machinery for cytotoxicity. Therefore, EVs were isolated from the cell media of CAR-T cells stimulated against cells expressing a specific antigen. These EVs were characterized for their content and functions. We observed that EVs isolated from stimulated CAR-T cells, exhibit similar content to their parental cells. Both cytokines (such as interferon gamma) and granzyme B were found to be higher when compared with EVs isolated from unstimulated CAR-T cells. We show that anti-HER-2 CAR-T EVs bounded and penetrated specifically into HER-2 expressing target cells. Furthermore, when analyzing HER2+ cells, co-cultured with either CAR-T cells or CAR T EVs isolated from stimulated CAR-T cells, we discovered elevated levels of caspase 3/7 activity compared to controls. However, while the CAR-T cells induced massive apoptosis during the first 24h, CAR-T EVs required 60 – 90 h. In summary, CAR-T EVs provide a novel potent immunotherapy approach that may be effective against solid tumors.

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Topic: *ASa03 Engineered T cell therapy for solid tumours*

PHARMACOLOGICALLY INHIBITING MYCN IMPROVES L1CAM-DIRECTED CAR T CELL EFFICACY AGAINST MYCN-AMPLIFIED NEUROBLASTOMA

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Current treatment protocols have only limited success in pediatric patients with neuroblastomas harboring amplifications of the central oncogene, *MYCN*. Adoptive T cell therapy significantly improved treatment of B cell malignancies. However, L1CAM-targeting CAR T cells achieved only limited response against refractory/relapsed neuroblastoma in an ongoing trial. Here we investigated how oncogenic MYCN levels influence tumor cell response to CAR T cells, as one possible factor limiting success in trials. High MYCN levels were elicited in the SK-N-AS neuroblastoma cell line harboring the normal diploid *MYCN* complement using a tetracycline-inducible system to allow comparison of differential MYCN expression in the same cell background. MYCN induction significantly reduced L1CAM target antigen expression on tumor cells, reducing L1CAM-CAR T cell activation and cytokine release and impairing *in vitro* tumor cytotoxicity. We confirmed diminished L1CAM expression in a cohort of primary neuroblastomas with high *MYCN* expression. Indirectly inhibiting MYCN *in vitro* using the aurora a kinase inhibitor, increased L1CAM expression on the neuroblastoma cells and restored L1CAM-CAR T cell effector function. MLN8237 synergistically enhanced L1CAM-CAR T cell-directed killing of neuroblastoma cells overexpressing MYCN. Combination therapy in immunodeficient NSG mice harboring SK-N-AS-MYCN^{ind} xenografts reduced PD-1 and Tim3 expression on CD8$^+$ T cells infiltrating the tumor compared with CAR T cell monotherapy, but survival was only marginally improved. These data suggest that while additional factors relating to oncogenic MYCN act to impair L1CAM-CAR T cell effector function, combining L1CAM-CAR T cell therapy with pharmacological MYCN inhibition may benefit patients with high-risk neuroblastomas harboring *MYCN* amplifications.

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Topic: *ASa03 Engineered T cell therapy for solid tumours*

CAR-T CELLS AND EXO-CAR T CELLS AS NOVEL TREATMENT FOR PEDIATRIC CENTRAL NERVOUS SYSTEM MALIGNANT TUMORS

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Pediatric CNS malignant tumors are the main cause of cancer-related death in children. Current treatments have resulted in prolonged free survival rates (60-70%). However, outcomes for children with unrespectable, relapsed, or refractory tumors, remain dismal. Additionally, those who survive may present long-life neurologic irreversible sequelae caused by the effects of surgery, chemo/and radiotherapy in the developing brain. Chimeric-Antigen-Receptor (CAR) T cells are an attractive therapeutic approach with potential to target tumor cells while sparing the surrounding healthy brain. However, there are still some hurdles for a successful CAR T cell therapy for pediatric brain tumors: the lack of specific Tumor Associated Antigens (TAAs), the immunosuppressive Tumor Microenvironment (TME), the difficulty to trespass the Blood Brain Barrier (BBB) and the need to avoid inflammation and neurotoxicity. NKG2D CAR T cells

target up to 8 different NKG2D ligands that are upregulated in CNS tumors and in Myeloid Derived Suppressor Cells and could overcome the tumor heterogeneity and hostile TME. Additionally, *in vitro*, we have observed NKG2D CAR T cells target glioblastoma, medulloblastoma and atypical teratoid/rhabdoid tumor cell lines. Recently, it has been shown that CAR T cells release small extracellular vesicles (exosomes) that maintain the TAA recognition and anti-tumor properties of their parental CAR T cells. These exosomes-derived CAR (Exo-CAR) present several advantages over CAR T cells including 1) a nanoscale size that facilitates trespassing BBB, 2) lack of expression of inhibitory molecules as PD1: providing enhanced resistance to the immunosuppressive TME, 3) inability to release inflammatory cytokines and thus minimizing the risk of Cytokine Released Syndrome (CRS) and Immune effector Cell Associated Neurotoxicity Syndrome (ICANS). In this study, we aim to isolate, characterize and test the anti-CNS tumor ability of Exo-NKG2D CAR both *in vitro* and in a stereotaxic mouse model, as a novel therapeutic approach to treat pediatric CNS tumors.

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Topic: *ASi03 Engineered T cell therapy for solid tumours*

EVOLVING MULTIPLEXED SHRNA TO GENERATE TAILORED CAR T CELL THERAPY

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Introduction: Manipulating protein expression to generate cells with a specific desired phenotype is one of the central goals of engineered cell therapy. Short hairpin RNA (shRNA) is a well-established approach to reduce protein expression through the targeted degradation of messenger RNA transcripts. However, the use of shRNA in Chimeric Antigen Receptor (CAR) T cell therapy has been limited. We have previously shown to multiplex up to three shRNAs: CD3zeta, CD95, and B2M in BCMA targeting CAR T to ensure the inhibition of Graft versus Host Disease, remove susceptibility to killing, and increase cell persistence by avoiding Host versus Graft. An attraction of the shRNA approach is to express multiple shRNA from the same vector that can regulate protein expression thereby optimizing CAR T cell phenotype.

Methods: Retroviral vectors encoding a CAR targeting a well-studied antigen (BCMA) co-expressing multiplexed shRNAs were generated. The multiplexed shRNA molecules were inserted within a microRNA framework to enable expression from a single PolII promoter. Functional assessment of target knockdown in T cells along with retroviral titers was determined.

Results: We have recently demonstrated the ability to simultaneously downregulate up to three different targets using a single vector strategy that encodes a BCMA-specific CAR and a selection marker. Next to these three targets, work is ongoing to further expand the platform and include additional shRNA targets, which will lead to an increase in CAR T survival, persistence and efficacy, without any negative effects on CAR T cell properties. Multiplexing shRNA within a single vector format is a highly attractive approach to generate CAR T cells with bespoke, desired phenotypes. Here we will present the latest results in the shRNA multiplexing platform development. These results demonstrate that multiplexed shRNA is an attractive strategy to regulate the expression levels of multiple targets simultaneously.

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Topic: *ASa03 Engineered T cell therapy for solid tumours*

CD137 AS A TARGET FOR TUMOR IMMUNOTHERAPY BY CHIMERIC ANTIGEN RECEPTORS

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CD137 (TNFRSF9, 4-1BB) is a potent costimulatory receptor on activated effector T cells, and CD137 agonists strongly enhance anti-tumor immune responses. The CD137 signaling domain is part of many chimeric antigen receptors (CARs). However, CD137 is also part of a negative feedback mechanism that prevents overstimulation by transferring CD137 from T cells to CD137 ligand (CD137L)-expressing antigen presenting cells (APC) via trogocytosis, upon which the CD137-CD137L complex is internalized and degraded. Regulatory T cells (Treg) express high levels of CD137 and use CD137 trogocytosis to quench T cell activity. We also found ectopic expression of CD137 in several cancers as it facilitates their escape from immune surveillance. Thus CD137 may be used as a target against certain cancer cells and Treg. For proof of concept, we generated an anti-CD137 CAR that targets CD137 cell surface expression, and armed it on the NK cell line KHYG-1. When anti-CD137 CAR-NK cells came in contact with the CD137-expressing nasopharyngeal carcinoma cell line HK-1, they became activated as evidenced by an increase in IFNγ and TNF secretion. Activation of the anti-CD137 CAR-NK cells enabled them to specifically and time-dependently kill CD137-expressing HK-1 cells in vitro. Further, anti-CD137 CAR-KHYG-1 cells limited the growth of human CD137-expressing nasopharyngeal carcinoma cells in vivo in a murine xenograft.

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SYNTHETIC PROMOTERS TO INDUCE IMMUNE-EFFECTORS IN THE TUMOR MICROENVIRONMENT

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Cancer immunotherapies are highly potent and are gaining wide clinical usage. Adoptive transfer of immune effector cells, such as CAR-T, brings great potency, but also the risk of cytotoxicity. We developed synthetic promoters for the induction of genes of interest under the control of inflammation and hypoxia-induced signals that are associated with the tumor microenvironment (TME). We termed this methodology Chimeric-Antigen-Receptor-Tumor-Induced-Vector (CARTIV). Here I present data of CARTIV based on promoter-responsive elements (PREs) of IFNγ, TNFα, and hypoxia. CARTIV manifests synergistic activity in cell lines, inducing gene expression according to multiple TME factors. We also demonstrate CARIV activity primary human T-cells. We further developed a method to fine-tune promoter attributes, involving turning ON/OFF stimulating factors, followed by sequential cell sorting and sequencing. Enriched promoters identified seventeen candidates, which were re-cloned and validated functionality, leading to the identification of two

CARTIVs with lower backgrounds and higher induction ratios. We further combined the hypoxia element, demonstrating additional modular improvement. The CARTIV platform can improve the therapeutic window of CAR T-cells and other engineered immune cells, providing TME-focused activity.

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Topic: *ASa03 Engineered T cell therapy for solid tumours*

TARGETING SNX9 RESCUES CD28-MEDIATED T CELL EXHAUSTION FOR CANCER IMMUNOTHERAPY

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Tumor-specific T cells are frequently exhausted by chronic antigenic stimulation. To explore new pathways for reinvigoration of anti-tumor immune functions, we developed a human *ex vivo* exhaustion model by repetitive antigenic stimulation of primary CD8 T cells. This results in T cells that resemble patient-derived T cells in tumors on a phenotypic and transcriptional level. Through a targeted CRISPR/Cas9 screen, we discovered that sorting nexin 9 (SNX9) was a mediator of T cell exhaustion. We found that SNX9 amplifies CD28-mediated activation of NFAT, NR4A1, and TOX, thereby accelerating terminal differentiation and dysfunction. Ablation of SNX9 maintains effector functions, induces memory differentiation, and improves tumor rejection by adoptively transferred T cells. In melanoma patients, SNX9 expression was associated with resistance to immune checkpoint blockade. Our findings highlight that targeting excessive co-stimulation is a strategy to prevent T cell exhaustion and to generate protective anti-tumor immunity.

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Topic: *ASa03 Engineered T cell therapy for solid tumours*

ISOLATION AND CHARACTERIZATION OF RNF43 NEO-EPITOPE SPECIFIC TCRS FOR T CELL THERAPY IN GASTROINTESTINAL CANCER

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Adoptive transfer of patient-derived autologous T cells modified to express tumor-specific T cell receptors (TCRs) represents an attractive treatment approach in cancer immunotherapy. Especially if antigens with exclusive expression in the tumor, such as frameshift (fs)- mutation-derived neo-antigens, are targeted, on-target/off-tumor toxicity should be minimized. Further, highly functional TCRs against mutation-derived neoantigens should not get deleted by central tolerance from the repertoire of healthy donors, thus providing a natural safety profile for therapeutic application. Here, we focus on neo-epitopes derived from the tumor suppressor gene Ring Finger Protein 43 (RNF43). RNF43 is frequently mutated in gastrointestinal tumors with high occurrence of fs-mutations, and loss of function enhances tumor growth. We identified HLA-A*02:01 and HLA-B*07:02 restricted epitopes from RNF43 fs-mutations by *in silico* predictions and *in vitro* proteasomal processing assays. We developed a platform for the isolation of extremely rare epitope-specific T cell populations from peripheral blood of healthy donors via single cell sorting on combinatorial pMHC multimer stained CD8+ naive T cells. Subsequent single cell sequencing of sorted multimer specific T cells retrieves the TCR sequence for re-expression via orthotopic TCR replacement (OTR). CRISPR/Cas9-guided endogenous TCR knock-out and knock-in of candidate TCRs enables near physiological re-expression of the TCR of interest. OTR-engineered T cells are thoroughly characterized for target specificity via pMHC multimer staining. Neo-epitope specific TCRs are evaluated for peptide sensitivity, identifying potent TCR candidates that show cytokine release and TCR downregulation after peptide stimulation. Cancer cells harboring the fs-mutation leading to neo-epitopes are recognized and targeted by the cytotoxic potency of TCR candidates. In summary, we developed a platform that can supply neo-epitope reactive TCRs from the naïve repertoire of healthy donors against any neo-epitope target of interest. As proof of principle, we identified highly functional RNF43 neo-epitope specific TCRs with potential for therapeutic application in gastrointestinal cancer.

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Topic: *ASa03 Engineered T cell therapy for solid tumours*

SENSING OF SOLUBLE ANTIGENS WITH ADAPTER CAR™ T CELLS

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Chimeric antigen receptor (CAR) modified T cells showed tremendous success in the treatment of hematopoietic malignancies, ultimately leading to FDA approval of pharmaceutical products. In contrast to the treatment of liquid tumors CAR T cell based therapy of solid tumors appears to be more challenging due to complexity and heterogeneity of the tumor microenvironment (TME) architecture.

CAR T cells are typically designed to respond to surface-bound antigens expressed by malignant cells and not to soluble antigens, e.g. chemokines or cytokines.

Here, we devise a novel technique to redirect CAR T cells to a specific soluble factor. Sensing of the soluble factor is mediated by adapter molecules, more specifically a tagged antibody. In addition, the ability to integrate multiple stimuli by a single CAR entity, while maintaining control over the CAR T cell function at the same time, represents a major advantage of using the Adapter CAR™ technology for this approach. We were able to successfully demonstrate adapter-dependent soluble antigen sensing by CAR T cells *in vitro*.

Sensing of soluble molecules with CAR T cells will on the one hand allow to investigate fundamental questions by comparing systemic to TME-confined soluble molecular cues and

their potential to promote immunosurveillance. On the other hand, new CAR T cell-based TME targeting approaches can be developed, which may help to improve safety and efficacy of solid tumor therapy.

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Topic: *ASa03 Engineered T cell therapy for solid tumours*

SIALYL-THOMSEN-NOUVEAU DIRECTED CAR-T CELLS TARGETING SOLID TUMORS

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Clinical trials have shown the potency and tolerability of CAR-T cells in hematological malignancies. Solid tumors have proven to be more difficult to treat by CAR-T cells. The availability of highly tumor-specific antigens is limited and tumor cells are often protected by stromal cells and pro-tumorigenic immune cells thereby creating an immunosuppressive tumor microenvironment. In this project, CAR-T cells are developed against solid tumors targeting the tumor-specific *Sialyl-Thomsen-Nouveau* antigen (STn). This glycan is of pathological origin and expressed in more than half of all analyzed solid tumors. Its expression is associated with poor prognosis and invasiveness preventing antigen loss. Additionally, loss of antigen expression is further prevented by the redundancy of STn-synthesizing enzymes. STn-directed, first-generation CAR-T cells have already been tested in a clinical setting but failed to induce clinically significant responses. State-of-the-art CAR-T cells in combination with novel humanized anti-STn antibodies with improved specificity might overcome previous limitations. We generated a panel of conventional and innovative CAR designs including CD28, OX40, 4-1BB, TruC (T-cell receptor fusion construct) and TAC (T-cell antigen coupler) and tested them *in vitro* and *in vivo*. All formats initially show potent killing *in vitro* but differed in their behaviour upon repeated stimulation with target cells. Non-conventional CAR designs lacking costimulatory domains had a reduced killing capacity in prolonged and repetitive killing assays. Multiplex analysis of secreted cytokines showed that these non-conventional CD3-epsilon based designs only produced minimal levels of IL-2, IL-4 and IL-6 and lower levels of TNF-α and IFN-γ compared to those including costimulatory domains. Taken together, STn is a promising new target for CAR-T cells and further pre-clinical analysis will define the best format that can be moved forward into clinical development.

P040
Topic: *ASa04 Non-viral vectors and transposons*

GENETIC MODIFICATION OF T-CELLS FOR ADOPTIVE CELL THERAPIES USING NON-VIRAL S/MAR DNA VECTORS

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Adoptive transfer of T-cells engineered with personalized or off-the-shelf T-cell receptors (TCRs) is an attractive therapeutic strategy for the treatment of cancer. Currently, transgenic T-cells are most commonly engineered using viral vectors. However, immunogenicity, the potential risk for stable integration into the genome and subsequent malignant transformation, as well as cost- and time-consuming large-scale manufacturing, limit their widespread application in cancer immunotherapy, particularly in a personalized setting. We have previously developed a non-viral DNA vector platform for the stable genetic engineering of T-cells based on Scaffold/Matrix-Associated Regions (S/MAR). S/MAR DNA-nanovectors (nS/MAR) replicate autonomously and extra-chromosomally in the nucleus of dividing human primary cells without affecting their behaviour and molecular integrity. Moreover, the low-cost and easy manufacturing production of this vector offers additional advantages over the currently used viral vector, transposon and CRISPR/Cas systems for the development of cellular therapies. Here, we refined the nS/MAR DNA system to generate engineered primary human T-cells from an HLA-$A2^+$ donor expressing a TCR specific for the melanoma antigen MART-1, currently tested in clinical trials. We first compared the performance of 5 strong or weak promoters to investigate their capacity to modulate TCR-transgenic T-cell functionality. Every promoter produced viable and functional transgenic T-cells; however, the EF-1 alpha core promoter resulted in the highest (89%) transfection efficiency of the MART-1 TCR and increased cytokine production when co-cultured with the MART-1/HLA-$A2^+$ Mewo melanoma cells. Furthermore, we optimised a GMP manufacturing clinical scale protocol to rapidly generate MART-1 TCR-transgenic T-cells. By combining the CliniMACS® Prodigy and MaxCyte®ExPERT platforms for the electroporation of 4E8 transgenic T-cells, we achieved a transfection efficiency of 86% and the production of clinically relevant recombinant T-cells in only 6 days. Altogether, we show that S/MAR DNA vectors represent a safe technology for T-cell engineering with key advantages for large-scale manufacturing.

P041
Topic: *ASa04 Non-viral vectors and transposons*

AN OPTIMIZED FULLY RNA-BASED PLATFORM FOR CRISPR/CAS9-MEDIATED DISRUPTION OF NATIVE T-CELL RECEPTORS FOR T-CELL THERAPIES

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Genetic engineering of T cells for adoptive T-cell therapies has marked a turning point in personalized immunotherapy. Recently, the field of T-cell engineering and CRISPR-mediated genome editing crossed paths, providing researchers with a powerful and precise tool to modify primary human T cells to increase their therapeutic potential. For example, T cells are gene edited via CRISPR/Cas9 to knock out the expression of T-cell receptor (TCR)α and TCRβ chains of native TCRs in antigen-specific TCR-engineered T cells to promote on-target

effects by avoiding TCR mispairing between native and introduced TCRs, and to prevent graft-versus-host disease in allogeneic T-cell therapies. Viral methods to introduce Cas9 raise concerns about persistent Cas9 expression that could lead to off-target editing. Thus, non-viral methods that involve transient expression of Cas9, such as those using Cas9 ribonucleoproteins or *Cas9* mRNA, benefit from a better safety profile. Compared to Cas9 ribonucleoproteins, in-house production of *Cas9* mRNA using plasmid vectors usually requires less resources and more easily accessible infrastructure, making mRNA electroporation a desirable method for non-viral T-cell engineering. Here, we adapted and further optimized recently published CRISPR/Cas9 and primary T-cell expansion protocols for complete native TCR knockout using a fully RNA-based platform, reducing the number of days needed to produce TCR-deficient T cells, the number of electroporations, and the amount of *Cas9* mRNA and native TCR-targeting guide RNAs used, without loss of efficiency. Our optimized one-week single-electroporation protocol resulted in high numbers of stably edited T cells with knockout efficiencies reaching 91% on average 48 hours after TCR disruption. Further qualifying this adapted CRISPR/Cas9 protocol, functionality of TCR-knockout primary T cells was evaluated for their capacity to recognize tumor cells via tumor antigen-specific TCRs introduced using mRNA electroporation. In summary, this RNA-based CRISPR/Cas9 strategy provides a versatile platform for rapid multiplex genome engineering of primary T cells.

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Topic: *ASa04 Non-viral vectors and transposons*

ORTHOTOPIC T CELL RECEPTOR REPLACEMENT - NEXT-GENERATION ENGINEERING OF T CELLS FOR THERAPY

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T cells can be redirected to an antigen of choice by transgenic insertion of a new T cell receptor (TCR). Conventionally, such engineering is performed by viral transduction. With the advent of CRISPR/Cas9, precise gene editing has now become feasible. This talk will present how CRISPR/Cas9-mediated orthotopic TCR replacement (OTR) can be used for next-generation engineering of T cells for therapy. OTR can be applied with higher throughput and flexilibity than conventional engineering, eliminates the endogenous TCR, introduces the transgenic TCR in a highly defined manner, generates TCR-transgenic T cells with near-physiological behaviour, and leads to more reliable and therefore predictable T cell responses.

P043

Topic: *ASa05 Allogeneic/universal donor cells*

NOVEL CRISPR/CAS9-BASED ALLOGENEIC CAR-T CELLS TARGETING NKG2D LIGANDS: A POTENTIAL UNIVERSAL CELL THERAPY AGAINST CANCER

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Chimeric antigen receptor (CAR)-T cell therapy represents a breakthrough in cancer treatment, showing impressive clinical efficacy even in patients with relapsed or refractory advanced-stage tumors. However, only autologous CAR-T cell therapies against hematological malignancies are currently commercially available, and they have limitations that restrict their clinical utility, such as high costs, manufacturing delays, and antigen-negative tumor relapses. In order to overcome these drawbacks, we developed a novel CAR-T cell therapy, useful for allogeneic application and directed against different types of cancer, by combining CRISPR/Cas9-based gene-editing to eliminate the expression of TCR and HLA-I (key players of graft-versus-host disease and therapy rejection, respectively) with viral integration of the NKG2D-CAR. This atypical CAR recognizes eight different ligands that are absent or rarely expressed in normal tissue but overexpressed in many solid and hematological tumors. Therefore, NKG2D-CAR-T cells are theoretically less prone to resistance and potentially useful against a wide range of cancer types. For the production of allogeneic NKG2D-CAR-T cells, we firstly isolated T cells from peripheral blood of healthy donors. After T cell activation, Cas9 ribonucleoprotein and specific guide RNAs were used in a multiplex manner to disrupt the *TRAC* and *B2M* genes. TCR-deficient T cells were then purified and NKG2D-CAR expression was induced by lentiviral transduction. The CAR-T cell product was characterized by flow cytometry and tested for antitumor activity *in vitro*. Using our manufacturing procedure, we obtained an allogeneic NKG2D-CAR-T cell product with $48.9 \pm 3.6\%$ double knockout (TCR-/HLA-I-) T cells, of which $62.3 \pm 3.5\%$ express the NKG2D-CAR. Furthermore, its antitumor activity was demonstrated against human cervical cancer HeLa cells (52% cytotoxicity after 72 hours of co-culture in an effector:target ratio of 4:1). Additional research is currently underway to further develop this novel universal CAR-T cell therapy to make it available in the clinical setting.

P044

Topic: *ASa05 Allogeneic/universal donor cells*

ANTIGEN-SPECIFIC REDIRECTION OF OFF-THE-SHELF NK-92 CELLS USING THE UNIVERSAL CAR PLATFORM ''UNICAR''

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Modifying of immune effector cells with chimeric antigen receptors (CARs) has revealed a promising therapeutic potential for targeting cancer, especially with CAR-modified T cells. However, the use of other immune cells like primary NK cells or NK cell lines appeared as another advantageous approach that can be combined with CAR technology. Unlike T cells, established NK cell lines can be used allogenically as an off-the-shelf product with reduced risk of toxicities. We have established previously a modular Universal CAR platform (UniCAR) which can be switched on/off and allows the flexible targeting of various tumor antigens. This system consists of two parts, the UniCAR-expressing immune effector cells and a target module (TM). The UniCAR-immune cells cannot recognize surface antigens but are only redirected with the TM which contains an antigen-binding moiety on one hand and an epitope recognized by the UniCAR molecules on the other hand. Here, we provide a proof of concept for using the UniCAR system in combination with the NK-92 cell line to target disialoganglioside GD2-expressing tumors. The UniCAR NK-92 cells induced increase in lysis of neuroblastoma and melanoma cell lines in the presence of scFv- or human IgG4-based TMs, associated with specific release of pro-inflammatory cytokines. Moreover, UniCAR NK-92 cells were shown to be functional in eradicating GD2-expressing tumors in experimental mice. In order to investigate *the in vivo* half-life of the scFv- and IgG4-based TMs, they were radiolabeled with ^{64}Cu and detected using PET imaging. Dynamic PET scanning has shown that the IgG4 format increased the half-life of the TM to around 24 folds in comparison to the scFv-based TMs. In summary, UniCAR NK-92 provides an off-the-shelf universal platform that can be combined with various antibody formats, and can be easily expanded for therapeutic use.

P045

Topic: *ASi05 Allogeneic/universal donor cells*

CD33-TARGETING PRIMARY CAR-NK CELLS DISPLAY POTENT ANTITUMOUR ACTIVITY AGAINST ACUTE MYELOID LEUKAEMIA

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Acute myeloid leukaemia (AML) is the most common acute leukaemia in adults. Although significant advances have been achieved in treating younger patients with AML, prognosis in the elderly who account for the majority of new cases remains poor. Natural killer (NK) cells are specialized effectors of the innate immune system and exhibit potent anti-tumour activity against AML. Promising clinical outcomes have been achieved for AML patients in adoptive immunotherapy with alloreactive NK cells. However, remissions induced by adoptively infused NK cells are only transient, which is partly due to the inhibitory signals deployed by tumour cells. Here we genetically engineered peripheral blood-derived primary human NK cells by using baboon envelope pseudotyped lentiviral vectors (BaEV-LVs) to express second generation CARs carrying different single chain Fv (scFv) antibody fragments specific for CD33, which is highly expressed on the bulk of AML tumour cells and leukaemia stem cells (LSCs). Functional characterisation of CD33 CAR-NK cells revealed high CAR expression and specific cytotoxicity against CD33-expressing but otherwise NK-resistant tumour cells. The CAR construct with the best performance was further tested in a xenograft mouse model. Potent *in vivo* antitumour activity of CD33 CAR-NK cells was observed, leading to a marked reduction of leukemic burden in mice without observable side effects. Since CD33 is upregulated in activated NK cells and can induce fratricide in CD33 CAR-NK cell, an optimised electroporation protocol was developed for efficient CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 (CRISPR-associated) ribonucleoprotein (RNP) complex delivery into primary human NK cells without impairing their functionality and phenotype. Our data suggest CD33-CAR NK cells as a promising approach for treatment of AML and the robust and efficient gene editing method established in this study for primary human NK cells may pave the way to develop more potent NK cell-based immunotherapies against cancer.

P046

Topic: *ASa05 Allogeneic/universal donor cells*

DEVELOPMENT OF AN OFF-THE-SHELF BCMA/CD19 CAR-NK CELL THERAPY FOR B CELL MALIGNANCIES

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Background: Chimeric antigen receptor T-cell therapy has proven to be a valuable new treatment option for patients with B cell malignancies. However, by applying selective pressure, outgrowth of antigen-negative tumor cells can occur, eventually resulting in relapse. Subsequent rescue by administration of CAR-T cells with different antigen-specificity indicates that those tumor cells are still sensitive to CAR-T treatment and points towards a multi-target strategy. Due to their natural tumor sensitivity and highly cytotoxic nature, NK cells are becoming a compelling alternative to T cells, especially considering the availability of an off-the-shelf unlimited supply in the form of the clinically validated NK-92 cell line.

Methods: Given our goal to develop a flexible system whereby the CAR expression repertoire of the effector cells can be rapidly adapted to the changing antigen expression profile of the target cells, electrotransfection of CD19/BCMA-CAR mRNA was chosen as CAR loading method in this study. We

confirmed the functionality of CAR NK-92 against tumor cell lines and primary patient samples. In order to test the clinical applicability of the proposed cell therapy product, the effect of irradiation on CAR NK-92 proliferation and functionality was investigated.

Results: Co-electroporation of CD19 and BMCA *CAR* mRNA was highly efficient, resulting in 88.1% dual-CAR NK-92 cells. The killing capacity of dual-CAR NK-92 exceeded 60% of single and dual antigen expressing cell lines, as well as primary tumor cells, at low effector:target ratios, matching that of single-CAR counterparts. In terms of CD107a degranulation, and IFN-γ and granzyme B secretion, dual-CAR NK-92 significantly outperformed single-CAR NK-92. Furthermore, our results confirm that 10 Gy irradiated dual-CAR NK-92 cease to proliferate and are gradually cleared without a significant impact on their killing capacity.

Conclusions: We established a readily accessible and flexible platform for the generation of highly functional multi-targeted CAR-NK cells.

P047

Topic: *ASa06 Rare, autoimmune and infectious diseases as cell therapy targets*

DEVELOPMENT OF ANTISENSE OLIGONUCLEOTIDES FOR IMMUNOMODULATION

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Autoimmune diseases such as Rheumatoid Arthritis (RA) are complex and involve many different cell types. The current treatment options available are restricted to small molecule drugs and Biologics, typically exerting its therapeutic activity by interfering with exact protein structures. Notably, only a fraction of the proteins in the body are predicted to be druggable by such drug modalities. Antisense Oligonucleotides (ASOs) are synthetic DNA-like molecules that are designed to manipulate expression of their target genes at the post-transcriptional level. As ASOs have the potential to target any gene, thus greatly expanding the universe of potential drug targets, it is a viable drug candidate to modulate aberrant pathways in diseases like RA. In my project, I present a discovery platform, identifying and validating potential drug targets to modulate T cells based on CRISPR-mediated genome editing, and the subsequent design, synthesis and validation of ASOs against these chosen targets. In particular, we are currently evaluating ASOs against the gene LCP2, which we have validated to be a good target to modulate over-active T cells. I have shown that these ASOs can modulate T cell behavior in human cell lines and human primary cells, setting the stage to expand this project to patient material and mouse models to further validate the potential of ASOs as a therapeutic approach for immune diseases. Though focused currently on limited gene targets, this paves the way to answer the overarching questions forming the basis of my PhD: what is a good drug target, and how can technological developments transform the field of drug development?

P048

Topic: *ASa06 Rare, autoimmune and infectious diseases as cell therapy targets*

ENGINEERING OF REGULATORY T CELLS BY MEANS OF MRNA ELECTROPORATION IN A GMP-COMPLIANT MANNER

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Regulatory T cells (Tregs) are crucial in inducing and maintaining tolerance. This unique capacity of Tregs in combination with proof-of-principle in preclinical studies, highlights the potential clinical use of Tregs for the treatment of autoimmunity and transplant rejection. Although proven to be safe and well tolerated in the first clinical trials, only modest clinical results were observed. In this regard, it has been hypothesized that current challenges lie in the development of antigen-specific Tregs. Here, we present an innovative, GMP-compliant, manufacturing protocol for Tregs applicable in a clinical-grade setting allowing efficient and safe redirection of Treg specificity. First, a soluble polymer conjugated with antibodies to CD3 and CD28 and high amounts of exogenous IL-2 for *in vitro* Treg expansion resulted in a more than 70-fold and 185-fold increase of a pure population of $CD4^+CD127^-CD25^{hi}$ Tregs and $CD4^+CD127^-CD25^+CD45RA^+$ Tregs, respectively. Next, expanded Tregs were engineered by means of TCR-encoding mRNA electroporation to generate antigen-specific Tregs. This resulted in an expression of the newly introduced TCR in up to 85 % of Tregs. Moreover, we did not observe a negative effect on the phenotype of Tregs, as demonstrated by the expression of FOXP3, Helios, CTLA-4 and CCR4, nor on the TSDR methylation status. Importantly, mRNA-engineered Tregs were still capable to induce *in vitro* suppression of effector T cells and produced anti-inflammatory, but not pro-inflammatory, cytokines when activated. In conclusion, our findings demonstrate that high numbers of stable and functional Tregs can be obtained with high purity, and successfully engineered with an antigen-specific TCR, in a GMP-compliant manner. We envisage that this clinical-grade protocol will provide solid basis for future clinical application of mRNA-engineered Tregs.

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Topic: *ASa06 Rare, autoimmune and infectious diseases as cell therapy targets*

ENGINEERING HUMAN CD4 LYMPHOCYTES WITH RESISTANCE TO HIV-1 VIA MT-C34 PEPTIDE KNOCK-IN INTO CXCR4 LOCUS

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One way to restrain HIV-1 spread, which occurs during antiretroviral therapy (ART) cessation, is to increase the population

of HIV-resistant CD4 lymphocytes that can be engineered via co-receptor knockout or expression of different antiviral genes. Peptides from the heptad repeat 2 (HR2) domain of gp41 are potent inhibitors of HIV-1 fusion, especially when designed to express on the cell surface. However, their therapeutic potential has only been examined using lentiviral vectors (LV). Earlier, we have developed CRISPR/Cas9-based fusion inhibitory peptide knock-in (KI) technology for the generation and selection of HIV-1-resistant T cells. Cell surface delivery of the peptide was achieved by constructing a chimera between the peptide and CD52. Here, using Cas9 plasmid or ribonucleoprotein electroporation and cell sorting with an antibody raised to MT-C34 peptide, we generated CD4 lymphocytes with KI of MT-C34 peptide at *CXCR4* locus. Concomitantly, we generated MT-C34 expressing CD4 lymphocytes by LV transduction. The degree of cell protection from HIV-1 infection was determined using single- and multiple-round replication assays. The levels of MT-C34 surface expression, lymphocyte proliferation kinetics, and cytokine production (IFNγ and TNFα) were analyzed using immunofluorescence, flow cytometry, and ELISA, respectively, and estimated for lymphocytes generated via KI or LV transduction. Comparative analysis of lentiviral and HDR-based MT-C34 delivery strategies in CD4 lymphocytes showed that LV offered efficient transgene delivery with low cell mortality rates, whereas CRISPR/Cas9 KI into *CXCR4* gene provided higher and uniformed expression of MT-C34 and better protection against HIV-1. Both techniques generated functional lymphocytes capable to proliferate and produce cytokines. Thus, KI-based antiviral peptide delivery opens up new perspectives for engineering HIV resistance, although further improvements in HDR efficiency are needed in order to meet clinical requirements. This work was supported by grant 075-15-2019-1661 from the Russian Ministry of Science and Higher Education.

P050

Topic: *ASa06 Rare, autoimmune and infectious diseases as cell therapy targets*

CHIMERIC AUTOANTIBODY RECEPTOR T CELLS TARGETING AUTOREACTIVE B CELLS IN N-METHYL-D-ASPARTATE (NMDA) RECEPTOR ENCEPHALITIS

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NMDA receptor (NMDAR) encephalitis is the most common autoimmune encephalitis causing psychosis, epileptic seizures and cognitive impairment. Current treatment options are based on broad immunosuppression or non-selective antibody removal, resulting in often treatment-limiting side effects or insufficient responses. Disease-defining pathogenic autoantibodies bind the NMDAR leading to its internalization and subsequent synaptic alterations. Here, we developed NMDAR-specific Chimeric autoantibody receptor (NMDAR-CAAR) T cells to selectively target the source of disease-causing autoantibodies, anti-NMDAR-specific B cells. NMDAR-CAARs consist of an extracellular NMDAR autoantigen fused to intracellular 4-1BB/CD3ζ domains. After coculture with Nalm6 or K562 cells expressing anti-NMDAR-specific B cell receptors (BCRs), human NMDAR-CAAR T cells released interferon-gamma and granzyme B as markers of T cell effector function and killed target cells in an antigen-specific manner with high specificity. Furthermore, target cell encounter *in vitro* induced proliferation of NMDAR-CAAR T cells. In a NOD/Shi-*scid*/IL-2Rγ^{null} (NOG) mouse model, treatment with NMDAR-CAAR T cells led to depletion of Nalm6 B cells expressing an anti-NMDAR BCR and sustained reduction of autoantibody levels with no notable off-target toxicity. For the first time, this strategy could provide a highly selective treatment of a severe neurological autoimmune disease with reduced side effects, faster remission and better long-term prognosis. These results will the pave the way for clinical trials of CAAR-T cells in patients with antibody-mediated neurological disorders and can be expanded to a broader spectrum of antibody-mediated diseases.

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Topic: *ASa06 Rare, autoimmune and infectious diseases as cell therapy targets*

MHC CLASS II-RESTRICTED T-CELL-RECEPTORS PROVIDE HELP FOR T-CELL-THERAPY OF HEPATITIS B VIRUS INFECTION

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Hepatitis B virus (HBV) infection remains a severe health problem with current treatment options being unable to achieve viral clearance. Since virus eradication is known to be accompanied by a strong T-cell response in patients with resolved infection, adoptive T-cell therapy represents a promising therapeutic approach. We recently demonstrated that $CD8^+$ T cells grafted with T-cell receptors (TCR) restricted by MHC class I have the potential to cure HBV infection *in vitro* and *in vivo*. Nevertheless, also $CD4^+$ T cells are known to play an important role in resolving HBV infection. Mechanistic details of their helper function in HBV infection, however, or their advantage for adoptive T cell therapy remain poorly understood. We identified, cloned and characterized a library of MHC class II-restricted TCRs from HBV-specific $CD4^+$ T cells, specific for eight different epitopes from the HBV envelope, core or polymerase protein. The HBV core-specific TCR 1C11 and envelope-specific TCR 1E1 were chosen for further functional studies, based on their broad therapeutic applicability due to a strong functional avidity and a favorable MHC restriction. Upon interaction with HBV peptide- or protein-loaded monocyte-derived dendritic cells in a triple coculture system, HBV-specific TCR-transduced $CD4^+$ T cells increased proliferation and IFN-g secretion of $CD8^+$ T cells, in particular at lower target concentrations. A helper effect of HBV-specific MHC class II-restricted $CD4^+$ T cells was also observed with regards to $CD8^+$-mediated killing of HBV-expressing hepatoma cells, especially when $CD8^+$ T cells were transduced with a low-affinity HBV-specific MHC class I-restricted TCR. These data suggest that MHC II-restricted TCRs will be useful for elucidating $CD4^+$ T cell help in HBV infection with potential benefit for T cell therapy.

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Topic: *ASa06 Rare, autoimmune and infectious diseases as cell therapy targets*

CHIMERIC AUTOANTIBODY RECEPTOR (CAAR) T CELLS AS INNOVATIVE TREATMENT OF ACHR ANTIBODY POSITIVE MYASTHENIA GRAVIS

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Myasthenia gravis is a chronic neuromuscular disorder that causes weakness of skeletal muscles leading to disability. In the majority of patients, the disease is induced by autoantibodies that target the nicotinic acetylcholine receptor (AChR), causing internalization of the receptor, complement activation and receptor blockade, thereby interfering with physiologic neuromuscular transmission. The AChR is composed of five different subunits, with the α1- and (to a lesser extent) the β1-subunit representing the predominant autoantigens. Current treatment options comprise immunosuppressive therapy with potentially harmful side-effects, that remains insufficient in a subgroup of patients. We therefore developed AChRα1- and AChRβ1-specific Chimeric autoantibody receptor (-CAAR) T cells to deplete exclusively the pathogenic autoantibody producing B cells. AChR-CAARs consist of an extracellular domain of the respective autoantigen and intracellular 4-1BB/CD3ζ domains. The efficacy of AChR-CAAR T cells was tested with different assays in vitro. After co-culture with hybridoma cells expressing anti-AChR-specific B cell receptors (BCRs), human AChR-CAAR T cells secreted interferon-gamma and granzyme B reflecting T cell effector function. In line, AChR-CAAR T cells expressed activation markers CD25 and CD69 upon co-culture with respective target cell lines. AChRα1-CAAR T cells killed α1-reactive target cells mAb35, G10, and D6 with high specificity. Accordingly, AChR β1-CAAR T cells selectively killed the β1-reactive cell line B3. Furthermore, AChRα1-CAAR T cells proliferated when stimulated by target cell encounter. This innovative therapeutic strategy could lead to a highly specific and effective treatment of Myasthenia gravis patients circumventing the problems of current immunosuppressive treatments.

P053

Topic: *ASa07 New approaches in lymphocyte engineering*

NUCLEASE-FREE ENGINEERING OF B-CELLS AGAINST HIV

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B-cell engineering via the CRISPR/Cas9 technology is a promising avenue in the fight against HIV. It has recently been shown that adoptive transfer of B cells engineered to express HIV-broadly neutralizing antibodies (bNAbs) can facilitate the production of HIV-neutralizing antibody titers in immunocompetent mice. Nevertheless, engineering using CRISPR/Cas9 may lead to genotoxicity. Here, we developed site-specific B cell engineering without nucleases, based only on endogenous class switch recombination (CSR) induced breaks. We use recombinant adeno-associated viral vectors to introduce genes coding for the anti-HIV bNAb 3BNC117 and GFP into CSR induced breaks at the IgH locus of B cells. Our cassette contains a GFP open reading frame, followed by a 2A peptide and a single-chain version of 3BNC117, with only the variable part of the heavy chain provided. A full, membranal BCR presentation would be possible only upon successful integration into the IgH locus. The introduced cassette was encoded under a derivative of a murine IgH promoter, which is active only upon on-target integration in proximity to the endogenous enhancers. Engineered splenic B cells were GFP-sorted and propagated on feeder cells expressing CD40L and BAFF, for activation and proliferation. Four days post-sorting, 57% of the cells expressed GFP, out of which 73% bound the HIV antigen gp120, demonstrating efficient nuclease-free B cell engineering. We also detected up to 4 ug/ml of 3BNC117 as a secreted IgG in cell culture media. Our method may provide a safe alternative to the rising field of CRISPR-based B-cell editing against the rapidly evolving HIV.

P054

Topic: *ASa07 New approaches in lymphocyte engineering*

ENGINEERED ADENOVIRAL VECTORS EFFICIENTLY TRANSDUCE PRIMARY MURINE LYMPHOCYTES IN VITRO AND IN VIVO

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Ex vivo engineering of lymphocytes is a challenging and costly procedure limiting the clinical application of cell therapies. Selective *in vivo* engineering of lymphocytes would therefore be of utility to the field. Our lab has developed genetically modified adenoviral (Ad) vectors retargeted to a variety of tissue types and demonstrated the use of Ad delivered CRISPR-Cas to achieve gene knock-in *in vivo.* On this basis we endeavored to develop Ads capable of efficiently transducing B and T cells, a target typically resistant to Ads due to the lack of the human Ad serotype 5 receptor (CAR). We therefore utilized a transgenic mouse model ubiquitously expressing the receptor for human Adenovirus 5 (hCAR) and found that *in vivo* and *ex vivo* human Ad5 vectors could achieve meaningful gene transfer to B and T cells, indicating they are susceptible to Ad uptake when the correct receptor is expressed. To extend this concept, we then screened genetically modified Ads developed in our lab targeting alternate receptors and found several candidates capable of achieving efficient gene transfer to C57BL/6 murine primary B and T cells *ex vivo*. We injected a lead infectivity enhanced vector in C57BL/6 mice and found it was capable of driving eGFP expression in statistically meaningful numbers of B and T cells. These studies form the basis of future development towards a genetically targeted Ad vector platform to deliver the CRISPR/Cas machinery to lymphocytes *in vivo* and achieve selective cell engineering.

P055
Topic: *ASa07 New approaches in lymphocyte engineering*

DASATINIB ENHANCES GENE DELIVERY BY T CELL-TARGETED LENTIVIRAL VECTORS THROUGH CD3 UPREGULATION

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CD3-targeted lentiviral vectors (LVs) mediate selective targeting of human T lymphocytes *in vitro* as well as *in vivo* while simultaneously activating the targeted cells. Previously, we have demonstrated sensitivity of receptor-targeted LVs towards receptor endocytosis resulting in reduced gene delivery efficiency. Indeed, CD3-LV causes downmodulation of the TCR:CD3, given its strong agonistic effect on T cells. Cross-linking of CD3:TCR induces CD3 phosphorylation by kinases such as LCK, leading to T cell activation and downregulation of TCR:CD3. We therefore hypothesized that inhibition of CD3 phosphorylation by Src/Abl tyrosine kinase inhibitors such as dasatinib results in enhancement of gene delivery by T cell-targeted LVs. Remarkably, dasatinib treatment of T cells prior to incubation with CD3-LV increased reporter gene delivery by 3- to 10-fold. The transduction-enhancing activity of dasatinib was concentration dependent, starting at 6.25 nM and reaching its maximal effect between 50-100 nM. Moreover, dasatinib increased binding of LV particles to the target cells for up to 24h. When combined with the delivery of the CD19-CAR gene, dasatinib increased CAR T cell numbers by 4- to 9-fold. Importantly, dasatinib-mediated inhibition of T cell activation during CD3-LV transduction did not interfere with the killing activity of the resulting CAR T cells. Tumor cell killing was equally efficient as that of vehicle-treated counterparts, exhibiting killing in the presence of an 8-fold excess of tumor cells. Furthermore, CAR T cells generated in presence of dasatinib showed a more naïve phenotype and were less exhausted, even when applied in long-term killing assay. Our data suggest that dasatinib prevents CD3-LV-induced phosphorylation and TCR:CD3 intake, increasing the amount of CD3-LV bound to the cell surface. This allows efficient CD3-LV cell entry, resulting in substantially increased transduction efficiencies. This is the first description of dasatinib as transduction enhancer, an activity particularly relevant for CAR T cell generation with CD3-LV.

P056
Topic: *ASa07 New approaches in lymphocyte engineering*

SPEEDINGCARS: ACCELERATING THE ENGINEERING OF CAR T CELLS BY SIGNALING DOMAIN SHUFFLING AND SINGLE-CELL SEQUENCING

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Chimeric antigen receptors (CARs) consist of an extracellular antigen-binding region fused to intracellular signaling domains, thus enabling customized T cell responses against target cells. Due to the low-throughput process of systematically designing and functionally testing CARs, only a small set of immune signaling domains have been thoroughly explored, despite their major role in T cell activation, effector function and persistence. Here, we present speedingCARs, an integrated method for engineering CAR T cells by signaling domain shuffling and functional screening by single-cell sequencing. Leveraging the inherent modularity of natural signaling domains, we generated a diverse library of 180 unique CAR variants, which were genomically integrated into primary human T cells by CRISPR-Cas9. Functional and pooled screening of the CAR T cell library was performed by co-culture with tumor cells, followed by single-cell RNA sequencing (scRNA-seq) and single-cell CAR sequencing (scCAR-seq), thus enabling high-throughput profiling of multi-dimensional cellular responses. This led to the discovery of several CAR variants that retained the ability to kill tumor cells, while also displaying diverse transcriptional signatures and T cell phenotypes. In summary, speedingCARs substantially expands and characterizes the signaling domain combinations suited for CAR design and supports the engineering of next-generation T cell therapies.

P057
Topic: *ASa07 New approaches in lymphocyte engineering*

MONITORING EARLY TRANSCRIPTIONAL PROFILES OF CAR T CELL PRODUCTS GENERATED WITH CONVENTIONAL OR CD8-TARGETED LENTIVIRAL VECTORS

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The complicated and multifactorial manufacturing process of chimeric antigen receptor (CAR) T cell products involving gene delivery often by lentiviral vectors (LVs) can potentially affect clinical efficacy and emerge issues ranging from compromised product quality to post-transfusion side effects. Investigating gene expression profiles via single-cell RNA sequencing (scRNA-seq) can substantially improve our understanding about CAR T cell products. In this study, we designed an approach to monitor early events of transduction on CD19-CAR T cells generated with either the conventional VSV-LV or the CD8α-targeted CD8-LV. LV-exposed human donor PBMC were evaluated using a panel of 400 immune-response related genes including LV-specific probes. The resulting data revealed a trimodal expression for the *CAR* and *CD8A* demanding for a careful distribution-based identification of CAR T cells and CD8+ lymphocytes in scRNA-seq analysis. The fraction of T cells expressing high *CAR* levels was in agreement with flow cytometry results. More than 97% of the cells transduced by

CD8-LV expressed the *CD8A* gene. Remarkably, the majority of the potential off-target cells were in fact on-target cells identified by the expression of *CD8B* resulting in a target cell selectivity of above 99%. This is the first study proving the excellent specificity of receptor-targeted LVs by scRNA-seq. Transduced CD8+CAR+ cells had an increased expression of genes associated with proliferation, activation, cell exhaustion, as well as regulatory genes of TCR signaling and inhibitors of apoptosis. Moreover, we identified genes associated with negative regulation of T cell activation and proliferation, along with IFN-induced and pro-apoptotic genes to be upregulated in non-transduced CD8+CAR- cells. The data suggest gene profile alterations and the activation of intrinsic mechanisms possibly influencing the transduction efficiency of LVs. Recent whole transcriptome analysis confirmed the initial findings and appears to provide further insights.

P058

Topic: *ASa07 New approaches in lymphocyte engineering*

EX VIVO GENERATED THYMIC NK CELLS AS A POTENTIAL CELLULAR THERAPY IN NEUROBLASTOMA

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The clear, however subtle, survival benefit of high-risk Neuroblastoma (HR-NBL) patients treated with immunotherapy shows both the potential of, and the medical need to improve immunotherapy in NBL. NK-cells are particularly important in eradication of NBL, as they target NBL via both missing-self cytotoxicity, as HR-NBL often lacks MHC-I expression, as well as via their important effector function during dinutuximab-based immunotherapy. Nonetheless, the cytotoxic NK-cell ratio is significantly decreased at diagnosis and during therapy, indicating the need to boost NK-cell cytotoxicity. Hence, adoptive NK cell therapy might stimulate the eradication of NBL cells in HR-NBL patients. Autologous $CD34^+$ stem cell rescue after high-dose chemotherapy provides an opportunity for cell therapy-based immune boosting strategies. We here explore an *ex vivo* OP9/DL1 co-culture protocol in which $CD34^+$ cells are differentiated into NK-cells. After four weeks of co-culture, $CD56^{bright}$ intracellular $CD3e^+$ ($cyCD3e^+$) NK-cells were generated, expressing similar levels of natural cytotoxicity receptors, superior granzyme levels, similar cytotoxicity against the MHC-I- K562 line, and at least similar targeting of NBL cells compared to healthy-donor NK-cells. The high cytotoxicity and $CD56^{bright}cyCD3e^+$ phenotype of these cells is consistent with a thymic NK-cell phenotype. It is suggested that the high degree of plasticity in MHC-I expression in NBL allows alternate evasion of both cytotoxic T- and NK-cells. We hypothesized that introduction of a NBL-specific T-cell receptor (TCR) in co-culture derived $cyCD3^+$ NK-cells may provide a solution for MHC-I plasticity-mediated immune evasion, as the endogenous CD3 expression may allow surface translocation of the TCR without the need of CD3 co-introduction. Indeed, introduction of a tumor-specific (PRAME) TCR in $CD34^+$ cells results in the generation of $CD56^{bright}TCR^+$ NK-cells with intact missing-self cytotoxic capacity and TCR-dependent killing. This study shows the potential of non-engineered and TCR-engineered $CD34^+$ stem cell-derived NK-cells as a cell therapy to improve HR-NBL immunotherapy.

P059

Topic: *ASa07 New approaches in lymphocyte engineering*

CHARACTERIZATION AND MODULATION OF ANTI-ABTCR ANTIBODIES AND THEIR RESPECTIVE BINDING SITES AT THE BTCR CHAIN TO ENRICH ENGINEERED T CELLS

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T cell engineering strategies offer cures to patients and have entered clinical practice with chimeric antibody-based receptors; $\alpha\beta$T cell receptor ($\alpha\beta$TCR)-based strategies are, however, lagging behind. To allow a more rapid and successful translation to successful concepts also using $\alpha\beta$TCRs for engineering, incorporating a method for the purification of genetically modified T cells, as well as engineered T cell deletion after transfer into patients, could be beneficial. This would allow increased efficacy, reduced potential side effects, and improved safety of newly to-be-tested lead structures. By characterizing the antigen-binding interface of a good manufacturing process (GMP)-grade anti-$\alpha\beta$TCR antibody, usually used for depletion of $\alpha\beta$T cells from stem cell transplantation products, we developed a strategy that allows for the purification of untouched $\alpha\beta$TCR-engineered immune cells by changing 2 amino acids only in the TCRβ chain constant domain of introduced TCR chains. Alternatively, we engineered an antibody that targets an extended mutated interface of 9 amino acids in the TCRβ chain constant domain and provides the opportunity to further develop depletion strategies of engineered immune cells.

P060

Topic: *ASa07 New approaches in lymphocyte engineering*

PHOTOPORATION OF NK92 CELLS WITH BIODEGRADABLE POLYDOPAMINE NANOSENSITIZERS AS A PROMISING STRATEGY FOR THE GENERATION OF CAR-NK CELL THERAPIES.

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Adoptive T cell therapy is one of the fastest-growing branches in cancer immunotherapy. However, as therapies are still expensive, labor-intensive, and have severe side-effects, there has

been growing interest in the use of NK cells. As NK cells are part of the innate immune system, they offer a possibility for allogenic therapies with fewer side-effects. To induce the expression of CAR constructs, NK cells must be transfected in advance. To avoid the safety hazards and limited cargo capacity of viral vectors, more efforts have gone into optimizing physical transfection methods. Our laboratory, in particular, has shown that nanoparticle-sensitized photoporation is an effective method to transiently permeabilize T cells and to allow influx of gene-modifying effector molecules. While non-degradable gold nanoparticles are commonly used for photoporation of cells, we recently found that biodegradable polydopamine nanoparticles can be used instead. In this project, we investigate the use of photoporation with polydopamine sensitizers to deliver different macromolecules in NK92 cells with the promise of the generation of CAR-NK products. Polydopamine nanoparticles (PDNPs) were synthesized through oxidative polymerization and characterized. Delivery efficiency was initially evaluated using FITC dextran 500 kDa (FD500) as a function of PDNP concentration, number of cells, and laser fluence. Quantification of delivery efficiency was assessed by flow cytometry and confocal microscopy. Optimized conditions obtained using FD500 were subsequently used for transfecting NK cells with eGFP-mRNA. PDNPs were successfully synthesized with a hydrodynamic diameter of 465 ± 6 nm, confirmed by DLS and SEM imaging. Preliminary results have shown delivery efficiencies of FD500 in NK92 cells up to 70% with cell viability of 65%. Photoporation with polydopamine nanosensitizers has already proven effective for mRNA transfections of human T cells. Here we show that the same concept can be used to deliver macromolecular effector molecules in NK cells, paving the road towards CAR-NK production.

P061

Topic: *ASa07 New approaches in lymphocyte engineering*

TRANSIENT EXPRESSION OF A CD123-DIRECTED CHIMERIC ANTIGEN RECEPTOR FOR SAFE IMMUNOTHERAPY OF ACUTE MYELOID LEUKEMIA

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T cells engineered with chimeric antigen receptors (CARs) have shown great success in treating selected hematological malignancies. However, approved CAR T cell therapies are patient-individually manufactured by viral transduction, which is not only time- and cost-intensive but also might bear safety issues. The transfer of *in vitro* transcribed (IVT) CAR-encoding mRNA into T cells is therefore pursued as a promising alternative to viral and non-viral DNA-based gene modifications. For acute myeloid leukemia (AML), a transient CD123-targeted CAR T cell therapy has a great potential for an improved safety profile but still requires intensive research. Therefore, to provide an optimal mRNA for T cell modification, the effect of mRNA processing on translation efficiency was analyzed in a cell-free *in vitro* translation system, in which the 5′ cap and the 3′ poly(A) tail could be confirmed as important structures for an efficient translation. Particular nucleoside modifications were shown to reduce the immunogenicity of IVT mRNA but coincidently decreased translation efficiency. The best ratio between immunogenicity and translation efficiency was achieved by n1-methylpseudouridine-modified IVT mRNA as a further optimization. After lipid nanoparticle-based transient transfection of T cells with the anti-CD123 CAR-encoding mRNA, the CAR was expressed by 73-94% of the cells and the transient CAR T cells showed potent cytotoxicity exclusively against CD123+ AML cell lines *in vitro* comparable to stable CAR T cells, which paves the way for *in vivo* testing. The data give insights into the challenging research pathway towards mRNA-based CAR T cell therapeutics. After successful *in vitro* and *in vivo* lab scale research, the transient anti-CD123 CAR T cells will undergo upstream processes for a good manufacturing practice-compliant generation to develop an effective and safe therapy for AML.

P062

Topic: *ASi07 New approaches in lymphocyte engineering*

P329G-CAR-T: A NOVEL ADAPTOR CAR-T CELL PLATFORM RECOGNIZING THE P329G MUTATION IN THERAPEUTIC IGG1 ANTIBODIES FOR ADOPTIVE T CELL THERAPY APPLICATIONS

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The field of chimeric antigen receptor (CAR) T cells has yielded promising results for the treatment of hematological malignancies and multiple approaches are being developed for use in various tumor indications. Universal or modular CARs that do not directly recognize the tumor target antigen itself, but instead are binding to an adaptor molecule facilitating interaction of tumor and CAR-T cells, have recently attracted major interest. Here, we describe the novel modular P329G-CAR-T platform that does not rely on haptens or tags attached to a targeting antibody, but instead recognizes the P329G mutation in the Fc portion of IgG1 antibodies, a mutation frequently applied to silence the Fc effector function of clinical stage therapeutic antibodies. Crystal structure analysis of the anti-P329G Fab fragment in complex with a P329G-Fc region revealed 1:1 binding stoichiometry and surface plasmon resonance analysis determined an affinity of 15 nM. Selective recruitment of P329G CAR-T cells in the presence of the respective P329G-containing antibodies and potent dose dependent tumor cell lysis by transduced T cells as well as IFNg release and subsequent T cell activation was demonstrated for multiple tumor antigens including CD20, CD33, HER2, FOLR1, and mesothelin. P329G-CAR-T activity was found comparable to the activity mediated by the respective direct scFv-based CAR-T cells in terms of kinetics and potency of killing. Furthermore, similar activity was determined in comparison to CD16 based CAR-T cells using the same IgG1 framework but engaging via the Fc-CD16 interaction. Taken together, P329G-CAR-T cells mediate potent and specific tumor cell killing using various adaptor molecules. This approach allows control of CAR-T activity and potential side

effects by titrating the adaptor molecule, and opposed to CD16-based CAR-T cells, P329G-CAR-T cells do not bind endogenous immunoglobulins. Based on these data *in vivo* studies to investigate efficacy and safety of the approach are currently ongoing.

P063

Topic: *ASa07 New approaches in lymphocyte engineering*

SATB1 AND HIVEP2 CO-REGULATE HUMAN TREG CELL IDENTITY AND FUNCTION

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Regulatory T cells (Tregs) are crucial to maintain a balanced immune response by suppressing effector T cells (Teffs) via different mechanisms including the expression of immunosuppressive CTLA-4, TGF-β and IL-10. The Treg master transcription factor (TF) FOXP3 is fundamental for their cellular function and maintenance. However, it remains unclear which other TFs act in parallel or in concert with FOXP3 to establish human Treg cell identity. To identify TFs shaping the identity of human Tregs we performed Cas9 ribonucleoprotein screens targeting different TFs under pro-inflammatory stimulations followed by single cell RNA-seq of the ''hits''. We could identify a large gene module that is co-regulated by the TFs HIVEP2 and SATB1 after IL-12 conditioning (Schumann et al., 2020). Loss of SATB1 or HIVEP2 results in Treg phenotypic destabilization. The KO Tregs express less Treg-specific markers like CTLA-4 and FOXP3 and show increased levels of pro-inflammatory IL-2 and IL-4 in comparison to control Treg cells. Interestingly, *SATB1/HIVEP2* double KO Tregs show mainly the *SATB1* single KO phenotype with the here tested FACS-markers. For some markers a boosting effect by creating a pro-inflammatory cytokine environment (e.g. IL-12, IL-4 conditioning) was shown. To distinguish Treg-specific from general CD4+ T cell effects we also generated KO Teffs. Interestingly, *SATB1* and/or *HIVEP2* KOs in Teffs lead to an opposite cytokine profile in comparison to Treg cells and an upregulation of FOXP3. Our results revealed that HIVEP2 seems to be involved in the upregulation of IFNγ release in Tregs and Teffs. To identify the target genes of HIVEP2 and SATB1 we are now applying ATAC sequencing. A better understanding of human Treg cells are transcriptionally ''wired'' can potentially help us to identify new targets for immunotherapies against autoimmune diseases or cancer.

P064

Topic: *ASa07 New approaches in lymphocyte engineering*

CRISPR-ENGINEERED GAUCHER'S MODEL PROVIDES A POWERFUL TOOL TO EVALUATE THE SAFETY AND EFFICACY OF PROTEIN REPLACEMENT THERAPY AND NANOMEDICINE

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Lysosomal storage diseases (LSDs) are a group of 50 inherited metabolic disorders that have a devastating effect and impose a heavy burden on both the patient and the health care system. However, a lack of genetic models has greatly hampered the development of novel therapeutics for the treatment of LSDs[1]. Here, we describe a CRISPRE-Cas9 strategy to establish a disease model of human Gaucher's disease, the most common type of LSDs, and use it to evaluate the efficacy of both established enzyme replacement therapy and newly developed nanomedicine. First, we demonstrate the strategy and feasibility of a total ablation of the beta glucocerebrosidase (GBA) in human haploid HAP1 cells by CRISPRE-mediated genome editing. We show a successful disruption of GBA gene by insertion of 757-bp DNA fragment in coding Exon 6 and corresponding loss of GBA activities (>95% reduction) in engineered cells as compared to those of parental HAP1. Characterizations of GBA-ablated cells show no significant alterations in cell morphology and growth rate but the amount of lysosomes increases by 43% in engineered cells, consistent with the pathological change of Gaucher's cells. Further, addition of recombinant hGBA to the culture medium of engineered cells can restore GBA activities in a dose-dependent manner, suggesting utility of this disease model for the evaluation of enzyme replacement therapy. Finally, we show that both the disease and parental HAP1 can successfully uptake exosomes, a novel type of nano-medicines that can potentially treat various LSDs, including Gaucher's disease[2]. Currently we are evaluating both the safety and efficacy of our exosome-based therapy for the treatment of Gaucher's disease by using CRISPR-engineered human cells.

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P065

Topic: *ASa07 New approaches in lymphocyte engineering*

TREATMENT OF GAUCHER DISEASE USING BETA-GLUCOCEREBROSIDASE-LOADED EXOSOMES

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Exosomes are cell-derived nano-sized extracellular vesicles that hold a great promise as biocompatible vehicles for targeted delivery of larger therapeutic molecules such as proteins and enzymes. However, an exosome-based strategy to deliver preloaded enzymes into targeted intracellular action sites is still lacking. Here we describe an enzyme replacement strategy based on enzyme-loaded exosomes for the treatment of Gaucher disease, one of the most common lysosomal storage diseases. By fusing the therapeutic β-glucocerebrosidase (GBA) and green fluorescence protein (GFP) with tetrapanin CD63 [1, 2], we were able to incorporate therapeutic GBA into exosomal compartments in living 293T cells. We demonstrated that GBA-loaded exosomes were eventually released into culture medium. Isolation and characterizations of exosomes from conditioned medium confirmed successful loading of GBA enzymes without significant alterations on the particle size and distribution of engineered exosomes as compared to non-modified controls. Further, addition of enzyme-loaded exosomes to the

culture medium of human HAP1 cells resulted in intracellular delivery to the endosomal compartments of recipient cells, the targeted action sites of GBA enzymes. Currently we are evaluating both the safety and efficacy of this exosome-based therapy for the treatment of Gaucher disease in a GBA-null cell model [3].

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P066

Topic: *ASa07 New approaches in lymphocyte engineering*

ACCELERATING DEVELOPMENT AND IMPROVING ACCESS TO CAR- AND TCR-ENGINEERED T CELL THERAPY IN EUROPE

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T2EVOLVE is a breakthrough alliance of academic and industry leaders in cancer immunotherapy that started in January 2021, under the European Union's Innovative Medicines Initiative (IMI). The key objective of T2EVOLVE is to accelerate development and to increase access of cancer patients to immunotherapy with reprogrammed immune cells. Reprogramming is accomplished by genetic engineering with a T cell receptor (TCR) or synthetic chimeric antigen receptor (CAR). Combined expertise of leading researchers in the field aims for identification of gaps and methods to improve efficacy, toxicity and engineering of modified T cells.

Granting EU patients access to the best available medical care, while providing guidance on the implementation of this novel treatment into the EU healthcare system in a sustainable way, is the strategic objective of T2EVOLVE. Moreover, patient involvement will ensure that the perspectives of cancer patients are at the center, in the research setting as well as along the cancer care continuum.

P067

Topic: *ASa07 New approaches in lymphocyte engineering*

MICROBIAL SHORT-CHAIN FATTY ACIDS ENHANCE CD8+ T CELL-MEDIATED CANCER IMMUNOTHERAPY

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Recently, we identified the short-chain fatty acid (SCFA) pentanoate as a low-abundant commensal bacterial metabolite. In this study, the effects of the SCFAs pentanoate and butyrate on $CD8^+$ cytotoxic T lymphocyte (CTL)-mediated anti-tumor immunity have been analysed. These induced the metabolic reprogramming of murine and human CTLs towards elevated glycolysis by increasing the activity of the mTOR complex. Furthermore, the enhanced cytotoxic capacity of $CD8^+$ T cells was achieved by SCFA-mediated inhibition of class I histone deacetylases (HDACs) promoting production of effector molecules such as perforin, granzyme B, TNF-a and IFN-g by CTLs. Moreover, we showed that pentanoate treatment led to histone hyperacetylation at the promoter regions of the master transcription factors EOMES and T-bet, highlighting that epigenetic modifications were mechanistically involved in inducing the CTL-associated phenotype. Importantly, the SCFA treatment of antigen-specific CTLs and chimeric-antigen receptor (CAR) T cells enhanced their capacity to reduce tumor progression in syngeneic tumor models. Collectively, we demonstrate that SCFAs as commensal-derived epigenetic and metabolic modifiers have a therapeutic potential in the context of adoptive cancer immunotherapy and might be used to improve the efficacy of engineered T cell products.

P068

Topic: *ASi07 New approaches in lymphocyte engineering*

FLOW CYTOMETRIC ASSAYS FOR CAR T CELL MANUFACTURING AND PATIENT MONITORING, INVOLVING SPECIFIC CAR DETECTION REAGENTS, STABILIZED PRE-FORMULATED COCKTAILS, AND AUTOMATED DATA ACQUISITION AND ANALYSIS

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Unprecedented efficacy in targeting cancer has been demonstrated using adoptive cellular therapy with genetically engineered chimeric antigen receptor (CAR) T cells. However, the CAR T cell manufacturing process is highly complex and has extensive demands on personnel and infrastructure, which is still a major obstacle for their routine clinical use. Using a device for automated cell processing, the CliniMACS Prodigy, helps to overcome these hurdles, allowing generation of CAR T cells in a single automated and closed system. For assessment of CAR T cells during the manufacturing process and also for patient immunomonitoring, we aimed to establish a set of flow cytometric assays prepared from recombinant antibodies along with CAR detection reagents, e.g. for CD19, CD22, or BCMA. These assays were to be complemented by stabilized pre-mixed cocktails of those and additional tools for automated data acquisition and analysis.

P069

Topic: *ASa07 New approaches in lymphocyte engineering*

A NOVEL GENE-EDITED REGULATORY T CELL PRODUCT RESISTANT TO TACROLIMUS

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Thus far, the ultimate treatment of end-stage organ failure is solid organ transplantation (SOT), which needs permanent immunosuppressive treatment to prevent allograft rejection. To circumvent adverse effects of the immunosuppression, first clinical studies adoptively applying regulatory T cells (Treg) found in-vitro-expanded Treg to be safe and possibly effective for transplantation allowing a reduction of immunosuppressants. However, we still do not have enough experience to use solely adoptive Treg therapy and wean the immunosuppressive drugs completely.

In this study, we developed a GMP-compatible production process for the manufacture of Treg products which are resistant to the commonly used immunosuppressant Tacrolimus. Consequently, $CD8^-$ $CD4^+$ $CD25^+$ $CD127^-$ Treg were isolated from peripheral blood with high purity by a flow cytometry-based sorting system.

After the first round of expansion, the cell-intrinsic adaptor protein that is required for the immunosuppressive function of Tacrolimus (FKBP12) was knocked out with high efficiency by CRISPR-Cas9 technology, aiming for a Treg product, which is resistant to Tacrolimus. Following another expansion phase, the gene-edited Treg products were phenotypically, functionally and qualitatively analyzed in vitro. Increased MFI of FoxP3 and almost full demethylation of TSDR of Treg products compared to the negative fraction of the T_{reg} sort cultured under equal conditions proved that gene-edited Treg products preserved their phenotype entirely after expansion. Also, pro-inflammatory cytokine production of Treg products measured after stimulation showed that proportions of IL2- and IFNg-producers were low in Treg products. Furthermore, we exposed Treg products to Tacrolimus: CITEseq, expansion and proteomic data illustrate that gene-edited Treg products showed resistance to Tacrolimus while they were was suppressed by alternative immunosuppressants (safety switch). Moreover, we investigated off-target activity by NGS-amplicon-sequencing of in silico-predicted off-targets.

Consequently, we developed a GMP-compatible minimally manipulative gene-editing approach, which may assist to further promote the success of adoptive Treg therapy in the SOT setting.

P070
Topic: *ASi09 Other*

AUTOMATED AND SCALABLE CLOSED-SYSTEM PLATFORM FOR CAR-T CELL ISOLATION AND ACTIVATION

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Chimeric antigen receptor T cell (CAR-T) therapy has rapidly advanced from preclinical research to clinical application for personalized medicine. However, wide application of this technology requires a streamlined manufacturing process to achieve reproducible high quality, clinical-grade products. To accomplish this, several challenges must be addressed. Manual, high-touch processing can introduce contamination, inconsistencies, and long throughput times that results in increased cost. Thermo Fisher Scientific's upcoming Gibco™ CTS™ DynaCellect Magnetic Separation System and fit-for-purpose consumables have been developed for automation of T-cell isolation and activation using magnetic beads, such as CTS Dynabeads™ CD3/CD28. The automated instrument performs cell separation and bead removal in a closed system by utilizing an integrated magnet-rocker and fluidic paths. Using the Gibco™ CTS™ DynaCellect Cell isolation kit in combination with CaptureSelect™ N-Ethyl Biotin Anti-CD4 Conjugate, we achieve >90% isolation efficiency of CD3+CD28+ cells with >95% purity, and the automated isolation protocol has no effect on the viability of the isolated T cell population when compared to that of the input cells. Furthermore, automated bead removal using the Gibco™ CTS™ DynaCellect bead removal kit resulted in >85% target cell recovery without affecting cell viability. The system is highly scalable allowing up to 1-liter of reaction volume for the isolation of up to 10 billion target cells with throughput time of approximately 100 minutes. Similarly, bead removal is achieved at a flow rate of 50 mL/min for rapid processing of large volumes of expanded cells. Therefore, Gibco™ CTS™ DynaCellect Magnetic Separation System can improve the cell therapy manufacturing process by combining ease of use with automation and scalability for the upstream isolation and activation of T cells, and subsequent bead removal.

P071
Topic: *ASa09 Other*

EARLY DIVERSIFICATION DURING CHRONIC INFECTION GENERATES EXHAUSTION-RECEPTIVE AND -RESISTANT T CELL SUBSETS

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CD8 T cells responding to chronic viral infection acquire an exhausted state that critically limits their effector function but also prevents immunopathology. It has recently been proposed that exhaustion first emerges in $TCF1^+$ memory precursors (MPs) that later generate exhausted $TCF1^-$ effector subsets. However, single-T-cell fate mapping upon infection with acute vs. chronic strains of lymphocytic choriomeningitis virus

(Armstrong vs. Clone-13), indicates that selective exhaustion of MPs should be insufficient to curtail the emergence of highly cytolytic TCF1$^-$KLRG1$^+$ terminal effectors (TEs). In fact, single-cell RNA sequencing and early adoptive re-transfers reveal that the bulk of TEs derive from TCF1$^-$KLRG1$^-$ early effectors (EE) that have already separated from MPs before the onset of exhaustion. While both MPs and EEs, generated during Armstrong infection, can be re-directed towards T cell exhaustion, mature TEs fail to upregulate the exhaustion-inducing transcription factor TOX and retain antiviral effector functions even when exposed to LCMV Clone-13. Or work shows that a clearer understanding of the early dynamics of T cell exhaustion hold relevance for the effective design of immunotherapies against chronic viral infections and malignancies.

P072
Topic: *ASa09 Other*

DEVELOPING AND IMPROVING A NATURAL KILLER CELL BASED MODEL AGAINST MULTIPLE MYELOMA

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Natural Killer (NK) cells are a promising adoptive cellular therapy against cancer due to their ability to lyse infected or transformed cells in an antigen-independent manner. Human allogenic NK cells produced from healthy donor peripheral blood cells should be expanded and activated with specific feeder cell and cytokines to become a viable treatment, generating expanded NK cells (eNK). Previous results from our research group showed a moderate cytotoxicity effect of eNK cells over several Multiple Myeloma (MM) cell lines, which could be increased combining the eNK cells with the anti-CD38 monoclonal antibody Daratumumab, approved for use in MM patients. However, there's often a noteworthy variability between each series of experiments because of the donor's genetic background. To avoid that variability, we chose the highly cytotoxic cell line NK-92 as a more stable model. However, NK-92 cells are unable to induce Antibody Dependent Cell Cytotoxicity (ADCC) since these cells do not express CD16. We transduced NK-92 cells with the high affinity CD16 variant and a fluorescent reporter using lentiviral supernatant. The new cell line obtained, NK-92-CD16, showed more cytotoxicity alone and especially in combination with Daratumumab than both parental cells and eNK cells. In order to maximize their cytotoxic potential and clinical use, work is in progress to transfect these NK-92-CD16 cells with an anti-SLAMF7 chimeric antigen receptor (CAR).

P073
Topic: *ASa09 Other*

T CELL STEMNESS DURING CHRONIC INFECTION IS MAINTAINED BY ONLY A SUBCOMPARTMENT OF TCF1+ CELLS

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Exhausted CD8 T cell responses to chronic viral infections or tumors are thought to be maintained in a stem cell-like fashion, with TCF1$^+$ precursors (TPEX) continuously replenishing more terminally exhausted subsets (TEX) that mediate residual effector functions. However, it is currently unknown whether all TPEX are functionally equivalent or whether additional heterogeneity exists within the TCF1$^+$ T cell compartment. By using single-cell RNA sequencing focused selectively on TPEX combined with RNA-velocity analysis of *in situ* developmental dynamics, we identify a small subcompartment among TCF1$^+$ cells that is localized at the origin of differentiation trajectories. Single-cell adoptive transfer and *in vivo* fate mapping reveals key stem cell features restricted to this subcompartment, like self-renewal and the potency to generate diverse effector subsets, but also stable PD-1 expression following population re-expansion. Importantly, control of viral replication upon adoptive T cell transfer and proliferative expansion upon PD-1 checkpoint inhibition originates exclusively from these T exhausted stem cells, making them attractive targets for new immunotherapeutic strategies.

P074
Topic: *ASa09 Other*

NEOADJUVANT PD-1/PD-L1 INHIBITORS FOR RESECTABLE HEAD AND NECK CANCER

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Objective: To assess the efficacy and safety of neoadjuvant immunotherapy for resectable HNSCC.

Data Sources and Study Selection: Electronic databases; PubMed (MEDLINE), EMBASE, the Cochrane Library and clinicaltrials.gov were systematically searched for published and ongoing cohort studies and randomized controlled trials for evaluating neoadjuvant immunotherapy for resectable HNSCC, the search results generated studies from 2015 to July 2021.

Data Extraction and Synthesis: Two investigators independently identified and extracted articles for potential inclusion. Random and fixed models were used to achieve pooled odds ratios. All results are presented with 95% confidence intervals (CI). Data quality was assessed by means of the Cochrane Collaboration's Risk of Bias Tool.

Main Outcomes and Measures: The primary outcomes were efficacy, evaluated by major pathological response (MPR) and pathological complete response (pCR) in the primary tumors and lymph nodes separately, and safety, assessed by preoperative grade 3–4 treatment related adverse events (TRAEs) and surgical delay rate.

Results: A total 344 patients from 10 studies were included. In 8 studies neoadjuvant immunotherapy only was administered, and the other 2 studies combined immunotherapy with neoadjuvant chemo/radiotherapy. The overall MPR rate in the primary tumors from studies reporting on neoadjuvant immunotherapy only, was 9.7% (95%CI 3-18.9%) and the pCR was 2.9% (95%CI 0-9.5%). Preoperative G3-4 TRAEs were reported at a

rate of 8.4% (95%CI 0.2-23.2%) and surgical delay at a rate of 0% (95%CI 0-0.9%). There was a favorable effect of neoadjuvant immunotherapy for all outcome measures. The subgroup analyses did not find one specific anti-PD-1/PD-L1 agent to be superior to another, and the favorable effect was demonstrated by either immunotherapy alone or in combination with anti-CTLA-4.

Conclusion and Relevance: Neoadjuvant anti-PD-1/PD-L1 immunotherapy for resectable HNSCC is well tolerated and may confer therapeutic advantages implied by histopathological response. Long term outcomes are awaited.

P075
Topic: *ASa09 Other*

ENDOSOMAL SIGNALING OF ANTIGEN RECEPTORS

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The recognition and the killing of targets by the T lymphocytes is based on the interaction of T cell antigen receptors (TCR) with tumor or pathogen-derived antigenic peptides bound to major histocompatibility complexes (MHC) of target cells. TCR - MHC interaction takes place at plasma membrane of T cells and is followed by the endocytosis of activated TCR, which was initially considered to be a mechanism of signal extinction. This initial view was challenged by the discovery that the internalized TCR is able to signal from endocytic compartments. We found that the endocytic compartment compatible with TCR signaling is tagged by Insulin Responsive AminoPeptidase (IRAP), whose deletion compromises T cell function. Our results indicate that TCR signaling triggered from plasma membrane is sufficient for IL-2 secretion and proliferation, but the effector T cell survival and their ability to infiltrate solid tumors requires endosomal TCR signaling. Our unpublished results showed that the endosomal signaling also applies to some ''TCR-like'' receptors, such as the engineered chimeric antigen receptors (CAR). Using *in situ* biotinylation coupled with quantitative mass-spectrometry we identified signaling pathways that seems to be targeted exclusively from endocytic signaling platforms. Our results might open new ways for engineering better CARs, whose signaling is more similar to that of endogenous TCR.

P076
Topic: *ASa09 Other*

HUMANISATION OF LARGER NSGS-MOUSE COHORTS FROM SINGLE CORD BLOOD DONOR MATERIAL FOR MORE STANDARDIZED IN VIVO TESTING OF ENGINEERED T CELLS

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Basic murine models allow the study of general questions of the immune system *in vivo*. Additionally, more advanced humanised mouse models give hope for the development of improved therapies against infectious diseases and cancer, as they mirror the human immune system closest. However, umbilical cord blood derived $CD34^+$ stem cells are rare and standardised models usually use high amounts of stem cells per mouse to guarantee robust humanisation efficacies, which hinders the generation of larger humanised mice cohorts from single donor material and consequently excludes the possibility to compare various experimental groups in the same setting. Here we demonstrate that optimising the $CD34^+$ single-donor cell dose in NOD-*scid* IL2Rgamma^{null}-3/GM/SF (NSG-SMG3) mice enables the generation of big animal cohorts with sufficiently high humanisation rates. By titrating the injected $CD34^+$ cell numbers, we detected an optimal threshold of 15,000 human $CD34^+$ stem cells resulting in high humanisation levels at 60 % together with succesfull differentiation of all important immune cells comparable to mice treated with the high dose standard. As a proof of concept, we used our protocol to evaluate the development of chimeric antigen receptor (CAR)-related side-effects in a cytokine release syndrome mouse model. It was sensitive enough to allow the discrimination of the toxicity potential of three different CAR-T cells and the control group and group sizes could be enlarged to eight animals per group by even using stem cells from just one donor. In summary, our findings allow the generation of 30 or more humanised mice from a single stem cell donor with high and nearly identical humanisation levels. The reported workflow also enables smaller humanisation experiments from only low $CD34^+$ starting material and can easily and cheaply be implemented for critical translational research in immunotherapies or lymphocyte engineering approaches.

P077
Topic: *ASa09 Other*

INFLUENCE OF CD19 DENSITY AND AFFINITY ON CAR T CELL ACTIVATION: A COMBINATORIAL ANALYSIS OF DIFFERENT PARAMETERS AND THEIR IMPACT ON CAR ACTIVITY

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Despite the great success of CAR T cells in the clinics and the rapid growth of the CAR T cell field in recent years, surprisingly few studies have investigated the impact of binding affinity on

CAR activity. To address this important question in a systematic manner, we are currently setting up an experimental system in which the effect of CAR affinity, antigen density and the co-stimulatory domain (4-1BB vs. CD28) can be measured in a single experiment. As a model system we chose the benchmark scFv FMC63, on which four out of five currently approved CAR T cell therapies are based. We have engineered a set of CD19 variants that bind to the FMC63 scFv with a broad range of affinities (low pM to high nM). The antigen density in our experimental model system will be controlled by transfecting Jurkat target cells with varying amounts of mRNA encoding those CD19 variants. Primary human T cells will be transduced with genes encoding two of the clinically approved CARs, tisagenlecleucel (CD19-4-1BBζ) and axicabtagene ciloleucel (CD19-CD28ζ). Importantly, keeping the CAR design, and especially the scFv binding site constant, will allow to assess the impact of CAR affinity without changing the CAR molecule itself. Together, we expect that this study will contribute to the understanding of CAR T cell activity and its regulation by binding affinity and antigen density. Finally, this experimental system will provide a valuable tool set for further comprehensive studies on different CAR features supporting rational CAR design.

P078
Topic: *ASa09 Other*

DISSECTING THE INTERPLAY OF TCR AVIDITY OF NAIVE CD8+ T CELLS AND TCR RECRUITMENT AFTER ANTIGEN EXPOSURE BY ENGINEERING POLYCLONAL REPERTOIRES

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During an immune response, $CD8^+$ T cells are recruited out of naïve antigen specific precursor populations. T cell receptor (TCR) avidity is considered to be a major determinant of this process. However, it is still not understood which $CD8^+$ T-cell repertoire is present as a precursor population and how it is shaped after antigen exposure. Here we dissected the composition of SIINFEKL-specific TCR repertoires before antigen exposure and investigated TCR avidity-dependent recruitment within a defined polyclonal TCR repertoire *in vivo*. We generated a library of SIINFEKL-specific TCRs isolated via major histocompatibility complex (MHC) class I multimers from naïve C57Bl/6 mice and performed in-depth characterization of functional and structural avidity by TCR re-expression in naïve primary murine T cells and human Jurkat triple parameter reporter cells. Utilizing the Jurkat reporter cells, we screened the TCR (cross)reactivity landscape against single position mutations of the cognate epitope for the entire TCR library, thereby studying 5320 unique TCR-pMHC interactions. Finally, we engineered a traceable SIINFEKL-specific polyclonal T-cell repertoire representative of a physiological avidity distribution in retrogenic mice, and were able to follow T cell recruitment and expansion at a single-TCR resolution *in vivo* after infection with recombinant *Listeria monocytogenes* expressing the model antigen ovalbumin. Our data revealed a perfect TCR CDR3 diversity and a highly diverse avidity distribution in the naïve antigen-specific precursor repertoire with very few clones exhibiting high avidity against the cognate epitope. Upon infection, exclusively high avidity TCRs were recruited in an efficient manner from the naïve repertoire. Furthermore, TCR avidity against the cognate epitope correlated with avidity against epitope mutations. Understanding TCR avidity-dependent T cell fate has major implications for improvement of cellular therapeutic strategies and vaccine design.

P079
Topic: *ASa09 Other*

ASSESSMENT OF WNT/B-CATENIN SIGNALING PATHWAY MODIFICATIONS IMPACT ON TH17 CELLS DIFFERENTIATION - IN VITRO STUDY ON COMPANION DOG MODEL

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Th17 cells are characterized by high production of IL-17 and expression of retinoic acid-related orphan receptor (RORγt). It was shown that adoptively transferred Th17-polarized cells mediated eradication of melanoma in a murine model. We have developed the Th17 cells differentiation protocol from $CD4^+$ T lymphocytes isolated from companion dogs (*Canis lupus familiaris*), since dog model is very useful for comparative medicine. Unlike transplantable xenograft rodent models, canine tumors occur spontaneously and share similar genetics, epidemiology, prognosis factors and clinical outcomes with human tumors.

The aim of our study was to determine the effect of selected factors modifying the Wnt/β-catenin signaling pathway on the differentiation and the memory phenotype of canine Th17 lymphocytes.

We isolated $CD4^+$ T cells from peripheral blood of companion dogs and activated them using epoxylated magnetic beads coated with anti-canine CD3 and CD28 antibodies. We used a previously developed cocktail of cytokines for type 17 polarization with the addition of 1) indomethacin a suppressor of β-catenin, 2) TWS119 an activator of Wnt pathway or 3) XAV-939 an inhibitor of β-catenin translocation to the nucleus.

Our research showed that administration of indomethacin enhanced IL-17 production, TWS119 inhibited canine Th17 differentiation, whereas XAV-939 had no impact on Th17 cells polarization. Additionally, more than 75% of Th17 cells acquired effector memory phenotype ($CD44^{high}CD62L^{low}$) after stimulation with indomethacin and XAV-939.

Overall our findings indicate that Wnt/β-catenin signaling pathway modifications may be used to support canine Th17 differentiation *in vitro* in order to potentially improve their antitumor activity upon adoptive transfer and enhance ability to eradicate tumors in pet dogs.

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P080
Topic: *ASa09 Other*

POTENTIAL CORRELATION BETWEEN THERMOSTABILITIES OF ANTIGEN BINDING DOMAINS AND CAR EXPRESSION LEVELS

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Most commonly single chain variable fragments (scFvs) are used as binding entities within CAR T cells. Though, a number of drawbacks have been associated with their use including CAR clustering, tonic signaling and T cell exhaustion (Long et al., 2015; Salzer et al., 2020). Thus, within this project a range of 15 potential alternative binder scaffolds were tested as binding entities. Plotting the thermostabilities (reflected in the midpoint of thermal denaturation, T_m) of the domains against their expression data within a CAR backbone revealed a putative correlation, which led to further investigation of this matter. In fact, thermostability and expression levels have been shown to be linked in yeast and mammalian cells before (Kowalski et al., 1998; Wang et al., 2013; Jain et al., 2017). In order to explore this relationship, a set of destabilized mutants based on 3 of those scaffolds were created. Using the FoldX software, specific positions for mutations were chosen, whilst leaving the surface properties (charge, hydrophobicity) largely unaltered. Biochemical analysis of those novel mutant proteins revealed monodispersity and a decrease in midpoint of denaturation up to 25°C. Expression of those mutant proteins in a 4-1BB-CD3ζ CAR backbone supports the trend of decreasing expression levels with decreased thermostability, but will need further investigation in order to draw a definite conclusion.

P081
Topic: *ASa09 Other*

BRIDGING THE GAP – ATMP PROCESS DEVELOPMENT ACCELERATING FROM BENCH- TO BED-SIDE

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Cell, gene and tissue products intended for human therapy are classified as Advanced Therapy Medicinal Products (ATMPs) in the European Union (EU). The rapid increase in clinical studies using ATMPs leads to a bottleneck problem: upscaling and automation of ATMP production. Currently, ATMP production is highly manual and therefore time-consuming and expensive. As an example, the manufacturing of patient-individual chimeric antigen receptor (CAR)-modified T cells (e.g. Kymriah®) is more than 250.000 € per patient. As the first GMP facility that has produced clinical-grade CAR-T cells Kymriah® in Europe, the Fraunhofer IZI established its in-house ATMP process development unit to bridge the gap between lab scale research and production according to Guidelines on Good Manufacturing Practice (GMP). Technologies studied in lab can be directly transferred to upscaling and automation processes in the GMP Process Development Unit to provide a GMP-ready process for subsequent GMP-compliant manufacturing. ATMP production processes to be optimized include antigen-specific T cells, CAR-T cells, CAR-NK cells, dendritic cells, mesenchymal stem cells (MSC), induced pluripotent stem cells (iPSC), and tissue engineering products. In the development unit, process adjustments can be tested and optimized flexibly and cost-efficiently. The impact of new devices, media, seed densities and freezing protocols on the GMP process is also investigated here. This poster gives insights into ATMP development processes, equipment, and technologies of value for academic as well as industry partners.