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International Conference on Lymphocyte Engineering
12–14 September 2023
Munich, Germany

ICLE 2023 – Oral Presentations

O001 / No. 183

Topic: AS02 Currently ongoing clinical trials

PHASE 1/2 MULTICENTER CLINICAL TRIAL OF DONOR SPECIFIC TOLERANCE INDUCTION IN LIVING DONOR LIVER TRANSPLANTATION VIA ENGINEERED T CELLS WITH SUPPRESSING FUNCTIONS

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Introduction: Patients with organ transplants must take immunosuppressants continuously for the rest of their lives to control rejection, and are at risk of fatal side effects such as serious infections and carcinogenesis. These immunosuppressants inhibit not only graft rejection as well as all the necessary immune response to maintain homeostasis, therefore selective immune-suppression targeted to immune rejection is the ideal. We have recently reported that seven liver transplant patients out of 10 have been achieved transplant tolerance over 10 years by donor antigen specific Treg cell therapy induced by the blocking the interaction between CD80/86 and CD28. We developed cell products as regenerative medicines and protocol of clinical trial for evaluating safety and efficacy in the liver transplantation.

Methods: Based on the previous research data, we have considered amount of lymphocytes as raw material and dose of cell product. Moreover, based on the discussion of the Pharmaceuticals and Medical Devices Agency in Japan, the study design, evaluation period and endpoints were examined.

Results: We have been conducting a single-arm, open-label, four transplant center clinical trial employing engineered Treg in HLA mismatched liver transplant recipients. This trial is composed of phase 1 as safety cohort (n=3) and phase 2 (n=7) as efficacy cohort with step-wise weaning and withdrawal of immunosuppression in 18 months after transplant. Endpoints include safety and operational transplant tolerance over a year.

Conclusions: This trial, which is currently recruiting, will provide clinical evidence of safety and efficacy of our engineered Treg cell product to induce liver transplant tolerance.

O002 / No. 184

Topic: AS02 Currently ongoing clinical trials

GD2.CAR T-CELLS AUGMENTED WITH A CONSTITUTIVELY ACTIVE INTERLEUKIN-7 RECEPTOR FOR TREATMENT OF PEDIATRIC CNS TUMORS

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Background: We employ GD2-directed CART-cells (GD2.CARTs) modified to express a constitutively active interleukin-7 receptor (C7R) to increase GD2.CART survival and function independent of external cytokines against GD2-expressing CNS malignancies.

Methods: In a Phase I study, we investigated the safety and efficacy of intravenous GD2.CART therapy for pediatric patients with diffuse midline glioma (DMG) or other GD2-expressing recurrent CNS tumors. The first treatment cohort received GD2.CARTs without C7R, while subsequent cohorts received GD2.CARTs co-transduced with C7R (C7R-GD2.CARTs) at two dose levels. Results: Twelve patients were treated without dose limiting toxicity. The first cohort (n=3) received GD2.CARTs without C7R at 1×10^7 cells/m². No toxicity was observed and all 3 patients had clinical neurological improvement lasting approximately 3-4 weeks before disease progression. The next two cohorts received C7R-GD2.CARTs at 1×10^7 cells/m² (n=3) and 3×10^7 cells/m² (n=6). Tumor diagnoses included recurrent medulloblastoma (n=2) and DMG (n=7). Mild tumor inflammation associated neurotoxicity was observed in 7 of 9 (78%) patients, which was controlled with anakinra without need for corticosteroids. CRS was observed in 5 of 9 (56%) cases, resolving with tocilizumab. Eight of 9 patients receiving C7R-GD2.CARTs had clinical improvement or stability for more than 8 weeks (up to 12 months at last follow-up) and received repeat cell infusions at 6 week intervals (range 2-4 cycles). Partial radiologic responses were confirmed for 2 of 7 (29%) patients with DMG.

Conclusion: Intravenous treatment with C7R-GD2.CARTs was well tolerated in children with CNS tumors. The duration of clinical improvement was extended by C7R co-expression.

O003 / No. 148

Topic: AS03 Novel clinical approaches and combination therapies

COMBINATION OF TCR-T CELLS COENGINEERED TO SECRETE SIRP-ALPHA DECOYS AND ANTI-TUMOR ANTIBODIES TO AUGMENT MACROPHAGE PHAGOCYTOSIS

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It is widely held that developing immunotherapeutic strategies for harnessing innate immunity is critical for improving responses to adoptive T-cell therapy (ACT). One promising target is the CD47/SiRP α 'don't eat me' axis, which deters macrophage-mediated phagocytosis of tumor cells. Although highly upregulated by many tumors, CD47 is expressed by virtually every cell-type. Hence, antibody treatment is limited by antigen sinks and toxicity. We thus coengineered TCR-T-cells to secrete high-affinity SiRP α (CV1) decoys directly in the tumor microenvironment (TME). We began with a Winn assay and observed significantly improved control of tumor outgrowth by TCR-T-cells coengineered to secrete CV1-Fc. However, in subcutaneous tumor models, the treatment failed due to T-cell depletion. We demonstrated that the coengineered TCR-T-cells become coated in decoy and susceptible to phagocytosis by both murine and human macrophages. We reasoned that we could instead co-administer anti-tumor antibodies comprising an active Fc tail with TCR-T-cells genetically modified to secrete CV1-monomer. We achieved significantly improved phagocytosis of tumor cells upon co-culture of macrophages with CV1-monomer and antibodies. Moreover, co-administration of Avelumab (anti-PD-L1) and Cetuximab (anti-EGFR) favorably reprogrammed the TME and significantly improved the A375 tumor control upon ACT. CD47 blockade on the T-cells by CV1-monomer also led to treatment failure in NSG mice. Importantly, however, T-cells coengineered with CV1-monomer or CV1-nullFc were not phagocytosed by human macrophages, presumably due to the presence and recognition of other 'don't eat me' signals. Taken together, we conclude important promise for the clinical translation of this combinatorial ACT strategy potentiating both adaptive and innate immunity.

O004 / No. 143

Topic: AS04 Superpowered lymphocytes

EMPOWERING CYTOTOXICITY OF CAR T CELLS TO ENHANCE ANTI-CANCER RESPONSES

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Initial clinical responses with chimeric antigen receptor (CAR) T cells in cancer patients were highly encouraging. However, primary resistance and relapse after therapy remain a problem that prevent durable remission. One of the underlying causes is the resistance mechanisms in cancer cells that limit effective killing by CAR T cells. We have developed a technology, coined optimized killing (OK) technology, that boosts tumor cell killing by CAR T cells, thereby bypassing resistance mechanisms in tumor cells. Using this technology we reveal that BCMA CAR T cells equipped with a granzyme B-NOXA fusion construct improves killing of multiple myeloma (MM) cell lines and patient cells in vitro and in MM xenograft models in vivo. In this approach, the granzyme B sequence allows

localization of pro-apoptotic NOXA into granzyme B-positive cytotoxic granules in BCMA CAR T cells. After tumor cell recognition granzyme B, together with NOXA, is secreted into the synapse between the BCMA CAR T cell and the tumor cell. NOXA enters tumor cells through perforin pores and specifically binds and inhibits pro-survival protein MCL-1. Since MM cells critically depend on MCL-1 expression, its inhibition effectively induces apoptosis. Importantly, our technology can be used with any type of engineered T or NK cell and for any type of cancer where apoptosis resistance in tumor cells hampers killing efficacy. It can enhance tumor cell killing of existing engineered T or NK cell strategies and maximize their success rate. As a consequence it may improve cancer treatment and prevent relapse.

O005 / No. 160

Topic: AS05 Non-viral CAR/TCR engineering

PERSISTENT GENETIC MODIFICATION OF HUMAN HEMATOPOIETIC STEM AND PROGENITOR CELLS USING S/MAR DNA NANOVECTORS

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Transplantation of HSPCs electroporated with drug resistance inducing genes would ensure effective tumor suppression by applying respective anticancer therapy while maintaining sufficient hematopoiesis in cancer patients. Effective strategies for the persistent genetic modification of HSPCs are needed for this treatment approach. Recombinant integrating viruses are a potent and widely used tool. However, their random integration into the genome introduces a risk of insertional mutagenesis. Their innate immunogenicity presents another impediment to clinical application. To achieve the goal of an efficient and safe generation of stably transfected cells of a variety of cell types, we have developed a non-viral DNA vector platform based on Scaffold/Matrix-Associated Regions (S/MAR). The episomal maintenance of S/MAR DNA nanovectors (nS/MAR) eradicates the risk of insertional mutagenesis, providing a major advantage compared to already clinically applied approaches using CRISPR/Cas9 systems or viral vectors. Additionally, with its unlimited capacity, even large therapeutic transgenes or other sophisticated genetic elements can be delivered with nS/MAR. Our study demonstrates the feasibility of using nS/MAR for persistent genetic modification of K562 and HSPCs. In contrast to K562, the transfection efficiency of primary human HSPCs was lower, with scope for improvement. However, our results demonstrate the ability of nS/MAR to transfect HSPCs even from poor-quality cryopreserved leukapheresis material. These findings provide a promising first step towards the genetic modification of HSPCs with nS/MAR and their usage in hematopoietic cell transplantation for the treatment of cancer patients. Further studies are ongoing to determine the optimal conditions for efficient modification of HSPCs including electroporation and expansion protocols.

O006 / No. 156**Topic:** AS06 Universal donor cells & advanced TCR engineering**REDIRECTING TCR SPECIFICITY IN REGULATORY T CELLS TOWARD CLASS I HLA-RESTRICTED ISLET ANTIGENS**R. Porret¹, P. Guillaume², O. Alfageme-Abello¹, A. Alcaraz Serna¹, L. Ermellino¹, E. Lana¹, E. Stefanidis², A. Semiliotof², G. Giordano Attianese², F. Lebreton³, V. Zoete⁴, E. Berishvili³, M. Irving², Q. Tang⁵, Y. Muller¹¹Centre Hospitalier Universitaire Vaudois (CHUV), Division Of Immunology And Allergy, Lausanne, Switzerland, ²Ludwig Institute for Cancer Research, University of Lausanne and Lausanne University Hospital, Department Of Oncology, Lausanne, Switzerland, ³Geneva University Hospitals (HUG), Cell Isolation And Transplantation, Geneva, Switzerland, ⁴Swiss Institute of Bioinformatics/ Ludwig Institute for Cancer Research, Department Of Oncology, Epalinges, Switzerland, ⁵University of California (UCSF), Diabetes Center, San Francisco, United States of America

Background Redirecting regulatory T cells (Tregs) specificity against class-I molecules abundantly expressed on pancreatic islets may represent a promising strategy to restore tolerance in type 1 diabetes. Herein, we study the contribution of the co-receptor CD8, which can be expressed as an $\alpha\beta$ -heterodimer or $\alpha\alpha$ -homodimer, in the activity of two previously described HLA-A2-restricted islet-specific TCRs.

Methods The TCR clones D222D(ZnT8₁₈₆₋₁₉₄) and 32(IGRP₂₆₅₋₂₇₃) were engineered into the TRAC locus of primary T cells and Tregs by homology-directed repair. In parallel, the CD4 co-receptor was swapped with either CD8 α or CD8 $\alpha\beta$ molecules into the CD4 locus. TCRs reactivities were challenged with 24 peptides derived from ZnT8 and IGRP. Transgenic cell lines expressing increasing HLA-A2 molecules were generated. Activation and CFSE-based T-cell suppression assays were performed.

Results Triple knock-out/in (TRAC, TRBC, CD4) efficiencies were 80.5% (+/- 17.2%) and 30.1% (+/- 12.2%) respectively. TCR-D222D was peptide-dependent and CD8 $\alpha\beta$ -dependent (EC50= 0.01mM vs. 0.03mM for CD4-to-8 $\alpha\beta$, 16mM for CD4-to-8 α , non-responsive for CD4^{KO} and wild-type conditions). TCR-32 induced CD25 and CD71 upregulation in a peptide-independent and HLA-A2-dependent manner (EC50=1044 HLA-A2 molecules vs. 1557 for CD4-to-8 $\alpha\beta$, 3505 for CD4-to-8 α) despite specific tetramer staining. Interestingly, the CD4 co-receptor inhibited downstream TCR signaling of clone 32. CD8 $\alpha\beta$ -TCR-D222D and CD8 α -TCR-32 engineered (eTCR) Tregs were successfully expanded (mean 19.5-fold) and suppressed significantly better than their polyclonal counterparts *in vitro*.

Conclusion We functionally validated two eTCRs Tregs for type 1 diabetes. Clone D222D was peptide-specific and CD8 $\alpha\beta$ co-receptor-dependent. In contrast, clone 32 was alloreactive against HLA-A2 molecules representing a better candidate for islet transplantation.

O007 / No. 102**Topic:** AS07 Beyond alpha-beta T cells**TCR-ENGINEERED INKT CELLS INDUCE ROBUST ANTI-TUMOR RESPONSE BY DUAL TARGETING CANCER AND SUPPRESSIVE MYELOID CELLS**G. Delfanti¹, F. Cortesi¹, A. Perini¹, L. Azzimonti², C. De Lalla¹, C. Garavaglia¹, M. Squadrito³, M. Consonni¹, F. Re⁴, H. Shen⁵, P. Dellabona¹, G. Casorati¹¹San Raffaele Scientific Institute, Experimental Immunology Unit, Division Of Immunology, Transplantation And Infectious Diseases, Milan, Italy, ²IDSIA, Supsi/usi, Manno, Switzerland, ³San Raffaele Telethon Institute for Gene Therapy (SRTIGET), Targeted Cancer Gene Therapy Unit, Milan, Italy, ⁴School of Medicine and Surgery, University of Milano- Bicocca, Bionanomedicine Center Nanomib, Monza, Italy, ⁵Houston Methodist Research Institute, Department Of Nanomedicine, Houston, United States of America

Adoptive immunotherapy with T cells engineered with tumor-specific TCRs holds promise for cancer treatment. However, suppressive cues generated in the tumor microenvironment (TME) can hinder the efficacy of these therapies, prompting the search for strategies to overcome these detrimental conditions and improve cellular therapeutic approaches. Invariant natural killer T (iNKT) cells are a T lymphocyte subset expressing a conserved semi-invariant TCR that recognizes lipid antigens presented by the monomorphic molecule CD1d, which actively participate in tumor immunosurveillance by restricting suppressive myeloid populations in the TME. We showed that harnessing iNKT cells with a second TCR specific for a tumor-associated peptide, generated bi-specific effectors for CD1d- and MHC-restricted antigens *in vitro*. Upon *in vivo* transfer, TCR-engineered iNKT (TCR-iNKT) cells showed the highest efficacy in restraining the progression of multiple tumors that expressed the cognate antigen compared to non-transduced iNKT cells, or CD8⁺ T cells engineered with the same TCR. TCR-iNKT cells achieved robust cancer control by simultaneously modulating intra-tumoral suppressive myeloid populations and killing malignant cells. This dual anti-tumor function was further enhanced when the iNKT cell agonist alpha-Galactosyl Ceramide (α -GalCer) was administered as a therapeutic booster through a platform which ensured controlled delivery at the tumor site. These preclinical results support the combination of tumor-redirected TCR-iNKT cells and local α -GalCer boosting as a potential therapy for cancer patients and point to iNKT cells as a cell platform for engineering with anti-tumor TCRs or CAR, providing an appealing alternative to conventional T cells for the treatment of solid and hematological malignancies.

O008 / No. 131**Topic:** AS08 In vivo T cell engineering**MULTI-OMICS INFORMED ENHANCEMENT OF CAR GENE DELIVERY BY RAPAMYCIN, INDUCED REDUCTION OF ANTIVIRAL MECHANISMS AGAINST T-CELL TARGETED LENTIVIRAL VECTORS**F. Charitidis¹, E. Adabi¹, N. Ho¹, A. Braun^{1,2}, C. Tierney³, L. Strasser³, F. Thalheimer^{1,4}, L. Childs⁵, J. Bones^{3,6}, C. Clarke^{3,7}, C. Buchholz^{1,2,4}¹Molecular Biotechnology and Gene Therapy, Paul-ehrlich-institut, Langen (Hessen), Germany, ²Deutsches Krebsforschungszentrum and German Cancer Consortium, (dktk), Heidelberg, Germany, ³Characterisation and Comparability Laboratory, National Institute For Bioprocessing Research And Training, Dublin, Ireland, ⁴Frankfurt Cancer Institute (FCI), Goethe University, Frankfurt am Main, Germany, ⁵Host-Pathogen Interactions, Paul-ehrlich-institut, Langen (Hessen), Germany, ⁶School of Chemical and Bioprocess Engineering, University College Dublin, Belfield, Dublin, Ireland, ⁷National Institute for Bioprocessing Research and Training, Fosters Avenue, Mount Merrion, Blackrock, Dublin, Ireland

Lentiviral vectors (LV) have become the dominant tool for stable gene transfer into lymphocytes including chimeric antigen receptor (CAR) gene delivery to T cells, a major breakthrough in cancer therapy. Yet, room for improvement remains, especially for the latest LV generations delivering genes selectively into T cell subtypes, a key requirement for in vivo CAR T cell generation. Towards improving gene transfer rates with these vectors, we conducted whole transcriptome analyses on human T lymphocytes after exposure to CAR-encoding conventional vector VSV-LV, and vectors targeted to CD8+ (CD8-LV) or CD4+ T cells (CD4-LV). Genes related to quiescence and antiviral restriction were found to be upregulated in CAR-negative cells exposed to all types of LVs. To further distinguish between cells that successfully convert into CAR T cells versus cells only bound by vector particles but not converting, ongoing analysis is using barcoded antibodies (Abseq) directed against the vector particle envelopes, the CAR, and 30 immune markers. Down-modulation of various antiviral restriction factors including the interferon-induced transmembrane proteins (IFITMs) was achieved with rapamycin as verified by mass spectrometry (LC-MS). Strikingly, rapamycin enhanced transduction by up to 7-fold for CD8-LV and CD4-LV without compromising CAR T cell activities, but did not improve VSV-LV. When administered to humanized mice, CD8-LV resulted in higher rates of GFP gene delivery as well as faster in vivo CAR T cell generation and tumor control. The data favor multi-omics approaches for improvements in gene delivery.

0009 / No. 130

Topic: AS09 Targeting non-malignant diseases

MRNA TECHNOLOGY FOR THE DEVELOPMENT OF CAR-T CELLS TARGETING FIBROTIC DISEASES

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Engineered T cells expressing chimeric antigen receptors (CARs) have already been proven to be an effective treatment in cancer immunotherapy. For this reason, recent research approaches are investigating the use of CAR-T cell therapies in non-malignant immune-mediated diseases such as infectious diseases, autoimmune or fibrotic diseases. Fibrotic diseases represent a number of different disorders characterized by the overgrowth, hardening, and/or scarring of various tissues caused by excessive deposition of extracellular matrix components. The main cellular mediators of fibrotic diseases are activated fibroblasts, which serve as the primary extracellular matrix-producing cells. While many surface antigens on activated fibroblasts have already been identified, the fibroblast activation protein (FAP) represents the most promising target. To date, the vast majority of CAR-T cells in development are based on stable genetic modification by viral vectors. However, the long-term consequences, for example due to off-target effects, are still poorly understood. An alternative to viral transduction is the transient modification by introducing an mRNA encoding the CAR protein into the T cells. Thus, mRNA-

based CAR cell therapies offer the possibility of a safe and pharmacokinetically controllable immunotherapy. Here, we present results for the reprogramming of T cells with CAR-mRNA directed against FAP for the treatment of fibrotic diseases. The work includes the optimization of the anti-FAP-CAR-mRNA for prolonged CAR expression and reduced immunogenicity. In addition, novel image-based cytotoxicity assays using live cell microscopy have been developed, providing new insights into the kinetics of CAR-T cell killing.

0010 / No. 52

Topic: AS09 Targeting non-malignant diseases

SELECTIVE DEPLETION OF ALLOREACTIVE B CELLS IN SOLID ORGAN TRANSPLANTATION: CHIMERIC ALLO-ANTIGEN-SPECIFIC T CELLS OVERCOMING REJECTION BY ANTIBODIES (CORA-TS)

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One major complication after solid organ transplantation (SOT) is antibody-mediated rejection (AMR) by anti-donor HLA antibodies, which is only indirectly addressed by modern immunosuppression. Direct B-cell depletion protocols are inefficient in preventing AMR and associated with an increased infection risk, emphasizing the need for a more precise targeting of alloreactive B cells. B cells with anti-donor HLA specificity are uniquely characterized by expressing the corresponding B-cell receptors (BCRs). As proof-of-concept, using anti-HLA-A*02 BCRs as target, we redirected T cells towards alloreactive B cells by introducing a novel chimeric receptor comprising a truncated HLA-A*02 molecule fused to intracellular 4-1BB/CD3 ζ signalling domains to generate T cells overcoming rejection by antibodies (CORA-Ts). Upon co-cultivation with B-cell lines expressing and secreting anti-HLA-A*02 antibodies, CORA-Ts were specifically activated (expression of CD25, CD69, CD137), released pro-inflammatory cytokines (e.g. IFN- γ , granzyme B), and exhibited strong cytotoxicity towards B cells with HLA specificity resulting in an effective reduction of anti-HLA-A*02 antibody release. In an allograft mouse model, CORA-Ts significantly reduced growth of anti-HLA-A*02 B cells. Genetic modification of the HLA-A*02 α 3 domain abrogated T-cell sensitization against the CORA receptor. Additionally, CORA-Ts were engineered to resist immunosuppressive treatment via CRISPR/Cas9-mediated knockouts of corresponding binding proteins. Our results demonstrate that CORA-Ts are able to specifically recognize and eliminate alloreactive B cells, and thus selectively prevent formation of anti-HLA antibodies even under immunosuppressive conditions. This suggests CORA-Ts as a potent novel approach to specifically

combat AMR and to improve long-term graft survival in SOT patients while preserving their overall B-cell immunity.

0011 / No. 181

Topic: AS10 Other

DEVELOPING NOVEL TARGETING CARs FOR GLIOBLASTOMA

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Glioblastoma is a devastating brain tumour with an alarmingly low survival rate of 5% beyond 5 years. Current treatments are insufficient, and new targeted therapies are urgently needed to improve patient outcomes. Chimeric Antigen Receptor (CAR) T cell therapy has revolutionized the treatment of some types of blood cancers by equipping a patient's immune cells with synthetic receptor that targets and destroys cancerous cells. However, there is a critical need for effective CAR T cell therapies for glioblastoma patients. Our research team has developed a pipeline to identify novel targets and generate and test CAR T cells for efficacy against glioblastoma. We first developed a novel CAR targeting the Epidermal Growth Factor Receptor Variant III (EGFRvIII), a specific mutation expressed in a subset of glioblastoma cases. Our new high-affinity CAR demonstrated highly effective anti-tumour activity in two orthotopic *in vivo* models of glioblastoma. Additionally, we have conducted comprehensive safety testing and confirmed that this therapy is likely to be safe for human use, even in tissues with high EGFR expression. We have mapped the epitope binding domain of the binder. Our successful development of this therapy showcases our capacity to take a recombinant protein and translate it into a functional CAR T cell therapy for glioblastoma. Moreover, we are expanding our research to identify novel glioblastoma targets using cell surface mass spectrometry. With this pipeline in place, we aim to iterate and develop further targeted CART cell therapies for glioblastoma patients and novel targets will be discussed.

0012 / No. 180

Topic: AS10 Other

INFECTED HEPATOCYTE CLEARANCE AND SUSTAINED TUMOR REGRESSION BY HBSAG-SPECIFIC TCR-T THERAPY FOR HBV-RELATED HCC

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Hepatitis B virus (HBV) infection accounts for 80% of virus-associated hepatocellular carcinoma (HCC) in Asia. HBV-DNA can integrate into the host cell genome, constantly produce viral antigens, and cause premalignant lesions and finally HCC, which can be targeted by HBsAg-specific TCR-T cells. A natural, high-affinity TCR was isolated from an HLA-A*02:01⁺ donor with resolved HBV infection (Wisskirchen, *JCI* 2019). Lentiviral TCR-transduction using a GMP-compliant protocol resulted in highly functional TCR-T cells with no detectable cross-reactivity in tissue panels tested. Furthermore, TCR-T cells showed a high target-specific anti-tumor activity in a preclinical mouse model transplanted with HBsAg+ hepatoma cells. Therefore, safety and efficacy of the HBsAg-specific TCR applied in an autologous cell product was assessed within an IIT (NCT05339321). A 54-year-old Chinese male with advanced HBV-related HCC was enrolled and received a single infusion of 7.9x10⁷/kg TCR-T cells after lymphodepletion. The treatment was well-tolerated and no major toxicity was reported. Serum HBsAg rapidly decreased from baseline 558 IU/mL to 0.08 IU/ml and remained barely detectable. Pre- and post-treatment biopsies indicated that the expression of HBsAg in hepatocytes was reduced from 10% to undetectable. The patient achieved a partial response using mRECIST score, with >50% decrease in the total diameter of target lesions. TCR-T cells persisted, and the tumor and antiviral response were maintained for at least 6 months. The clinical data demonstrated encouraging clinical proof-of-concept of HBsAg-specific TCR-T cell therapy in treatment of HBV-related HCC and warrants further evaluation of its safety and efficacy within a systematic phase I/II clinical trial.

ICLE 2023 – Poster Presentations

P001 / No. 64

Topic: AS01 Update on the approved therapies

DIRECT CELL REPROGRAMMING OF FIBROBLASTS TO NATURAL KILLER CELLS

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Natural killer (NK) cells are key innate lymphocytes with remarkable cytotoxic abilities that control cancer and viral infections independently of antigen-specificity. Indeed, NKs are the first iPSCs-derived hematopoietic cells to be tested in clinical trials against hematological tumors. However, limited persistence *in vivo* and complexity of differentiation protocol, pose roadblocks to widespread NK-based therapeutics. Direct cell fate reprogramming was successfully applied to reprogram myeloid immune cells, but instructor transcription factors (TFs) of lymphoid cells are not known. We hypothesize that direct reprogramming can be applied to efficiently generate NK cells contributing to a better understanding of the NK transcriptional network. To induce NK identity, we overexpressed NK canonical TFs – TBET, ETS1, NFIL3, EOMES, (TENE) – in mouse embryonic fibroblasts and profiled induced cells by single cell RNA-seq (scRNA-seq). TENE induced downregulation of fibroblast signature and upregulated the NK-associated genes *CD34*, *CD38* and *Itga2*. In human embryonic fibroblasts, TENE induced surface expression of CD34 and CD56, supporting the species conservation of the minimal transcriptional network for NK reprogramming. Prolonged time in culture and use of cytokine cocktails for iPSC-derived NK differentiation resulted in higher percentages of CD34+ cells, suggesting *in vitro* expansion of this population. Furthermore, to identify candidates that support NK reprogramming, we screened 48 TFs employing a barcoded TF approach coupled with scRNA-seq and identified RUNX, IKZF and STAT families as potential novel regulators. Overall, our findings contribute to understand the role of TFs in NK specification and present evidence for an alternative platform to generate patient-specific NK.

P002 / No. 90

Topic: AS01 Update on the approved therapies

DISTINCT ANTIBODY- AND T CELL-BASED IMMUNE RESPONSES TO SARS-COV-2 VACCINATION AND INFECTION IN PATIENTS TREATED WITH ANTI-CD19 CAR T CELL THERAPY

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Chimeric antigen receptor (CAR) T cell therapy targeting CD19 has become an established treatment in hematological patients and holds great potential in the field of autoimmune disease. Besides its ability to deplete malignant or self-reactive B cells, CD19 CAR T therapy also depletes the pool of naïve B cells responsible for protection against new pathogens, such as the SARS-CoV-2 coronavirus. To determine the effect of CAR T induced B-cell aplasia on adaptive immune protection, we conducted a study with over 20 α CD19 CAR T treated patients and monitored specific immune responses after vaccination and/or infection with SARS-CoV-2. T cell response to Spike, Nucleoprotein and Membrane protein peptide pools were determined by IFN γ Enzyme-linked immunosorbent spot (ELISpot) and flow cytometry. Antibodies to SARS-CoV-2 Spike and the Nucleoprotein in the serum were measured by Luciferase Immunoprecipitation System (LIPS) assay. Our results show the ability to generate strong T cell immunity despite persistent B-cell aplasia and suggest an inverse relationship between the virus-specific antibody titer and the frequency of virus-specific T cell responses in the patients. Further studies investigating the effect of SARS-CoV-2-specific immune status on infection severity are warranted. Monitoring T cell responses in CD19 CAR T patients may facilitate informed decisions regarding vaccination schemes for these patients, which will become increasingly relevant as the use of this therapy will further expand in hematologic malignancies and has started to extend towards the autoimmunity field.

P003 / No. 151

Topic: AS02 Currently ongoing clinical trials

3RD-GENERATION CD19-DIRECTED CHIMERIC ANTIGEN RECEPTOR T-CELLS (CARTS) FOR RELAPSED/REFRACTORY CHRONIC LYMPHOCYTIC LEUKEMIA (CLL) – UPDATE ON THE ONGOING ACADEMIC PHASE 1/2 TRIAL (HD-CAR-1)

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Introduction: Success of CD19-CARTs in CLL has been impeded by the disease-inherent T-cell dysfunction. Here, we show an update on HD-CAR-1, a 3rd generation CD19-CART, in patients with high-risk r/r CLL.

Methods: HD-CAR-1 is an IIT evaluating escalating doses of CD19-CARTs comprising 4-1BB and CD28 as costimulatory domains in patients with advanced B-cell malignancies. CART manufacturing, administration and monitoring were performed in-house. CLL patients were eligible if they had failed chemoimmunotherapy and at least one pathway inhibitor and/or alloHCT.

Results: Until February 2023, seven CLL patients were treated with HD-CAR-1, with a median age of 62 years and two to ten prior treatment lines. Three patients had also failed alloHCT, and six patients harbored TP53 abnormalities. In all patients, successful CART manufacturing was achieved and three different dose levels were administered. Rapid CART expansion was observed in six patients. CART peak levels correlated with the applied CART dose level. With a single case of CRS \geq III^o and no severe neurotoxicity, HD-CAR-1 toxicity was moderate. However, in one patient prolonged grade four neutropenia with neutrophil recovery on day +32 was observed. Responses were noticed in all seven patients. All five patients being treated at the highest dose level reached a CR, of whom four were minimal residual disease (MRD)-negative. With a median follow-up of 524 days (275-650), four of these five patients are alive with two of them being in ongoing CR.

Conclusions: Successful generation of homebrewed 3rd generation HD-CAR-1 CARTs was possible for heavily pre-treated patients with high-risk CLL and exerted a promising safety and efficacy profile.

P004 / No. 132

Topic: AS02 Currently ongoing clinical trials

IMPACT OF DIFFERENT Γ -CHAIN CYTOKINES ON CLINICAL CAR T CELL MANUFACTURING

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Although chimeric antigen receptor T cell (CAR-T) therapy is effective in treating B cell malignancies, a certain fraction of patients failed effective treatment response or relapse within a couple of months. A critical parameter influencing the effectiveness of CAR-T cell therapy is the T cell phenotype and differentiation status of the final product. Cytokines were shown to impact the final CAR T cell product in terms of e.g., maintenance of CD4⁺ subpopulations or higher fractions of favorable phenotype subpopulations, such as stem cell like (T_{SCM}) or central memory (T_{CM})-like CAR T cells. We have developed a manufacturing protocol for our switchable universal CAR-T platform, which is currently used to manufacture autologous universal CAR-T products for two clinical trials (NCT04230265, NCT04633148), using a combination of interleukin (IL)-2, IL-7, and IL-15. Over 40 manufacturing runs have been successfully completed until today. All products showed a beneficial phenotype with predominantly T_{CM} in the final product. To better understand the impact of different common

y-chain cytokines on product quality, we have tested different combinations of IL-2, IL-7, IL-15 and IL-21 in a down-scale model of our clinical manufacturing process. Our results demonstrate that factors like transduction rate, CD4/CD8 ratio, viability and expansion rate are significantly affected by the chosen cytokine regime, with sometimes opposite effects caused by certain cytokine combinations in the different parameters. This study demonstrates that the cytokine cocktail used for a manufacturing process must be carefully selected and a variety of parameters must be analyzed to determine an optimal combination.

P005 / No. 150

Topic: AS02 Currently ongoing clinical trials

THIRD-GENERATION CHIMERIC ANTIGEN RECEPTOR (CAR) T CELLS IN ADULT ALL AND NHL PATIENTS

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Background: Third-generation chimeric antigen receptor (CAR)-engineered T-cells (CARTs) might improve clinical outcome of patients with B-cell malignancies. Here we report a phase-1/2 investigator-initiated trial (HD-CAR-1) on a third-generation CART dose-escalating, investigator-initiated trial treating adult patients with acute lymphoblastic leukemia (ALL) or non-Hodgkin's lymphoma (NHL).

Methods: Refractory and/or relapsed (r/r) adult ALL or NHL patients received escalating doses of CD19-directed third-generation CARTs comprising the costimulatory domains CD28 and CD137 (4-1BB) after lymphodepletion with fludarabine and cyclophosphamide. Leukapheresis, manufacturing and administration of CARTs were performed in-house.

Results: 35 patients with r/r ALL or r/r NHL were enrolled. Patients had received a median of 4 (range 2 to 9) prior treatment lines, including allogeneic stem cell transplantation (alloSCT). For all patients, CART manufacturing was feasible. Patients were treated with one to 200 million CARTs/sqm. None of the treated patients developed any grade of immune effector cell-associated neurotoxic syndrome (ICANS) or a higher-grade (\geq grade III) cytokine release syndrome (CRS). Response and outcome were associated with the administered CART dose. At 3-year-follow-up, overall survival (OS)/progression free survival (PFS) was 70%/50 % and 50%/25% for NHL and ALL patients, respectively. Responders displayed higher frequencies of a specific memory-like T cell subset within the CART-product.

Conclusions: Third-generation HD-CAR-1 CARTs were remarkably safe and of promising efficacy. A specific subset of memory T cells within the CART product could predict response to treatment. Overall, HD-CAR-1 appears to be a promising step towards safe and effective ALL eradication.

P006 / No. 50

Topic: AS03 Novel clinical approaches and combination therapies

OVERCOMING THE CHALLENGES FOR CAR T-CELL IMMUNOTHERAPY AGAINST GLIOBLASTOMA BY IMMUNOMODULATION 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, thus, increasing efforts are being devoted to developing new therapeutic strategies, particularly in the field of CAR T-cell immunotherapy. CAR T-cell therapy in GBM, and in solid tumors in general, faces several challenges including the high level of heterogeneity, and the immunosuppressive tumor microenvironment (TME). Recently we have published our work on a novel CAR T against a specific antigen. We have shown that this CAR T acts against both mouse and human GBM cancer cells, yielding prolonged survival in mouse models. However, most animals eventually succumbed to the disease. and tumor analysis revealed a highly immunosuppressive TME. To overcome this hurdle and improve the efficacy of our novel CAR T we are working on a combination therapy strategy with an immunomodulatory agent that can revert the immunosuppressive milieu and reinvigorate T-cell effector mechanisms. In order to ensure a localized and safe expression of the agent at the tumor site, we are using a lipid-nanoparticle mRNA-based tool for *in vivo* targeted expression with promising preliminary results. Our *in vitro* studies showed that this agent promotes T-cell proliferation and function, and combination therapy *in vivo* resulted in prolonged survival of the mice. To conclude, even though GBM is a challenging candidate for CAR T-cell immunotherapy, we consider that the combination with nanomedicines, which can remodel the TME and overcome immunosuppression, represents a promising therapeutic approach.

P007 / No. 54

Topic: AS03 Novel clinical approaches and combination therapies

COMBINATORIAL TARGETING OF SOLID TUMOR ANTIGENS FOR CAR T CELL THERAPY

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Cellular therapies have become a newly established pillar of cancer therapy. This has dramatically revolutionized treatment paradigms for several leukemic diseases, especially in regard to B cell malignancies. While cellular therapies have been unprecedentedly successful against leukemia, they lack efficacy when it comes to solid tumors – an area of active research. One major roadblock is the lack of truly tumor-specific targets. In the setting of CAR T cell therapies for B cell leukemia, CD19 has been a most prominent target. However, CAR T cells are not able to differentiate between normal and malignant B cells. As a consequence, the patient will suffer from a more or less permanent B cell aplasia - an acceptable side effect that can be treated. For solid tumors the disintegration of healthy tissues by off-tumor CAR toxicity prevents effective treatment so far. Here, we present a potential solution by using the AdCAR™ technology for combinatorial targeting of solid tumor antigens. The AdCAR™ technology allows the usage of small antigen-binding molecules, like Fabs or Nanobodies, as titratable elements during CAR engagement, thus adding an additional lever for control of CAR T cell activity. We show that by the combination of different concentrations of two target binding molecules, CAR T cell cytotoxicity can be steered to only specifically kill cells, overexpressing the combination of targets. In summary, AdCAR T cells represent a versatile and effective option for specific tumor targeting.

P008 / No. 77

Topic: AS03 Novel clinical approaches and combination therapies

NKG2D-CAR MEMORY T CELLS TARGET PEDIATRIC T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA IN VITRO AND IN VIVO BUT FAIL TO ELIMINATE LEUKEMIA INITIATING CELLS

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Refractory/relapsed pediatric acute leukemia are still clinically challenging and new therapeutic strategies are needed. Interactions between Natural Killer Group 2D (NKG2D) receptor, expressed in cytotoxic immune cells, and its ligands (NKG2DL), that are upregulated in leukemic blasts, are important for anti-leukemia immunosurveillance. Nevertheless,

leukemia cells may develop immunoescape strategies as NKG2DL shedding and/or downregulation, which impairs NKG2D binding to NKG2DL and targeting of tumor cells. In this report, we analyzed the anti-leukemia activity of NKG2D chimeric antigen receptor (CAR) redirected memory (CD45RA⁺) T cells *in vitro* and in a murine model of human T-cell acute lymphoblastic leukemia (T-ALL) with Jurkat cells. We also explored how soluble NKG2DL (sNKG2DL) affected NKG2D-CAR T cells cytotoxicity and the impact of NKG2D-CAR T cells on Jurkat cells. *In vitro*, we found NKG2D-CAR T cells targeted leukemia cells and showed resistance to the immunosuppressive effects exerted by sNKG2DL. *In vivo*, NKG2D-CAR T cells controlled T cell leukemia burden and increased survival of the treated mice but failed to cure the animals. After CAR T cell treatment, Jurkat cells upregulated genes related to proliferation, survival and stemness, and *in vivo*, these Jurkat cells exposed to NKG2D CAR T cells exhibited functional properties of leukemia initiating cells. The data here presented show, that, NKG2D CAR T cells target T-ALL blasts *in vitro* and *in vivo*. However, for a successful clinical translation, combination of NKG2D CAR T cells with other therapeutic approaches specifically targeting the LICs subset may be needed to totally eradicate pediatric T-ALL.

P009 / No. 103

Topic: AS03 Novel clinical approaches and combination therapies

ANTI-TUMOR POTENTIAL OF NKG2D CAR T CELLS AGAINST PEDIATRIC MALIGNANT CENTRAL NERVOUS SYSTEM TUMORS

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Pediatric malignant Central Nervous System (CNS) tumors are the most common solid tumors, and the leading cause of cancer-related death in children, underlying the need for new therapeutic approaches. In this regard, CAR T cells have emerged as a new pillar of treatment for pediatric CNS tumors. The interactions between NKG2D receptor on immune effector cells and NKG2D ligands on tumor cells are essential for tumor immunosurveillance. In the present study, we have explored the ability of NKG2D CAR T cells to target pediatric brain tumors. By using Europium-TDA cytotoxicity assays *in vitro*, we found 6 out of the 6 tested CNS tumor cells lines were sensitive to NKG2D CAR T cells lysis, with a percentage of cytotoxicity $\geq 30\%$ when effector: target ratios of 20:1 were used. Furthermore, in 3D cultures, NKG2D CAR T cells showed ability to penetrate and eliminate glioblastoma tumor-spheres. In an orthotopic murine model of human glioblastoma, NKG2D CAR T cells injected intracranial reduced tumor burden but not when we used intravenous infusions. In addition, we have recently iso-

lated exosomes from NKG2D CAR T cells and we have found they maintain NKG2D expression on their surface. We are now planning to explore their anti-tumor potential against CNS pediatric malignant tumors in the near future. In sum, although very preliminary, our results show that NKG2D CAR and Exo-NKG2D CAR could be a promising therapeutic approach to treat these tumors.

P010 / No. 218

Topic: AS03 Novel clinical approaches and combination therapies

THE POTENTIAL USE OF CAR-T EXTRACELLULAR VESICLES (EVS) AS A THERAPEUTIC STRATEGY FOR SOLID TUMORS

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LB: Academic Abstract Body: Solid tumors remain the leading cause of cancer-related mortality in developed countries; mainly due to advanced stage diagnoses, leading to poor prognosis. CAR-T cells has remarkable success in the treatment of hematology cancers. However, have limited ability to traffic & infiltrate the solid tumor niche. A plausible explanation is the immunosuppressive tumor microenvironment. Furthermore, CAR-T cell therapy has been found to be toxic as well as difficult to manufacture.

EVS, a small membraned vesicle originated from cells, can interact with target cells via several mechanisms and transfer their cargo content of proteins, genetic molecules (DNA/RNA/miRNA) and etc. CAR-T EVs are a novel approach that combines the benefits of both EVs and CAR T cells. Aharon and Globerson Levin have previously shown that CAR-T EVs bind and kill target cells in a specific CAR dependent manner. Thus, administration of CAR-T EVs may facilitate the infiltration of solid tumor niches while reducing side effects and toxicity. This study aims to investigate CAR-T EVs mechanisms of action against solid tumors.

To examine the use of CAR-T EVs for the treatment of solid tumors, we are studying the specific mechanisms lays both in *in vitro* and *in vivo* in several adenocarcinomas. We will produce human and murine CAR-T cells as well as CAR-T EVs, characterize their functionality and evaluate the effects of CAR-T cells and EVs in adenocarcinomas murine models using several routes of administration in order to determine the appropriate path and protocol for maximum therapeutic effect.

P011 / No. 105

Topic: AS03 Novel clinical approaches and combination therapies

DEVELOPMENT OF A CLINICAL SCALE MANUFACTURING PROCESS FOR GENERATING ORGAN-TARGETED REVCAR REGULATORY T-CELLS

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*These authors contributed equally to this work. Clinical trials using polyclonal regulatory T cells (Tregs) have shown proof-of-safety and efficacy in the context of solid organ transplantation, autoimmune diseases and graft-versus-host disease (GvHD). However, recent pre-clinical studies indicate that antigen-specific Treg therapies using the chimeric antigen receptor (CAR) technology could exert a more localized immunosuppression making it a safer and more effective treatment option. A novel modular switchable CAR platform namely the RevCAR system has been developed by the Bachmann and Feldmann Lab in Dresden which renders the genetically modified T cells inert and requires the infusion of bispecific target modules (RevTMs) to be functional. In our collaborative work we show the feasibility of generating universal RevCAR Tregs as a therapeutic cell product for patients with inflammatory and autoimmune disorders. To achieve this, we have established a clinical scale GMP-compliant manufacturing process that starts with leukapheresis from a healthy donor followed by a two-step Treg isolation process i.e., magnetic-activated cell sorting (MACS) followed by GMP-compliant fluorescence-activation cell sorting (FACS). The isolated Tregs are transduced and expanded in a fully closed automated instrument until clinically relevant cell numbers are reached. The final product containing universal RevCAR Tregs is harvested for characterization using phenotypic and functional assays. We could successfully show proof-of-concept for our manufacturing process, which generated highly pure (>85%) and specific Treg cells with clinically relevant yield (fold expansion >90). We envision this novel immune-modulatory cell therapy product as a promising versatile treatment option for patients with inflammatory and autoimmune disorders.

P012 / No. 164

Topic: AS03 Novel clinical approaches and combination therapies

GPMB-TARGETING CAR T CELL THERAPY OF MIT/TFE-DRIVEN CANCERS

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Relapsed and metastatic paediatric and adolescent/young adult sarcomas have poor long-term outcomes. Some of which, like Alveolar soft part sarcoma (ASPS), have no standard-of-care systemic treatment options. Despite limited clinical experience, Chimeric antigen receptor (CAR)-T cell therapy has shown promising activity in relapsed/metastatic sarcomas. We have developed a novel 2nd generation 41bbζ chimeric antigen receptor (CAR) T cell therapy that targets a cell surface protein called glycoprotein NMB (GPNMB), which is highly expressed in several solid tumours, including ASPS. In preclinical testing, our CAR T therapy, called GCAR1, efficiently kills GPNMB-expressing cancer cells in culture and elicits durable regression of GPNMB-expressing solid tumors when xenografted into mice, including metastatic lesions in the brain. Motivated by these data, we produced clinical-grade GCAR1 lentiviral vector using good manufacturing practice (GMP) and validated a GMP-compliant GCAR1 cell manufacturing process on the Clin-iMACS Prodigy. We now aim to conduct a first-in-human, multi-centered, phase I clinical trial of GCAR1 in patients with relapsed/refractory GPNMB-expressing solid tumours and otherwise limited non-curative standard options.

P013 / No. 120

Topic: AS03 Novel clinical approaches and combination therapies

NOVEL RNA CAR-T CELLS AGAINST GBM-ASSOCIATED ANTIGEN PTPRZ1 SHOW ANTI-TUMOR EFFICACY BOTH IN VITRO AND IN VIVO

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In recent years, the therapeutic use of chimeric antigen receptor T cells has achieved significant success in the treatment of B cells malignancies. Despite promising results in mouse tumor models, a similar outcome hasn't yet been observed in solid tumors. Specifically, in glioblastoma (GBM) several clinical trials only showed a modest efficacy, partly due to the high tumor heterogeneity. In this setting, we aim to develop an "à-la-carte" CAR-T cell strategy that targets a panel of GBM antigens. We are also focused in used temporarily expressed RNA CARs, looking for a reduction of side effects and allowing multiple CAR-T cell infusions. As a first GBM target, we selected a highly-associated marker, especially with Glioma-initiating cells, PTPRZ1. Through a phage display screening, we generated 6 different new scFv against PTPRZ1 and cloned them into an RNA CAR plasmid. Then we selected the one (RRB471) with better cytotoxic activity against a GBM PTPRZ1-expressing cell line obtained in our institution. Using triple reporter Jurkat cell lines electroporated with PTPRZ1 targeting CAR, we show that CAR expression was detected for up to

7 days while tonic signaling was detected in less than 20% cells. In an orthotopic allogeneic tumor model using NSG mice, even only one dose of our anti-PTPRZ1 CAR-T cells was able to significantly increase the mice's overall survival. Our results point to validating the use of PTPRZ1 as an attractive new target in the GBM setting and our CAR against this antigen as a new promising anti-tumoral tool.

P014 / No. 174

Topic: AS03 Novel clinical approaches and combination therapies

CYTOKINE-ARMORED DENDRITIC CELL PROGENITORS SYNERGIZE WITH CAR-T CELLS IN SOLID TUMORS

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Dendritic cells (DCs) are specialized myeloid cells with the ability to uptake, process, and present antigens to T lymphocytes; they also generate cytokine and chemokine gradients that regulate immune cell trafficking, activation, and function. Monocyte-derived DCs (moDCs) pulsed with tumor antigens have been used as a platform for therapeutic vaccination in cancer. However, in spite of significant development and testing, antigen-loaded moDCs have delivered mixed clinical results. Here we present a DC therapy that uses a population of mouse or human DC progenitors (DCPs) engineered to produce two immunostimulatory cytokines, IL-12 and FLT3L. In the absence of antigen loading, cytokine-armoured DCPs efficiently differentiated into conventional type I DCs (cDC1) and inhibited tumor growth in melanoma and autochthonous liver cancer models. Tumor response to DCP therapy involved synergy between IL-12 and FLT3L and was associated with massive T and NK cell infiltration and activation, robust M1-like macrophage programming, and ischemic tumor necrosis. Mechanistically, anti-tumor immunity was dependent on endogenous cDC1 expansion and interferon- γ (IFN γ) production and signaling, but did not require CD8⁺ T cell cytotoxicity. In one application, cytokine-armoured DCPs synergized with antigen-specific CAR-T cells to eradicate intracranial gliomas in mice.

P015 / No. 96

Topic: AS03 Novel clinical approaches and combination therapies

TUMOR-DIRECTED MACROPHAGE GENE DELIVERY OF NATURAL AND SYNTHETIC CYTOKINES EMPOWERS CAR T CELLS IN THE GLIOBLASTOMA MICROENVIRONMENT TO THWART TUMOR GROWTH

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The immunosuppressive tumor microenvironment (TME) of glioblastoma represents a major roadblock to chimeric antigen receptor-T cells (CAR-Ts). While cytokines can counteract immune-suppression, their systemic administration entails risk of toxicities and counter-regulatory responses. We showed that TIE2-expressing macrophages (TEMs) can deliver interferon- α (IFN) specifically in the TME through a hematopoietic stem cell-based gene therapy (GT) to broadly reprogram it toward immune-activation, leading to tumor inhibition and prolonged survival of immunocompetent mice challenged with a novel glioblastoma model (mGB2), faithfully recapitulating the human disease. This approach is undergoing clinical trial for glioblastoma patients. To investigate whether the reprogrammed TME could favor CAR-T function, we generated B7H3-redirected CAR-Ts by optimizing a high-efficiency lentiviral-based transduction protocol of murine T cells. IFN GT ameliorated CAR_{B7H3}-T activation and reduced exhaustion, improving tumor growth control and survival with either systemic or loco-regional administration of CAR-Ts, in absence of toxicities. To corroborate the portability of our GT and boost CAR-Ts expansion, we investigated delivery of lymphotropic cytokines. GT with inducible interleukin (IL)-12 showed full tolerability and enhanced CAR_{B7H3}-T activity similar to IFN GT. We also established a private crosstalk between TEMs and CAR-Ts by implementing orthogonal IL-2/ receptor pairs. We achieved safe and selective potentiation of CAR-Ts, leading to improved function. Double-route CAR-T injection enhanced their basal anti-tumor activity, that was similarly potentiated by IFN and orthogonal IL-2 GTs and even furtherly when cytokines were co-delivered. TEM-based cytokine GT overcame some key limitations of CAR-Ts in a clinically-relevant glioblastoma model, rescuing T-cell function and synergizing in tumor inhibition while preserving full tolerability.

P016 / No. 84

Topic: AS03 Novel clinical approaches and combination therapies

EVALUATION OF CD318 AS TARGET FOR CAR T CELL THERAPY OF PANCREATIC DUCTAL ADENOCARCINOMA

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Pancreatic ductal adenocarcinoma (PDAC) is one of the leading causes for cancer-associated deaths and it accounts for around 90% of all pancreatic cancers. Unfortunately, patients diagnosed with PDAC have a poor 5-year overall survival prognosis of less than 10 %. Current treatment options are limited and mainly traditional treatments like surgical resection and chemoradiotherapy are performed. However, 80 % of all cases are unresectable, highlighting the need of new therapies for patients with PDAC. Chimeric antigen receptor (CAR) T cell therapy could be a new hope to treat PDAC. It is important to find a tumor-specific target with restricted off-tumor expression

for CAR T cell therapy, to ensure a differentiation between normal and PDAC cells. Here, we present CD318, the CUB domain-containing protein 1 (CDCP1), as a potential target for CAR T cell therapy in PDAC. CD318 is a transmembrane glycoprotein that contains three CUB-like domains and its serine protease mediated cleavage leads to cell growth and tumorigenesis. We show that CD318 has almost no off-tumor expression and based on mass spectrometry, RNA sequencing and antibody binding libraries, it is predicted to be a safe target for CAR T cell based therapy of PDAC. For healthy tissues with highest expression, we performed functional assays, to exclude off-tumor activity. Furthermore, a selection of fully human scFv binders with varying attributes and binding domains were chosen to target CD318 leading to tumor-specific killing and no off-tumor effects.

P017 / No. 155

Topic: AS04 Superpowered lymphocytes

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 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 / No. 163

Topic: AS04 Superpowered lymphocytes

ENHANCING TCR EFFICACY THROUGH CD3Z MODIFICATIONS

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Modified T-cells are currently being rapidly developed for use as immunotherapies, with chimeric antigen receptor (CAR) T-cells currently being used to treat patients with B-cell malignancies, via CD19 targeting. However, despite their success CAR T-cells are limited by their exclusive recognition of proteins present on the cell surface. One way to expand the range of target antigens is to utilise T-cell receptor (TCR) mediated recognition. The TCR interacts with peptides presented by MHC molecules allowing the recognition of intracellular as well as cell surface proteins. For efficient expression in T cells, the TCR α/β chains form a complex with four CD3 chains, one of these being CD3 ζ which is key in promoting signalling after TCR binding. Here we explore whether modifications to the CD3 ζ chain can enhance TCR signalling and T cell function. These modifications include the addition of the co-stimulatory domains CD28 or 4-1BB, at the membrane proximal or the membrane distal position of the intracellular tail of CD3 ζ . Our preliminary results show that the CD28 can improve TCR signalling and enhance T cell effector function. We will present a detailed analysis of how CD28 and 4-1BB signalling domains incorporated into two different positions of the CD3 ζ chain affect TCR stimulation and T cell effector function. The modification of the CD3 ζ chain provides an opportunity to improve TCR gene therapy by incorporating the conventional TCR signal 1 and co-stimulatory signal 2 in one molecular complex.

P019 / No. 100

Topic: AS04 Superpowered lymphocytes

ADAPTING ANTIVIRAL AND REGULATORY T CELL PRODUCTS FOR USE IN THE TRANSPLANTATION SETTING: RESISTANCE TO TACROLIMUS ACHIEVED BY A GMP-GRADE CRISPR/CAS9 TECHNOLOGY APPROACH

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Transplant recipients require lifelong immunosuppression to prevent organ rejection, which can adversely cause morbidities and death due to infections or cancer. Recently, adoptive T-cell therapy (ATT) using regulatory T-cells (T_{reg}) to prevent organ rejection or antiviral ATT to control viral infections have become attractive alternatives to standard medications. However, due to insufficient experience in the case of T_{reg} -ATT, still baseline immunosuppression including Tacrolimus must be maintained in organ transplant recipients, which can negatively impact T_{reg} and antiviral T-cell products (TCPs) resulting in limited efficacy and long-term performance. To improve performance of TCPs, we hypothesized that knockout of the adaptor protein for Tacrolimus, FKBP12, would allow sustained function even in the presence of immunosuppressants. Indeed, we generated T_{reg} and virus-specific TCPs, which are resistant to Tacrolimus by transfer of ribonucleoprotein complexes of Cas9 with a site-specific single guide RNA resulting in knockout of the target. We functionally assessed Cytomegalovirus-/SARS-Corona-Virus-2-/Epstein-Barr-Virus- or Influenza A-Virus-specific TCPs in the presence of distinct immunosuppressants upon virus-specific stimulation and revealed improved cytokine production and preferential transcriptomes in CITEseq. *In vitro* lung infection models are being established. Furthermore, we have evidence for improved expansion and T_{reg} stability in presence of Tacrolimus confirmed by proteomic and transcriptomic analyses. Importantly, an alternative immunosuppressant, cyclosporine A, serves as safety-switch. *In vivo* data imply improved graft condition in a humanized allograft rejection model after transfer of Tacrolimus-resistant T_{reg} . We investigated *in silico*-predicted off-targets-effects by NGS-amplicon-sequencing for safety assessment. We applied for a manufacturing license for Tacrolimus-resistant T_{reg} as prerequisite of translation of these GMP-compliant TCPs to a first-in-human application.

P020 / No. 95

Topic: AS04 Superpowered lymphocytes

ISOLATION OF ANTIGEN-SPECIFIC HUMAN REGULATORY T CELLS ENABLED BY GENOME EDITING

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Regulatory T (Treg) cells can potentially suppress immune responses to tissue antigens, thus preventing the development of autoimmunity. However, tumor infiltrating Treg cells may also suppress conventional T (Tconv) cells thus being detrimental for cancer immunotherapy. Despite fundamental knowledge on the mechanisms of Treg cell differentiation and function, their specificity for self-antigens remains largely unknown due to technical difficulties in expanding human Treg cells *in vitro* for their functional characterization. Here we describe a novel approach combining CRISPR/Cas9-based genome editing with antigenic stimulation and T cell cloning,

which allows improved *in vitro* expansion of human Treg cells and enables the study of their antigen specificity. Using this method, we interrogated the antigen specificity of circulating human Treg and Tconv cells isolated from healthy donors and autoimmune patients. We identified Treg cells specific for tissue-restricted antigens and microbial antigens. Antigenic stimulation coupled to single cell RNA sequencing allowed us to determine frequency, TCR $\alpha\beta$ paired sequences and gene expression profile of antigen-specific Treg and Tconv cells. Furthermore, analysis of serial blood samples from healthy donors revealed that self-reactive T cells persist for years in the circulating Treg cell repertoire of adult donors. Overall, our method allows the efficient isolation of antigen-specific human Treg cells and their deep characterization with regard to TCR $\alpha\beta$ sequences, cognate epitopes, and MHC restriction. The identification of the antigens recognized by human Treg cells advances our understanding of the mechanisms of immune tolerance to self, and offers new possibilities for the treatment of autoimmunity and cancer.

P021 / No. 126

Topic: AS04 Superpowered lymphocytes

SUPERIOR ANTI-TUMOR EFFICACY OF CD4+ CAR-T CELLS IN HEMATOLOGICAL MALIGNANCIES

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Background: Optimization of CAR T-cell (CART) therapy requires a better understanding of the optimal characteristics of the administered cell products, including the appropriate ratio of CD4+ and CD8+ T-cell subsets. The aim of our study was to further investigate the functionality of CD4 and CD8 expressing CARTs targeting a variety of hematologic malignancies and solid tumors.

Results: In a serial co-culture screen, we observed enhancing anti-tumor-efficacy of all CART products with increasing CD4/CD8 ratios which were mainly related to an increased proliferative capacity of CART products with higher CD4+ T cell content. Indeed, even at lower total amounts of T-cells pure CD4+ CARTs exhibited a superior anti-tumor efficacy compared to CD8+ containing CART products suggesting that the addition of CD8+ CARTs even diminished the functionality of CD4+ CARTs. Further mechanistic studies revealed that cytokines secreted by CD4+ CARTs could enhance the functionality of CD8+ CARTs whereas direct cell-to-cell interaction with CD8+ CARTs diminished the cytolytic and proliferative potential of CD4+ CARTs. *In-vitro*, depletion of CD8+ CARTs significantly improved the functionality of CD19-CARTs derived from patients treated within the HD-CAR-1 trial. Furthermore, pure CD4+ CARTs demonstrated superior anti-tumor efficacy in multiple NSG xenograft models targeting different hematologic malignancies.

Conclusion: Our data suggest that the CD4/CD8 ratio of CART products is crucial for their functionality and that CD4+ containing T cell products have superior anti-tumor efficacy over CD8+ CARTs. Our results warrant further exploration of pure CD4+ CAR T-cell products within future clinical trials.

P022 / No. 97

Topic: AS04 Superpowered lymphocytes

TURNING AN IMMUNOSUPPRESSIVE MARKER INTO A T-CELL ACTIVATING SIGNAL: USING THE REVCAR SYSTEM TO TARGET IMMUNE CHECKPOINTS

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Immunotherapy based on chimeric antigen receptor (CAR) T-cells has demonstrated remarkable therapeutic effects, particularly against some hematological cancers. A versatile adaptor CAR system called RevCAR, consisting of RevCAR T-cells and a bispecific target module (RevTM) has been developed to overcome severe side effects associated with conventional CAR T-cell therapy. As the activity of RevCAR T-cells can be steered based on the availability of RevTM, working as an on/off switch, the system can immediately be turned off if side effects occur. Furthermore, the RevCAR system is highly flexible, since the same RevCAR T-cell can be directed towards different tumor-associated antigens (TAA) simply by adding RevTMs with different specificities. However, the effectiveness of CAR T-cells against solid tumors remains limited particularly due to their immunosuppressive tumor microenvironment (TME). To overcome these hurdles, we have established a novel RevCAR system targeting immune checkpoint molecules such as PD-L1, which are frequently overexpressed by cancer cells to suppress immune responses. We have constructed novel RevTMs that can redirect RevCAR T-cells to kill tumor cells that express such immune checkpoint molecules. Furthermore, true AND gate tumor targeting was achieved by targeting a TAA in addition to PD-L1 in a combinatorial manner using our Dual-RevCAR system. In this way, targeting PD-L1 not only results in Dual-RevCAR T-cell activation but simultaneously blocks the immunosuppressive PD-L1/PD-1 axis. Altogether, we have turned an immunosuppressive marker into an immune-activating signal that might modulate the TME in a beneficial manner showing promise for the development of an effective immunotherapy against solid tumors.

P023 / No. 223

Topic: AS04 Superpowered lymphocytes

SYNTHETIC DUAL-COSTIMULATION FOR TCR AND TCR-LIKE TARGETED CELL THERAPIES

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LB: Academic Abstract Body: Adoptive cell therapies based on chimeric antigen receptors (CARs) have demonstrated potent anti-tumour efficacy in the clinic. Conversely, therapies involving T-cells endowed with T-cell receptors (TCRs), including TIL therapies, have so far been less successful. A key difference between CARs and TCR-based therapies is that CARs have autochthonous co-stimulation. We engineered a fusion receptor, based on the extracellular domain of CD80 and intracellular 4-1BB domain, 80BB. We hypothesized that this molecule can simultaneously deliver both CD28 and 4-1BB co-stimulation, upon binding to CD80 ligands CD28 and CTLA4, in a TCR-compatible manner. Using engineered T cells with a TCR or an HLA-Independent TCR (HIT), we illustrated that 80BB-endowed TCR T cells displayed increased proliferation and tumouricidal function *in vitro* and *in vivo*, ultimately leading to prolonged survival in aggressive B-ALL and melanoma models. Phenotyping and scRNA sequencing of bone marrow from *in vivo* models revealed greater T-cell accumulation and enrichment of genes associated with cytotoxicity and proliferation, and lower levels of inhibitory and exhaustion-associated genes in HIT^{80BB} cells. Further mechanistic investigations confirmed that 80BB can interact with both CD28 and CTLA4, leading to bi-directional signalling upon CD28 ligation. Polyclonal TILs endowed with 80BB expanded from a melanoma sample led to increased proliferation, glycolytic rates, respiratory capacities and improved repetitive tumouricidal functions *in vitro* and led to prolonged tumour control in a patient-matched disseminated melanoma model. Combining costimulatory features in one compact, antigen-agnostic synthetic molecule, 80BB is a promising tool to sustain CD3-dependent receptors in a wide range of targeted immunotherapies.

P024 / No. 61

Topic: AS04 Superpowered lymphocytes

DUAL TARGETING OF PD-L1 AND ERBB2 BY CAR-NK CELLS ENABLES SPECIFIC ELIMINATION OF SOLID TUMOR CELLS AND OVERCOMES IMMUNE ESCAPE VIA ANTIGEN LOSS

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Retargeting of natural killer (NK) cells with chimeric antigen receptors (CARs) can be a powerful approach to overcome NK-cell resistance of tumor cells. However, targeting a single tumor-associated antigen may be insufficient for some tumors to trigger effective NK-cell activation or result in the selection of antigen-loss variants and tumor immune escape. To overcome this hurdle, here we generated CAR-NK cells carrying two CARs that target the tumor-associated antigens PD-L1 and ErbB2 (HER2), respectively. NK-92 cells were transduced with lentiviral CAR

constructs, and their cytotoxicity against cancer cell lines of different solid tumor origins was compared to that of parental NK-92 and corresponding single-target CAR variants. Dual targeting significantly increased *in vitro* cytotoxicity against PD-L1 and ErbB2 double-positive tumor cell lines including breast, ovarian, pancreatic, lung and gastric cancer cells when compared to single-target CAR variants. These results were also confirmed with 3D spheroid tumor models. Off-target cytotoxicity was not observed. On a molecular level, this enhanced cell killing may be explained by synergistic activation of PLC γ and MAPK pathways. Incubation of cancer cells with IFN- γ further improved killing efficacy due to upregulation of PD-L1 expression. Furthermore, blocking experiments revealed that dual PD-L1/ErbB2-CAR NK-92 cells can overcome immune escape based on loss or inaccessibility of a single target antigen. Altogether, we showed that dual targeting of PD-L1 and ErbB2 improves efficacy of CAR-NK cells against otherwise difficult to treat tumors, and counteracts potential resistance and immune escape mechanisms of cancer cells.

P025 / No. 67

Topic: AS04 Superpowered lymphocytes

TURNING NKG2D CAR-T THERAPY INTO AN IMPROVED IL18 TRUCK AS A TREATMENT FOR PEDIATRIC CENTRAL NERVOUS SYSTEM TUMORS

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Central Nervous System (CNS) tumors are the most common solid pediatric tumors and the leading cause of cancer-related death in children. Although current therapies have improved their outcome, survivors present severe long-time sequelae from therapy. Chimeric Antigen Receptor (CAR)-T cells are a therapeutic alternative, but their application in solid tumors is still challenging. This is due to the scarce therapy infiltration, the lack of therapy persistence and the immunosuppressive tumor microenvironment (TME). NKG2D CAR-T has shown substantial benefit in osteosarcoma studies *in vivo*, with some limitations. This project aims to improve CAR-T cell therapy on pediatric CNS tumors. To overcome TME limitations, we are manipulating NKG2D CAR-T cells to release engineered interleukin 18 (IL18) versions upon CAR T activation. IL18 is a proinflammatory cytokine that recruits immune cells, activating NK and T cells. It will transform the immunosuppressive TME, allowing more immune cells to reach the tumor. Engineered IL18 is expected to increase the protein stability and release. Four IL18 versions have been cloned under the control of NFAT and PGK promoters. We have compared IL18 release through ELISA, observing that one of the IL18 modifications causes an increase in the amount of cytokine secreted. Their functionality is currently being tested through migration and NK activation assays. The more effective IL18 versions will be cloned in NKG2D CAR-T. Then, we will assess the therapy efficacy using

3D models. This interdisciplinary approach could improve current hurdles in CAR-T efficacy by generating a tool whose utility exceeds pediatric brain oncology itself.

P026 / No. 119

Topic: AS04 Superpowered lymphocytes

ENHANCING T CELLS EFFECTOR FUNCTIONS THROUGH CRISPR/CAS9 GENE KNOCK-OUT

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Chronic antigen exposure occurring in cancer or persistent infections causes T cells to enter into a dysfunctional state called exhaustion. Exhausted T cells progressively lose their effector functions, proliferation potential and upregulate inhibitory receptors on their surface. This condition is not only responsible of defective pathogen clearance, but can also reduce the efficacy of cancer immunotherapy treatments based on redirecting T cells to kill tumour cells. Therefore, there is much interest in understanding the key molecular mediators driving T cell exhaustion. Recently, CRISPR based screening approaches revealed several genes involved in the control of T cell dysfunctional state, among which RAS GTPase-activating protein (RASA2). Our aim is to study the role of candidate proteins involved in exhaustion, including RASA2, by applying CRISPR/Cas9 mediated gene knock-out in the respective coding genes. In particular, we started targeting human CD8 and CD4 T cell clones with known specificity for viral pathogens, and also *ex vivo* isolated polyclonal T cells. The phenotype of edited T cells will be characterized in terms of their specific functions including proliferation, killing activity, and cytokine production. The results of these studies may open the possibility to establish gene editing strategies to boost the efficacy of pathogen or tumor-specific T cell therapies.

P027 / No. 177

Topic: AS04 Superpowered lymphocytes

BEST OF TWO WORLDS: ENGINEERING NKT-CELLS TO GENERATE AN ALTERNATIVE ADAPTIVE CELL THERAPY STRATEGY AGAINST NEUROBLASTOMA

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Despite high-intensity, multi-modal therapy regimens, survival rates for high-risk neuroblastoma (HR-NBL) patients remain poor. The embryonal origins of NBL result in low immunogenicity, limiting therapeutic efficacy. Notably, absence of MHC-I expression greatly limits engagement of cytotoxic T-cells. We previously demonstrated upregulation of MHC-I expression in NBL through release of IFN- γ by activated NK-cells. Further, it is suggested that a high degree of plasticity in MHC-I expression enables alternate evasion of both cytotoxic T- and NK-cells. To circumvent immune evasion driven by MHC-I plasticity, we combined MHC-I restricted and NK-cell mediated cytotoxicity by introducing a NBL-specific TCR into naturally

occurring CD3+CD56+ natural killer T (NKT)-cells. These are a separate subset to invariant $\gamma\delta$ -chain NKT-cells. Extensive phenotypic analysis revealed NKG2D expression on all, and NKp46 and KIR2D expression on a subset of NKT-cells isolated from peripheral blood of healthy donors. Functional analysis showed that, like NK-cells, NKT-cells engage in missing-self cytotoxicity, recognize and kill a panel of NBL cells, and induce MHC-I expression on surviving NBL cells. An NBL-specific TCR, PRAME HSS1, was effectively introduced into NKT-cells, and peptide recognition and antigen-dependent cytotoxicity confirmed. Finally, we compared the cytotoxic capacity of wild-type and TCR-engineered NKT- and T-cells, observing a superior cytotoxic capacity in TCR-engineered NKT-cells. With this pilot study, we provide groundwork supporting the use of NKT-cells as a therapy source to improve the outcome for children with HR-NBL, and demonstrate that the combination of NK-mediated and MHC-I restricted cytotoxicity is a promising strategy to overcome MHC-I plasticity-related immune evasion in NBL.

P028 / No. 81

Topic: AS04 Superpowered lymphocytes

PROFILING NEOANTIGEN-SPECIFIC T CELL RECEPTOR ACTIVATION: MODERATE STIMULATION PATTERNS LINKED TO INCREASED T CELL RESILIENCE

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Neoantigens promise safe, highly personalized targets for adoptive transfer of T cell receptor (TCR)-transgenic T cells. Single-cell sequencing substantially advanced neoantigen-specific TCR (neoTCR) identification and characterization, however, single TCR determinants for most potent and simultaneously durable tumor rejection remain to be deciphered. In this study, we investigated activation profiles of several neoTCRs with previously determined reactivity against MS-validated neoantigens derived from a metastasized melanoma patient. These high-resolution activation patterns comprised transcriptomic data from single-cell TCR- and RNA-sequencing of neoepitope-specifically stimulated, CD137⁺-enriched peripheral-blood derived patient CD8⁺ T cells as well as functional in vitro characterization of donor-derived neoTCR-transgenic T cells. Our analyses revealed a spectrum of heterogenous signatures even between TCRs sharing MHC-

peptide specificity. Compared to a stronger/inhibitory activation pattern, more moderate stimulation resulted in stable cytotoxicity and coincided with higher frequencies in the patient. We expanded tumor infiltrating lymphocytes (TILs) derived from an in vivo xenograft model for neoTCR-transgenic T cells and re-injected these TIL-products (TIL-P) into new tumor-bearing hosts to compare rejection kinetics upon repeated tumor encounter. Upon rechallenge moderately activated neoTCR-transgenic T cells were linked to more sustained tumor control. Further in vivo and in vitro investigations on repeated killing and detailed assessment of TIL-P multifunctionality (e. g. cytokine profile, phenotype) suggested T cell resilience upon antigen rechallenge as a central factor for successful therapy with TCR-transgenic T cells. Overall, investigations like ours provide important results to establish engineering strategies for T cell products for clinical application.

P029 / No. 136

Topic: AS04 Superpowered lymphocytes

INVERTED LENTIVIRAL VECTOR COMPRISING INDEPENDENT PROMOTERS FOR ON-COMMAND GENE-CARGO DELIVERY BY TUMOR REDIRECTED T-CELLS

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Technological advances in cellular engineering are reshaping the clinical landscape. For example, CAR therapies targeting CD19 have conferred unprecedented responses against some advanced hematological malignancies, and TCR-engineered T-cells redirected against HLA-A2 restricted NY-ESO-1157-165 have shown important clinical promise against a variety of solid tumors. It is widely held, however, that the development of coengineering strategies to overcome suppressive barriers in tumors, along with tools enabling efficient multi-gene integration, are critical for improving the safety and efficacy of T-cell therapies against a broader range of cancers, and for a larger proportion of patients. We have developed a dual inverted lentiviral transfer vector comprising two functionally independent promoters, one driving constitutive expression of a tumor-directed receptor, and the second one enabling efficient activation-inducible (6xNFAT) expression of gene-cargo. Notably, we observed that in a dual forward vector configuration there was transcriptional interference in activated transduced T-cells, and in a bidirectional orientation there was promoter leakiness. We encountered obstacles to the production of dual inverted lentiviral particles and developed a GMP-compatible methodology enabling high titers. We have successfully coengineered T-cells using our dual inverted vector with either a TCR or a CAR, along with a variety of gene-cargo including a cytokine and miR-based shRNA. We also showed that gene-cargo encoded under 6xNFAT is specifically expressed by T-cells upon antigen encounter. Overall, our strategy allows a reduction in lentivirus vector production costs due to the use of a single all-encoding vector and higher titers generated, and it can facilitate better T-cell therapies reaching the clinic.

P030 / No. 127**Topic:** AS04 Superpowered lymphocytes**SPECIFIC TCR/CDR MUTATIONS POSITIVELY OR NEGATIVELY MODULATE THE FUNCTIONAL EFFICACY OF NY-ESO-1-REDIRECTED CD8 T CELLS IN A MUTUALLY EXCLUSIVE MANNER**

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Background: Little is known on the consequences of complementary-determining region (CDR) mutations on the overall TCR interactions to peptide-MHC and functional efficacy of tumor-redirection T-cells for adoptive T-cell therapy. Therefore, we generated 21 TCR variants from BC1 TCR specific for HLA-A2/NY-ESO-1 based on six individual or combinatorial substitutions within CDR3 α/β and/or CDR2 α/β , including two clinically used variants, CDR3 α Q95L+ α T96Y and CDR2 β G50A+ β A51E.

Results: TCR variants with dual or triple mutations displayed enhanced TCR-pMHC binding avidities, especially when combining CDR3 to CDR2 ones. Alongside, we observed the progressive acquisition of negative functional features, related to NY-ESO-1-independent TCR-HLA-A2 self-interactions and leading to the tonic reduction of global T-cell function. In contrast, TCR variants of intermediate, optimal binding avidities (above the wild-type BC1 TCR) showed preserved positive functional characteristics (survival, memory state, proliferation and target-cell killing) with only limited tonic/negative impact. A functional coefficient score was determined for each individual CDR point-mutation, revealing the following hierarchical order; α Q95L> β G50A> α T96Y> β A51E> α S53W> β A97L. The latter one represents the most detrimental mutation, also supported by *in silico* modeling, showing that the increased affinity mediated by the β A97L mutation was due to unfavorable interactions with self-HLA-A2.

Conclusions: The two clinically used TCR variants showed preserved T-cell functional efficacy and antigen specificity, whereas CDR3 β A97L alone or in combination had drastic negative impact on all functional properties, by favoring TCR-HLA-A2 self-reactivity over TCR-pMHC specificity. Together, CDR mutations positively or negatively modulate the functional efficacy of NY-ESO-1-redirection T-cells, in a mutually exclusive manner, with possible consequences for the clinical design of optimized anti-cancer TCRs.

P031 / No. 139**Topic:** AS04 Superpowered lymphocytes**USING AN ADAPTOR CAR SYSTEM TO TARGET FIBROBLAST ACTIVATION PROTEIN FOR DIAGNOSTIC AND THERAPEUTIC PURPOSES**L. Hoffmann¹, L. Rodrigues Loureiro¹, C. Neuber¹, L. Rupp², M. Kubeil¹, C. Hagemeyer³, M. Schmitz^{2,4,5}, A. Feldmann^{1,4,5}, M. Bachmann^{1,4,5}¹Helmholtz-Zentrum Dresden-Rossendorf, Institute Of Radiopharmaceutical Cancer Research, Department Of Radioimmunology, Dresden, Germany, ²Faculty of Medicine Carl Gustav Carus, TU Dresden, Institute Of Immunology, Dresden, Germany, ³Australian Centre for Blood Diseases,Central Clinical School, Monash University Melbourne, Melbourne, Australia, ⁴National Center for Tumor Diseases Dresden (NCT/UCC), Germany; German Cancer Research Center (DKFZ), Heidelberg, Germany, Faculty Of Medicine And University Hospital Carl Gustav Carus, Tu Dresden, Dresden, Germany; Helmholtz Zentrum Dresden-rossendorf (hzdr), Dresden, Germany, ⁵German Cancer Consortium (DKTK), Partner Site Dresden, Dresden, Germany

T-cells genetically modified to express chimeric antigen receptors (CARs) are playing a more and more important role in targeted cancer immunotherapy. However, these living drugs can also cause life-threatening side effects. To overcome such limitations and improve the safety of CAR T-cell therapy, adaptor CAR platforms such as the universal CAR (UniCAR) have been developed. This platform consists of the UniCAR T-cell and a target module (TM) cross-linkage effector and tumor cells. Here, we have established a novel UniCAR system targeting Fibroblast Activation Protein (FAP) that is highly expressed in the tumor microenvironment of epithelial cancers and a marker for cancer-associated fibroblasts. For that, we constructed two novel FAP-directed TMs possessing different sizes and pharmacokinetic properties, in which one is based on a single-chain variable fragment (scFv), and the other is based on an IgG4 backbone. We have shown that both TMs were able to bind to FAP-expressing cells and redirect UniCAR T-cells *in vitro* to monolayer and spheroid target cells inducing effective killing. Furthermore, we could show infiltration and activation of T-cells in the spheroid setting. Using *in vivo* models, the TMs were proven to be suitable to be used for PET imaging showing FAP-specific accumulation at the tumor site. Moreover, the immunotherapeutic effect of UniCAR T-cells in combination with FAP TMs was demonstrated using mouse models. In conclusion, in this work, we could show that the two novel anti-FAP TMs prove to hold great theranostic potential for diagnostic imaging and immunotherapy.

P032 / No. 196**Topic:** AS04 Superpowered lymphocytes**A NOVEL STRATEGY TO ENHANCE ADOPTIVE T CELL IMMUNOTHERAPY WITH MICRORNA-29A**N. Khatwani^{1,2}, C. Hopkins³, C. Rafie^{1,2}, X. Leng⁴, D. Kolonias⁵, J. Spiegel⁵, J. Fraietta⁶, E. Stelekati^{2,4}¹University of Miami Miller School of Medicine, Sheila And David Fuente Program In Cancer Biology, Miami, United States of America, ²Sylvester Comprehensive Cancer Center, Sylvester Comprehensive Cancer Center, Miami, United States of America, ³University of Pennsylvania, Cell And Molecular Biology Group, Philadelphia, United States of America, ⁴University of Miami Miller School of Medicine, Department Of Microbiology And Immunology, Miami, United States of America, ⁵Sylvester Comprehensive Cancer Center, Division Of Transplantation And Cellular Therapy, Miami, United States of America, ⁶University of Pennsylvania, Department Of Microbiology, Philadelphia, United States of America

Low persistence of chimeric antigen receptor (CAR) T cells and progressive T cell exhaustion pose significant challenges for CAR-T immunotherapy. In a mouse model of chronic infection, we recently demonstrated that microRNA-29a (miR-29a) attenuates exhaustion, enhances memory differentiation, and promotes long-term persistence of CD8 T cells, while retaining their

effector functions. In this study, we hypothesized that miR-29a could augment CAR-T cell responses by improving T cell persistence and functionality. We transduced CD3+ T cells isolated from healthy donor PBMC with a lentiviral construct expressing CD19-CAR, 4-1BB and miR-29a (CD19-CARbbz-miR29a) or with a control lentiviral construct expressing CD19-CAR and 4-1BB (CD19-CARbbz). Post-expansion CAR+ cells in both groups showed enhanced expression of effector markers (CD28, CD69, CD95 and granzyme B) suggesting equal effector differentiation. However, forced expression of miR-29a attenuated exhaustion, as CD19-CARbbz-miR29a CAR+ cells showed a significant decrease of exhaustion-related molecules (TIM-3, CD39 and TOX). Importantly, miR-29a promoted a memory/progenitor-like differentiation, as shown by increased IL-7Ra, CCR7 and CXCR5 expression. Subsequently, we hypothesized that miR-29a expression could associate with clinical responses to CAR-T therapy. RNA from pre-CAR-T leukapheresis products of DLBCL patients was isolated from sorted PD-1^{High} and PD-1^{Low} CD8 and CD4 T cells (n=5). Interestingly, miR-29a expression correlated with clinical responses, suggesting that pre-infusion miR-29a expression levels may affect responses to CAR-T therapy. In summary, our preliminary findings support the potential application of miR-29a as a novel strategy to enhance CAR-T responses and promote the clinical benefits of immunotherapy.

P033 / No. 89

Topic: AS04 Superpowered lymphocytes

FOURTH GENERATION CAR-T CELLS SECRETING A TLR MODIFIED LIGAND EXHIBIT AN ANTITUMOR PROFILE IN VITRO

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Immune escape, antigen heterogeneity and an immune-suppressive tumor microenvironment (TME) weaken the efficacy of CAR-T cell therapy in solid cancer treatment. To overcome these limitations, we aim to empower anticancer immunity by producing 4th generation CAR-T cells secreting a modified TLR ligand into the TME, to establish an inflammatory and immune-stimulating milieu. MC38 colon adenocarcinoma cells expressing a truncated human EGFR were produced as target and TLR ligand functionality was tested. CAR-T cells were obtained by transducing primary murine T cells with retroviral vectors carrying a cetuximab-based murine 3rd generation CAR and/or the TLR ligand. After establishing optimal transduction conditions, killing ability, cytokine release, TLR ligand secretion, and the activation status of CAR-T cells were assessed upon co-culture with MC38 cells. The secreted TLR ligand activated TLR when coupled with the IgK signal peptide alone or in combination with a repetition of arginines (polyR). The addition of the polyR helped the protein to overcome an impermeable cell monolayer, suggesting a transit through cell

membranes and better diffusion within the tumor mass. After 48 hours of co-culture, viability of target cells was reduced compared to control cells, indicating CAR-dependent target recognition and killing. Moreover, CAR-T cells expressed higher levels of the activation markers CD25 and CD69 and secreted the activation-induced cytokines IL-2 and IFN- γ . Finally, the TLR ligand produced by CAR-T cells showed TLR activation. As a new potential weapon against cancer, further characterization of our 4th generation CAR-T cells *in vitro* and *in vivo* will unravel their potential in anticancer immunotherapy.

P034 / No. 112

Topic: AS04 Superpowered lymphocytes

HIGHLY SPECIFIC CAR-T CELLS OVERCOMING THE IMMUNOSUPPRESSIVE TUMOR MILIEU IN LUNG CANCER: 4TH GENERATION TECHNOLOGY TARGETING CD176

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In the development of effective CAR-Ts against solid tumors, both choosing an appropriate tumor-associated target and overcoming the immunosuppressive tumor microenvironment (TME) remain major challenges. The oncofetal carbohydrate CD176, hidden in adult benign tissues by sialylation or prolongation with carbohydrates, is unmasked in 90% of carcinomas. Because of its low predicted "on-target/off-tumor"-toxicity, CD176 is an extremely promising target. To demonstrate this while also circumventing the immunosuppressive TME, we developed 4th-generation CD176-specific CAR-Ts, also known as T cells redirected for universal cytokine-mediated killing (TRUCKs). In addition to the constitutive CD176-CAR expression, these cells were engineered with an inducible cassette expressing either IL-12 (iIL12_CD176_TRUCKs), IL-18 (iIL18_CD176_TRUCKs), or EGFP as a control (iEGFP_CD176_TRUCKs). Following co-culture with different CD176⁺ lung carcinoma cell lines, all CD176_TRUCKs increased NF- κ B activity, became activated, released effector molecules (e.g. IFN- γ), and mediated effective cytotoxicity. They did not react towards CD176⁻ control cells, indicating safety. The inducible cytokines IL-12 and IL-18 were released by respective TRUCKs in a target-specific manner and clearly improved their functionality in comparison with iEGFP_CD176_TRUCKs. Precision-cut lung sections (PCLS) were generated from explanted human lung adenocarcinoma tissue and shown to express CD176. Using PCLS as *ex vivo* model, specific cytotoxicity of CD176_TRUCKs against tumor tissue but not against healthy tissue of the same lung was demonstrated. Thus, CD176_TRUCKs equipped with inducible

cytokines were shown to be highly functional and a promising strategy to overcome the TME. Based on the tumor-specific expression of CD176, CD176_TRUCKs have a high potential to effectively control lung carcinoma while avoiding “on-target/off-tumor”-toxicity.

P035 / No. 106

Topic: *AS04 Superpowered lymphocytes*

PRE-CLINICAL EVALUATION OF NOVEL CAR T CELLS TARGETING FOLATE RECEPTOR 1 EXPRESSING HIGH GRADE SEROUS OVARIAN CANCER

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Treatment of ovarian cancer remains challenging. A high unmet clinical need for targeted, efficient and persisting drugs still is a major issue for patients diagnosed with ovarian cancer. CAR (chimeric antigen receptor) T cells have shown efficient and persisting anti-tumor functionality in hematological malignancies. Therefore, we characterized FOLR1 as CAR T cell target, designed novel CAR T cells, and assessed these drug candidates preclinically in vitro and in vivo. We performed ultra-high content imaging on high-grade serous epithelial ovarian cancer as well as healthy tissue samples to profile the expression of the tumor-associated antigen FOLR1. Subsequently, we designed a series of CAR T cell candidates based on different monoclonal antibodies targeting FOLR1, in order to leverage on clinical experience with these binders, particularly safety aspects. FOLR1-directed CAR T cell constructs were evaluated against various ovarian cancer cell lines in vitro and in vivo. In this study we show high and consistent expression of the tumor-associated antigen FOLR1 on primary high-grade serous ovarian cancer samples. Moreover, FOLR1 expression is low, restricted, and polarized in healthy tissues. We generated diverse FOLR1-specific CAR T cells and characterized their efficacy and specificity in vitro and in an ovarian cancer xenograft model. CAR T cells rapidly and efficiently eradicate xenograft tumors. This anti-tumor response is accompanied by CAR T cell expansion, FOLR1-dependent cytokine secretion, and CAR T cell infiltration. These findings demonstrate the feasibility of a preclinical CAR T cell approach targeting FOLR1 as treatment option for ovarian cancer and potentially other FOLR1-expressing tumors.

P036 / No. 63

Topic: *AS04 Superpowered lymphocytes*

SYSTEMS BIOLOGY APPROACHES ALLOW INVESTIGATING THE SIGNALING AND METABOLIC REPROGRAMMING OF T CELLS

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Cancer immunotherapy is a promising approach for treating cancer, and adoptive cell therapy (ACT) has emerged as a particularly effective treatment for certain types of cancer. However, the tumor microenvironment (TME) can induce metabolic and signaling changes in T cells that impede their anti-tumor activity, leading to T cell dysfunction and reduced efficacy of ACT. To overcome these challenges, it is crucial to understand the metabolic and signaling rewiring occurring in T cells in the TME and during ACT. Seeking to study the transcriptional and metabolic reprogramming of T cells at a mechanistic level, we developed computational systems biology approaches to understand the metabolic and signaling states of T cells. First, we generate a systematically and consistently reduced version of the human genome-scale metabolic model Recon 3D. We then integrate into the model scRNA-seq data for T cells. The resulting T cell-specific metabolic model allows us to characterize the metabolic state of the T cells under several conditions. Finally, we build the downstream signaling networks of the T cell receptors and identify the metabolic transcription factors, their state, and the effects they have on the metabolic pathways. These methods and analyses provide a novel approach that will help us gain insight into the mechanisms underlying T cell biology in cancer and identify potential targets for therapeutic intervention. In addition, computational methods can aid in the design of engineered T cells to improve the development of effective immunotherapies.

P037 / No. 113

Topic: *AS04 Superpowered lymphocytes*

LMP2A-TARGETING TCR-ENGINEERED T CELLS WITH INDUCIBLE INTERLEUKIN-18 EXPRESSION TO TREAT EPSTEIN-BARR VIRUS-ASSOCIATED MALIGNANCIES

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Epstein-Barr virus (EBV) infects more than 90% of the population and remains in B-cell compartments life-long. While in healthy individuals strong immune responses control EBV reactivation, in immunocompromised patients, infections and reactivations can lead to severe EBV-associated malignant complications, such as post-transplant lymphoproliferative disease (PTLD). In latency stages II/III, latent membrane protein 2a (LMP2a) is expressed, which is associated with PTLD as well as various malignancies. Recently, a clinically protective TCR recognizing an LMP2a-derived peptide in context of HLA-A*02 was identified. Based on this, we developed TCR-engineered T cells and further equipped these with an inducible cassette for

locally restricted IL-18 release (LMP2a_iIL18_TCR-Ts), which was shown to convert T cells into pro-inflammatory effector cells, preventing function loss and exhaustion, and reshaping the immunosuppressive tumor microenvironment. LMP2a_iIL18_TCR-Ts were analyzed with respect to their memory phenotype, replicative capacity, state of activation and exhaustion as well as cytotoxicity towards EBV-infected HLA-A*02⁺ cells. They showed no signs of HLA cross-reactivity or recognition of an irrelevant HLA-A*02-restricted peptide. Sensitivity and specificity of LMP2a_iIL18_TCR-Ts was further confirmed using *in vitro* cytotoxicity models and Ca²⁺ flux analysis. Increased cytotoxicity was detected by 7-AAD staining, live cell imaging and target cell detachment in real-time impedance measurements when combined with inducible IL-18 expression. In conclusion, *ex vivo* isolated protective TCRs were redirected into T cells from third-party donors with the potential to attract innate immune cells and alter the tumor environment, thereby widening the applicability of T-cell therapy to refractory viral infections.

P038 / No. 175

Topic: AS04 Superpowered lymphocytes

BANANA LECTIN EXPRESSING CAR T CELLS ENHANCE ANTI-TUMOR ACTIVITY AGAINST HETEROGENOUS SOLID TUMORS

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To overcome the heterogenous and dynamic expression of tumor antigen expression in solid tumors and the physical barrier of stroma, we developed a CAR using a lectin binder (H84T BanLec) which recognizes patterns of aberrant glycosylation preferentially expressed on tumor cells and surrounding stroma. Aberrant glycosylation patterns are a widely present hallmark of cancer, therefore incorporating H84T BanLec as part of a CAR on T cells engineered to target other tumor antigens should boost the efficacy of current therapies. Previously, we showed that H84T CAR expressing T cells effectively target and eliminate pancreatic cancer and now demonstrate activity against non-small cell lung cancer and osteosarcoma cells in both monolayer cultures and 3-dimensional spheroids composed of tumor cells, cancer stroma, and inhibitory monocytes. When H84T CARs are co-expressed with a conventional scFv-derived CAR targeting HER.2 we observe superior anti-tumor activity using dual expressing HER.2/H84T CAR T cells compared to HER.2 CAR T cells alone. Similarly, in the presence of cancer-supportive stromal cells, GD2/H84T dual CAR T cells demonstrate greater cytotoxicity against osteosarcoma than do single CAR expressing T cells. We are elucidating the underlying mechanisms by which H84T CARs induce stromal disruption and enhance tumor infiltration in 3-dimensional and *in vivo* models. These findings suggest that H84T BanLec CAR T cells provide a tool to target the aberrant glycosylation patterns of malignant cells and their supporting stroma. This should provide a broadly effective treatment option for multiple heterogenous tumors, with H84T beneficially combined with CAR/TCR binders directed to “conventional” tumor antigens.

P039 / No. 209

Topic: AS04 Superpowered lymphocytes

UNBIASED CHEMOKINE RECEPTOR SCREENING REVEALS SIMILAR EFFICACY OF LYMPH NODE- AND TUMOR-TARGETED T CELL IMMUNOTHERAPY

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LB: Academic Abstract Body: Localization is a crucial prerequisite for immune cell function and solid tumors can evade immune control by modulating immune cell infiltration into the tumor stroma. Immunosuppressive cells like regulatory T cells are attracted while cytotoxic CD8⁺ T cells are excluded. Engineering CD8⁺ T cells with chemokine receptors is a potent strategy to turn this mechanism of directed immune cell recruitment against the tumor. Here, we utilized fluorescent tagging to track the migratory behavior of tumor-specific T cells engineered with a library of all murine chemokine receptors *in vivo*. We then asked whether chemokine receptor-mediated redirection of antigen-specific T cells into tumors or tumor-draining lymph nodes showed superior antitumoral activity. We found that both targeting approaches showed higher therapeutic efficacy than control T cells. However, multiple receptors conveying the same homing pattern did not augment infiltration. Instead, in the MC38 colon carcinoma model, antitumoral efficacy as well as lymph node vs. tumor-homing patterns were mostly driven by CCR4 and CCR6, respectively. Overall, our data, based on fluorescent receptor tagging, identify the tumor-draining lymph node and the tumor itself as viable targets for chemokine receptor-mediated enhancement of adoptive T cell therapy.

P040 / No. 133

Topic: AS04 Superpowered lymphocytes

A NON-CD25-BINDING IL-2 VARIANT FOR THE EXPANSION OR ENGINEERING OF T CELLS THAT CONFER SUPERIOR TUMOR CONTROL

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Interleukin-2 receptor (IL-2R) signaling functions as a rheostat governing many T-cell function and fate decisions, and it is critical to the efficacy of many cancer immunotherapies. For example, IL-2 is used for the robust expansion of tumor infiltrating lymphocytes, and it is administered at high doses in patients upon adoptive T-cell transfer (ACT) to support

persistence. Unfavorable properties of IL-2, however, including the terminal differentiation of effector T cells, induction of activation-induced cell death, expansion of regulatory T cells, and toxicity, have driven the development of IL-2 variants. Notably, although IL-15 can be exploited to promote stemness, the T-cells are metabolically inactive and do not sufficiently expand for ACT purposes. Taking systems biology approaches to evaluate scRNAseq, phospho-proteomic and metabolomic datasets, we comprehensively explored the impact of an IL-2 variant (IL-2v) that does not engage CD25 (IL-2Ra; *i.e.*, it is an IL-2Rbg agonist) on CD8⁺ T-cells. We characterized T-cell function, signaling and differentiation, as well as metabolic and transcriptomic programs associated with IL-2v versus wild-type IL-2 or IL-15 stimulation. Interestingly, we determined that the attenuated but persistent signaling driven by IL-2v generated a novel metabolically active stem-like memory state that ultimately conferred better T-cell engraftment, persistence and tumor control upon ACT in tumor-bearing mice. We further engineered T-cells with IL-2v and demonstrated superior tumor control as compared to T cells with enforced expression of IL-15, and without signs of toxicity. Taken together, we have presented a pipeline for the development and evaluation of cytokine variants for therapeutic purposes.

P041 / No. 115

Topic: AS04 Superpowered lymphocytes

VITAMIN C CONDITIONING CREATES STABLE CAR-T CELLS WITH SUPERIOR CYTOTOXIC CAPACITY AND METABOLIC FITNESS TO COMBAT THE IMMUNOSUPPRESSIVE TUMOR MICROMILIEU

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Despite the promising results of CAR-T-cell therapy, poor *in vivo* persistence and low potency especially in the treatment of solid tumors are challenges that remain to be addressed. It has been shown that the efficacy of adoptively transferred and genetically modified T cells can be modulated by *ex vivo* culture conditions. Vitamin C (vitC) is a micronutrient that influences the immune system by mechanisms such as the regulation of epigenetic processes and reactive oxygen species-induced oxidative stress. In this proof-of-principle study, we investigated the impact of vitC pre-conditioning on the phenotype and functionality of CD19-targeting CAR-T cells (vitC-CAR19-Ts vs. CAR19-Ts). Using multicolor flow cytometry, metabolic and multiplex assays, we analysed their activation and regulatory status as well as phenotypic characteristics and cytotoxic capacity during generation and upon target cell encounter. Strikingly, vitC-CAR19-Ts had a prominent effector memory phenotype and displayed increased long-term mitochondrial fitness compared to CAR19-Ts. Moreover, vitC-CAR19-Ts showed significantly enhanced cytotoxicity towards CD19⁺ but

not CD19^{KO} Nalm-6 cells. In line with that, significantly higher concentrations of granulysin were secreted by vitC-CAR19-Ts upon target cell recognition. RNA sequencing of vitC-CAR19-Ts and CAR19-Ts after target cell encounter revealed upregulation of GNLY (granulysin) as well as genes involved in T-cell activation, metabolism and epigenetic reprogramming upon vitC pre-conditioning. In conclusion, we could show that vitC pre-conditioning leads to CAR19-T-cell products with significantly enhanced cytotoxicity and fitness, which is potentially epigenetically imprinted. Therefore, vitC pre-conditioning is a promising strategy to generate stably improved T-cell products with superior cytotoxic and persistence capacity.

P042 / No. 135

Topic: AS04 Superpowered lymphocytes

BEYOND CRISPR: NON-GENE EDITING, MULTIPLEX GENE EXPRESSION TUNING FOR CELL-BASED IMMUNOTHERAPY

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Genome engineering technologies are very powerful tools in cell-based immunotherapy. However, their use for multiple gene edits poses relevant biological and technical challenges. shRNA-based cell engineering bypasses these criticalities, and represents a valid alternative to CRISPR-based gene editing. We developed a miRNA-based multiplex shRNA platform for easy, safe, efficient, and tunable modulation of multiple target genes simultaneously. Combination of highly efficient miRNA scaffolds into a chimeric cluster delivers up to 4 shRNA-like sequences. This cassette can be deployed together with other components, streamlining the generation of engineered CAR T-cells. The plug-and-play design of the platform allows swapping each target sequence without affecting performance. The expression of each target gene can be fine-tuned, up to achieving a functional KO, all without the need of gene editing, ensuring a high safety profile. To demonstrate the effectiveness of our approach, we simultaneously knocked down several co-inhibitory receptors in CAR T-cells. Co-inhibitory receptors play a key role in T-cell exhaustion and targeting them proved highly effective in some cancer indications. Still, many patients remain refractory to therapies against single immune checkpoints, advocating for multiple checkpoints targeting. However, co-inhibitory receptors are critical in maintaining T-cell homeostasis, and complete inhibition/ablation of multiple checkpoints may impair T-cell functionality or facilitate autoimmunity. Our strategy simultaneously prevents overexpression of three receptors (PD-1, LAG-3, and TIM-3) upon T cell activation, thus protecting from T-cell exhaustion, while granting enough expression to maintain homeostasis. Furthermore, combination with death receptor CD95 (FAS) knock-down protects T-cells from ligand-induced apoptosis.

P043 / No. 138

Topic: AS04 Superpowered lymphocytes

SPECIFIC IMMUNOTHERAPEUTIC TARGETING OF GLIOBLASTOMA USING THE SWITCHABLE REVCAR NK-92 SYSTEM

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Chimeric antigen receptor (CAR)-expressing immune cells, such as CAR Natural Killer (NK) cells and CAR NK-92 cell lines have shown promising therapeutic potential over the past years against solid tumors. Targeting Glioblastoma (GBM) with conventional CAR therapies is still challenging due to the sophisticated tumor microenvironment, antigen escape, and on-target/off-tumor toxicity. Therefore, our project aims to develop a safer and more flexible adapter CAR NK-92 platform, called the Reverse CAR (RevCAR) NK-92 system, which consists of two components, the RevCAR NK-92 cell expressing an extracellular short peptide epitope and a bispecific Rev Target Module (RevTM) that redirect the RevCAR NK-92 cells to tumor cells. Only upon this cross-linkage, redirected RevCAR NK-92 cells are activated to lyse tumor cells. The fibroblast growth factor-inducible 14 (Fn14) surface receptor is a promising target antigen overexpressed on GBM. Therefore, we have developed Fn14-specific RevTM to specifically redirect RevCAR NK-92 cells against Fn14-expressing GBM cells. Through *in vitro* and *in vivo* analyses, we assessed the cytotoxic effect of our system on GBM and showed for the first time that GBM cells were efficiently killed by redirected RevCAR NK-92 cells using the novel Fn14-specific RevTM at picomolar concentration, and that the tumor cell killing was associated with increased IFN γ secretion. Hence, these findings give an insight into the clinical potential of the RevCAR NK-92 system as a safe and specific immunotherapy against glioblastoma.

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Topic: AS04 Superpowered lymphocytes

A CHIMERIC SYNGENEIC MOUSE MODEL FOR HUMAN TCR EVALUATION AND DEVELOPMENT OF COENGINEERING STRATEGIES

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The adoptive transfer of TCR-engineered T-cells targeting the HLA-A2 restricted epitope NY-ESO-1₁₅₇₋₁₆₅ (A2/NY) has shown important clinical promise in the treatment of various solid tumors. Previously, by computational design, we developed a panel of affinity-enhanced A2/NY-TCR and demonstrated maximum function of TCRs in the upper range of natural affinity (~5-1 μ M). Human T-cells transduced with TCR 'DMb'

comprising 2 amino acids in CDR2-beta (G50A+A51E, K_D=1.9 μ M) demonstrated significantly improved tumor control as compared to wild-type in NSG mice engrafted with target tumors including A375 and Me275 melanoma. To evaluate the impact of endogenous immunity on tumor control by TCR-T-cells of clinical interest, and assess coengineering and combinatorial treatment strategies, we have developed a chimeric syngeneic mouse tumor model. Briefly, we engineered the murine B16 melanoma tumor cell line to express A2-containing amino acid replacements in alpha-3 in order to accommodate murine CD8 co-receptor binding. Ultimately, we determined that NY-peptide presentation was only possible if fused via a linker directly to the A2 complex (B16-A2/NY). We further efficiently retrovirally transduced murine T-cells to express hybrid TCRs comprising the human variable with mouse constant regions. We demonstrated that these hybrid DMb-TCR-T-cells specifically and efficiently kill B16-A2/NY tumor cells *in vitro*, and, upon transfer to HLA-A2 transgenic C57BL/6 mice, control tumors and reprogram the microenvironment. We next plan to coengineer the TCR-T-cells to overcome specific barriers. We conclude that our chimeric syngeneic mouse tumor model holds potential towards enabling more effective TCR-T-cell-based immunotherapies reaching the clinic.

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Topic: AS04 Superpowered lymphocytes

DEVELOPING THERAPY FOR ACUTE MYELOID LEUKEMIA WITH FIVE-GENE ENGINEERED T-CELLS EXPRESSING TRANSGENIC WT-1 TCR, GM-CSF LIGAND-BASED CAR, CD3X33 BITE AND EGFR SUICIDE GENE SYSTEM

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Cancer immunotherapy with transgenic T-cell receptor engineered T cells (TCR-T) enables targeting of intracellular tumor-specific antigens while the Chimeric antigen receptor-modified T cells (CAR-T) mediate tumor cell killing via recognition of surface antigens. In the case of acute myeloid leukemia (AML), the efficiency of cellular immunotherapy is limited by the lack of AML-specific surface antigens and TCR-T therapy via targeting tumor antigen WT-1 represents a promising alternative approach. However, the efficiency of TCR-T therapy primarily depends on the affinity of recombinant TCR towards the HLA/peptide antigenic complex. With our research we address need for development of molecular strategies to enhance the functionality of tumor-specific transgenic TCRs. Herein, we engineered T cells with piggyBac transposon expressing a T-cell receptor specific to WT-1 antigen (WT-1 TCR), NFAT-regulated Chimeric antigenic receptor specific to GM-CSF receptor (GMCAR), CD3xCD33 bispecific T-cell engager (BiTE) and truncated EGFR suicide gene system. Our findings show that NFAT-driven GMCAR significantly enhances antileukemic functions of WT-1 TCR-T in *in vitro* models with AML cell lines and primary AML cells but preserves their specificity to recognition of HLA-A2/peptide antigenic complex. By inserting the BiTE into the transposon, we equipped the TCR-T cells with ability to target CD33 antigen and recruit non-transfected bystander T cells, resulting in strong antileukemic function of

engineered T cells. The described piggyBac expression platform enables the insertion of multiple transgenes within a single vector. Additionally, each component can be easily replaced with constructs of different specificities to target other tumor antigens.

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Topic: AS04 Superpowered lymphocytes

RUNX1 NEOANTIGENS AS TARGETS FOR TCR GENE THERAPY IN ACUTE MYELOID LEUKEMIA

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About 10% of patients with acute myeloid leukemia (AML) present with mutations in Runt-related transcription factor 1 (RUNX1). RUNX1 mutations occur throughout the entire gene and are associated with poor survival. Frameshift mutations, caused by insertions and deletions in the 3' half of the gene, often create mutant RUNX1 (mRUNX1) proteins with C-terminal amino acids translated in the same alternative reading frame. Here, we investigated whether this alternative reading frame is a relevant target for immunotherapy. A construct with the c.883dupT mRUNX1 gene encoding p.S295FfsTer305 was introduced into EBV-B cell lines expressing common HLA alleles. Using peptide elution and mass spectrometry, we identified 9 mRUNX1 peptides in HLA-B*07:02. To validate mRUNX1 epitope presentation in AML, we created (AML) cell lines with endogenous RUNX1 frameshift mutations using CRISPR/Cas9. Parallel reaction monitoring (PRM) mass spectrometry on HLA eluates of mRUNX1 SIG-M5 and K562 detected 5 out of 9 identified mRUNX1 peptides. For the 4 most C-terminal peptides, peptide-MHC tetramers were synthesized to screen healthy individuals for mRUNX1-specific CD8+ T cells. We identified two T cell clones specifically recognizing a mRUNX1 epitope upon coculture with mRUNX1 peptide-pulsed EBV-B cells. We sequenced and cloned the T cell receptors (TCRs) of the two clones and transduced them into CD8+ T cells from healthy donors. Upon overnight coculture, mRUNX1 TCR-T cells showed reactivity towards 4 mRUNX1 AML cell lines whereas parental cell lines expressing wildtype RUNX1 were not recognized. In conclusion: we identified an HLA-B*07:02-restricted RUNX1 neoantigen that may be relevant for AML immunotherapy.

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Topic: AS04 Superpowered lymphocytes

STEPWISE SOLUTIONS TO IMMUNOSUPPRESSIVE TUMOR MICROENVIRONMENTS BY CAR-IMAC

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Immunosuppressive tumor microenvironment (TME) impairs chimeric antigen receptor (CAR)-immune cells. Four major barriers hamper CAR-immune cells. Lack of migration and sustainability are the main issues in conventional CAR-T cells.

We established CAR-iMac leveraging the robust generation of functional macrophages (iMac) from human pluripotent stem cells. iMac migrated in the tumor spheroids and sustained over two weeks without changing the B7-1 co-stimulatory ligand level in culture, overcoming the first two major barriers. The third barrier is that cancer cells silence CAR-iMac with do-not-eat-me signals. We replaced the loci of *SIRPA* and *SIGLEC10* with the CAR cassette. The resultant CAR-iMac is resistant to cancers' do-not-eat-me signals. We found that human hepatocellular carcinoma cell line Huh7 expressed CD47 and CD24, ligands of the signals, rendering escape from macrophages. Huh7 is a suitable model for measuring CAR-iMac's resistance to do-not-eat-me signals. The fourth barrier is the immunosuppressive milieu. We employed immune priming cytokines IL12 and IL18 to remodel TME into the tumoricidal niche. We engineered CAR-iMac to secrete IL12 and IL18. We confirmed that IL12 and IL18 induced NK cells to secrete interferon-gamma for cytotoxicity. We anticipate that CAR-iMac will enhance tumor-killing by immune priming NK cells. Altogether, stepwise solutions of migratory and sustainable macrophages, targeting do-not-eat-me signals, and immune priming cytokines will overcome four major barriers in CAR-immune cell therapy.

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Topic: AS04 Superpowered lymphocytes

'SPLICESEEK'- A NEW METHOD FOR CRISPR-BASED SCREEN OF ALTERNATIVE SPLICING EVENTS IN ACTIVATED T CELLS- REVEALS NOVEL TARGETS FOR CANCER IMMUNOTHERAPY

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The use of immune checkpoint blockade (ICB) has greatly improved the treatment of metastatic melanoma. However, many patients still do not benefit from ICB, and there is a pressing need to identify new immunotherapy targets. Immune receptors have distinct alternative splice isoforms, but these isoforms have not been extensively studied. For example, we have shown that PD-1 has two isoforms with opposite functions. The full-length isoform inhibits T-cell activation while the soluble form activates T-cells by blocking PD-L1, the ligand of PD-1. To better understand how splicing affects T-cell function and identify isoforms with potential use for cancer immunotherapy, we developed an innovative CRISPR alternative-splicing screening method - SpliceSeek. SpliceSeek consists of a guide library designed to disrupt splice sites and give preference to one splice variant. For a proof of concept we chose 150 genes encoding immunomodulators. Primary T-cells were transduced in parallel with a TCR against NY-ESO-1:157-163/A2 restricted epitope and the SpliceSeek library. Following, T-cells were sorted according to their IFN γ production, and favorable isoforms were identified using DNA sequencing of the SpliceSeek library. Five isoforms that showed improved cytokine secretion which did not occur after knock-out of their gene, were tested separately. Our screen discovered new immunomodulators isoforms

that increased IFN γ secretion, a hallmark for therapeutic qualities. These results suggest SpliceSeek has the potential to identify novel targets among an array of immune gene isoforms, and expand the arsenal of cancer immunotherapy.

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Topic: AS04 Superpowered lymphocytes

HARNESSING IMMUNOMETABOLISM TO REINVIGORATE THE EFFECTOR FUNCTION AND ANTITUMOR IMMUNITY OF CAR T CELLS IN MANTLE CELL LYMPHOMA

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Current CAR T-cell therapy uses autologous T cells engineered for each individual patient. However, tumor-imposed restrictions create a barrier to immune cells in the tumor microenvironment (TME). T cells from the patients predominantly displayed exhaustion phenotypes. T cells from the patients with MCL displayed exhaustion phenotypes as indicated by decreased T-cell activation markers and increased immune checkpoint. TCR stimulation failed to induce the expression of IFN γ and TNF α in these T-cells efficiently. Mitochondrial respiration was also impaired in MCL-derived T-cells. Metabolic rewiring or precondition T-cells in TME mimicking conditions would enhance T-cell metabolic fitness. Glutamine metabolism was upregulated in MCL cells, which may negatively regulate CD8⁺ T cells. Indeed, CD19-CAR T-cells cultured in glutamine-limited media, or treated with a glutamine inhibitor, displayed increased activation markers and diminished exhaustion signatures. Inactivation of T cell autophagy may enhance metabolic fitness and anti-tumor immunity. Pre-treatment of T cells in the co-culture with the autophagy inhibitor led to T-cell activation and elevated cytokine secretion. Induction of PGC1 α with the PPAR δ agonist increased mitochondria mass, reserve respiratory capacity, and ATP production, along with augmented effector functions and anti-tumor immunity. Ectopic expression of PGC1 α in T cells conferred similar metabolic and functional upgrade. CAR T cell metabolic activity, persistence and antitumor effect will be assessed on CAR T cells incubated with immune checkpoint-targeting BiTEs when they become available. Together, metabolic reprogramming by inhibiting glutamine metabolism or autophagy, and promoting mitochondria biogenesis may overcome the metabolic barrier in TME and enhance T-cell fitness to bolster the efficacy of immunotherapies.

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Topic: AS04 Superpowered lymphocytes

IMPACT OF CO-STIMULATION ON IN VITRO PERSISTENCE OF A MODULAR CHIMERIC ANTIGEN RECEPTOR PLATFORM

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T cells genetically modified to express chimeric antigen receptors (CARs) have shown encouraging clinical efficacy. Cytokine release syndrome and limited long-term persistence constitute some pitfalls of CAR-T cell therapies. While conventional CAR-T (conCAR-T) continuously target the tumor antigen, our modular reverse CAR (RevCAR) platform separates tumor recognition and T cell activation enabling switchable tumor targeting. Despite overcoming multiple limitations of conCAR-T, the long-term persistence of RevCAR-T requires further investigation. Numerous factors influence CAR-T persistence, such as CAR manufacturing and architecture. However, the choice of co-stimulation incorporated into the CAR design drastically impacts CAR-T characteristics. We focused on combining co-stimulatory domains (CSD) with distinct T cell activation properties into our RevCAR platform, designing four unique variants (28 ζ _{long-linker}, 28 ζ _{short-linker}, 28BB ζ , and ICOSBB ζ). The type of co-stimulation present in RevCAR-T did not impact the transduction efficiency and phenotype of the final CAR-T product. Of note, the addition of CSD negatively impacted surface RevCAR density leading to a functionally slower initial response kinetic of 3rd generation RevCAR-T. Next, we repeatedly challenged RevCAR-T with target cells for three weeks to investigate long-term anti-tumor response *in vitro*. Both CD28 ζ variants displayed equivalent cytotoxic efficacy despite a threefold lower RevCAR density of 28 ζ _{long-linker}, highlighting that RevCAR density was not the only influencing factor. However, RevCAR-T restimulation revealed prolonged functional persistence of 28BB ζ and ICOSBB ζ accompanied by decreased PD1 expression, lower terminal memory differentiation, and prolonged secretion of effector cytokines. Overall, in-depth *in vitro* characterization of RevCAR-T revealed sustained cytotoxic potential of RevCAR-T incorporating alternative and additional co-stimuli.

P051 / No. 28

Topic: AS05 Non-viral CAR/TCR engineering

AN ENHANCED TCBUSTER(TM) (TC-B-M(TM)) TRANSPOSASE HAS BEEN DEVELOPED FOR HIGHLY EFFICIENT AND ROBUST DELIVERY OF THERAPEUTIC CARGO FOR BOTH RUO AND CLINICAL APPLICATIONS

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Rapid development of genome engineering tools has driven a number of immune- and stem cell therapies in clinical trials with the goal of generating autologous and allogeneic therapeutics. A majority of these therapies use viral vectors for the delivery of therapeutic cargo. However, viral-mediated therapies carry the risk of immunogenicity, cargo size limitations, integration site risk, manufacture delays, and is highly cost prohibitive. We have developed a non-viral transposase-based editing platform to overcome current viral limitations, which allows for rapid cell manufacture, and reduced cell manufacturing cost. TcBuster is found in the red flour beetle and is a member of the hAT family of transposases. Using directed evolution, we engineered a

hyperactive mutant (TcBuster-M™) that has improved integration rates using less plasmid DNA transposon. Since TcB-M™ is less constrained by cargo size, we've designed large multicistronic plasmids for robust delivery of multiple proteins into various cell types, including primary T- and NK- cells, mesenchymal stem cells, and induced pluripotent stem cells (iPSCs). The improved TcB-M results in cargo integration rates of over 60% in primary T-cells, without sacrificing cell growth or clonal dominance concerns. Finally, we have conducted direct comparisons against lentiviral-engineered CAR-Ts, demonstrating TcB-M engineered CAR-Ts had higher integration percentage, comparable copy number per genome, and safer genome insertion loci. Overall, TcB-M is a proven non-viral gene editing technology delivering large or difficult therapeutic cargos in a variety of cell types. TcB-M thus reduces many of the viral-mediated editing hurdles, allowing the faster generation of crucial therapeutics to market.

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Topic: AS05 Non-viral CAR/TCR engineering

CLINICAL TRANSLATION OF CRISPR-CAS9-MEDIATED TCR ENGINEERING FOR THE TREATMENT OF VIRAL INFECTIONS

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The transfer of naturally occurring, virus-specific T cells has been successfully employed in treating life-threatening re-activations of latent viruses (like Cytomegalovirus (CMV)) in some immunocompromised patients. However, finding suitable seropositive donors is often difficult. On the contrary, engineering T cells using antigen-specific TCRs opens the possibility to develop versatile 'living drugs'. Moreover, we recently developed a CRISPR-Cas9-mediated TCR delivery called 'orthotopic TCR replacement' (OTR) that allows to generate near-physiological engineered T cells with highly predictable *in vivo* behaviours. To make such a TCR therapy broadly accessible, we aim at creating libraries of virus-specific therapeutic TCRs that can ensure the treatment of the vast majority of putative patients. Therefore, we determined the minimum number of Human Leukocyte Antigen (HLAs) molecules that allows to cover 95% of the worldwide population. Then we identified a pool of 9-mer peptides from the CMV antigen pp65 for those HLAs that we used for the isolation of CMV-specific TCRs by high-throughput single-cell sequencing based functional screening. In addition to an off-the-shelf TCR library, we are implementing the OTR approach into a GMP-conform T cell manufacturing process. By studying the kinetics of turnover and expression of the endogenous and transgenic TCR, respectively, we defined a 5 days-manufacturing process, which includes the use of small molecule inhibitors for boosting editing efficiencies. In summary, it is our goal to bring

OTR-engineered virus-specific T cells into the clinic, which will result in defined TCR-transgenic T cell medicinal products with predictable *in vivo* functions.

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Topic: AS05 Non-viral CAR/TCR engineering

PREVENTING TRANSLOCATIONS IN MULTIPLEX-EDITED ALLOGENEIC CAR T CELLS BY COMBINING DIFFERENT CRISPR NUCLEASES FOR SIMULTANEOUS KNOCK-IN AND BASE EDITING

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LB: Academic Abstract Body: Gene editing using CRISPR-Cas technology holds great promise for the development of off-the-shelf CAR T cell therapies. However, the simultaneous introduction of multiple genetic modifications can lead to genomic rearrangements and safety concerns. In this study we addressed this problem in an iterative way. First, we performed non-viral CRISPR-Cas9 knock-in and Cas9-derived base editing for DNA double strand break free gene silencing. This approach allows for the efficient generation of T cell receptor replaced CAR T cells while KO of *B2M* and *CIITA* disrupted the expression of human leukocyte antigen (HLA) class I and II in a single intervention. However, we observed guide RNA exchange between the editors by the presence of small insertions and deletions at the base editing target sites, as well as translocations between the edited loci. By Combining Cas12a Ultra for CAR knock-in and a Cas9-derived base editor, translocation rates were reduced significantly. The resulting triple-edited CAR T cells, displayed a translocation frequency comparable to unedited T cells. Functionally, the cell product demonstrated resistance to allogeneic T cell targeting, indicating a potential application for off-the-shelf CAR therapeutics. This single-step procedure for simultaneous non-viral gene transfer and efficient gene silencing, using distinct CRISPR enzymes for knock-in and base editing, provides a safe and effective strategy for multiplex gene edited CAR-T cells.

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Topic: AS05 Non-viral CAR/TCR engineering

SLEEPING BEAUTY PLATFORM FOR ENGINEERING DONOR-DERIVED CAR-ClK CELLS TOWARDS B-ALL MULTI-TARGETING

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Chimeric antigen receptors (CARs) have demonstrated remarkable success in the treatment of r/r lymphoid malignancies. Despite the impressive results, CAR T-cell failure and emerging CD19 negative relapses represent an unmet clinical need. On the other hand, the utilization of viral methods to engineer T cells with high costs and narrow availability limits their use. Our group has recently demonstrated the pre-clinical and clinical efficacy of donor-derived non-viral cytokine-induced-killer (CIK) cells transfected with a Sleeping Beauty (SB) transposon encoding a CD19CAR. CIK is an attractive cell source due to low GvHD incidence. Furthermore, the utilization of donor materials may overcome the autologous T-cell anergy related to previous extensive chemotherapy. In this study, we focused our interest on CD22 CAR and our previously reported BAFFR CAR, which target antigens expressed on both normal B lymphocytes and in B-cell neoplasms. We designed and cloned in a SB plasmid newly anti-CD22 and anti-BAFFR CAR molecules and we evaluated CAR expression, immunophenotype, exhaustion profile and in vitro killing activity of single and co-electroporated CAR T cells. Robust killing activity was observed for both anti-CD22, anti-BAFFR, and dual CAR T cells against the leukemic NALM6 cell line. In vivo testing using DAUDI and Raji leukemia mouse models demonstrated potent activity and persistence. In conclusion, multi-targeting donor-derived CARCIK cells engineered with the SB virus-free system may be a useful tool to prevent and treat B-ALL relapses.

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Topic: *AS05 Non-viral CAR/TCR engineering*

AN INDIVIDUALIZED, NON-VIRALLY ENGINEERED TCR-T CELL THERAPY TARGETING NEOANTIGENS FOR THE TREATMENT OF SOLID TUMORS

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CAR-T and TCR-T cell therapies for the treatment of solid tumors are often limited by a low prevalence or insufficient tumor-specificity of tumor-associated antigens. Mutant neoantigens, arising through somatic mutations, which are presented in an HLA-dependent manner, are tumor-specific and are present in most solid tumors across indications. An individualized TCR-engineered T cell therapy directed against neoantigens therefore represents a tumor-specific, indication- and HLA-agnostic approach that enables the simultaneous targeting of multiple antigens for a given tumor. Here we are presenting an end-to-end process that combines computational prediction of neoantigen presentation and immunogenicity with highly multiplexed functional avidity-based TCR discovery from peripheral blood of cancer patients. Applying this pipeline, we identify potent TCRs that are reactive against a panel of private neoantigens in a cohort of lung cancer patients. We further identify a subset of these TCRs to be among the most highly expanded TCR clonotypes within the tumor, suggesting that they can mediate an anti-tumor response. An efficient and flexible non-viral precision gene editing approach enables the targeted installation of neoantigen-reactive TCRs together with the removal of endogenous TCRs in autologous or allogeneic T cell populations. T cells engineered in this way express functional neoantigen-specific TCRs and maintain a favorable T stem cell memory phenotype. Exploiting the unique mutational landscape of a given cancer using an individualized TCR-engineered T cell therapy has the potential to be transformational for the treatment of solid tumors.

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Topic: *AS05 Non-viral CAR/TCR engineering*

A HIGH THROUGHPUT AND MULTIPLEXED SYSTEM FOR ANTIGEN SPECIFIC TCR IDENTIFICATION FROM THE NAÏVE REPERTOIRE

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Due to its virtually unlimited scope, the uncompromised T cell repertoire of healthy donors could display an unrealized potential of effective immune response. T cells with antigen specificity for tumor neoantigens provide an attractive source for adoptive cell therapy. However, neoantigen specific naïve T cells occur in extremely low frequency and often lack a high functionality profile. Thus, the search for TCRs in this setting demands high-throughput and multiplexed analysis strategies. Novel approaches in the field of MHC technology have expanded the capacity of epitope discovery. Combinatorial multimer stainings and spectral flow cytometry allow simultaneous identification of multiple antigen-specific T cells. This multiplexed approach could substantially increase the number of TCR candidates for adoptive cell therapy that can be obtained from a single donor. For a subsequent first screening of TCR specificity and sensitivity, we established a high-throughput system based on the Jurkat E6.1 cell line, which is commonly used to investigate TCR function. CRISPR-Cas9-mediated TCR delivery allows fast integration of TCR constructs. To enable enrichment of the transgenic TCR population, a CD3⁻ T cell receptor beta constant region knock-out Jurkat cell line was generated. Successful integration of the transgenic TCR construct in the constant region of the T cell receptor alpha gene will re-establish CD3 expression in the KI⁺ fraction, thus allowing an isolation targeting CD3-expressing cells. In summary, the platform for TCR functional validation allows to orthotopically introduce TCRs in Jurkats and validate TCR functionality based on reporter gene expression upon stimulation within the timeframe of 1-2 weeks.

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Topic: *AS05 Non-viral CAR/TCR engineering*

EFFICIENT TCR SILENCING BY MRNA-BASED EPIGENETIC EDITING OF THE TCR ALPHA LOCUS

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LB: Academic Abstract Body: Allogeneic chimeric antigen receptor (CAR) T-cell therapies have emerged as groundbreaking approaches for cancer immunotherapy. In the last years, the high cost and limitation by autologous T-cell availability have driven the development of allogeneic CAR T-cell therapies. One key challenge in allogeneic CAR T-cell therapies is the prevention of graft versus host disease from the cellular product. To overcome this hurdle, T-cell receptor (TCR) knockout technology has been widely employed, which mostly relies on the CRISPR/Cas system. Although CRISPR/Cas is a convenient and efficient system, the introduced double stranded breaks can lead to unwanted chromosomal rearrangements and induce apoptosis through the DNA repair pathway. The novel method of epigenetic

editing applies DNA-binding domains such as dCas9 fused to epigenetic effector domains, which can modify histone modifications or DNA methylation at the target locus. Thereby, genes can be silenced or activated without interfering with the DNA sequence. Here, we exploit epigenetic editing to silence the TCR alpha locus. We achieved efficient TCR silencing by transiently delivering CRISPR-based epigenetic editors and their guide RNAs as mRNA into the Jurkat model cell line. Future work will focus on the translation of this technology to primary and CAR T-cells as well as probing various epigenetic editors for tunable TCR silencing. This work provides a proof-of-principle for an innovative and safe editing approach for TCR-depletion in allogeneic CAR T-cell therapies.

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Topic: AS05 Non-viral CAR/TCR engineering

PRECISE GENOME EDITING TO ENABLE NEXT-GENERATION T CELL THERAPIES

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Genome editing has enabled the discovery and development of innovative therapeutic approaches for the treatment of a wide spectrum of human disorders, ranging from infection to cancer. The CRISPR-Cas9 system has been an enabling tool in the engineering of primary T cells to support a paradigm shift in T cell-based immunotherapies, more importantly, next-generation chimeric antigen receptor (CAR)-T cells. To this end we have harnessed the power of Precise Genome Editing (PGE) to generate T cell therapies with improved efficacy and/ or safety profiles. With an end-to-end process starting from “clinical-grade” sgRNA design to stringent sgRNA off-target profiling as well as phenotypic, functional, and genetic validation. We have established optimised workflows for non-viral and viral PGE strategies, allowing us to accurately edit human T cells via endogenous gene Knock-Out (KO) and/or exogenous transgene Knock-In (KI). Together these strategies enable the manufacture of next-generation T cell therapies through the of engineering human T cells to express exogenous Chimeric Antigen Receptors (CARs) and/ or alternative T Cell Receptors (TCRs), as well as through the KO of endogenous genes to phenotypically enhance cell therapies. PGE offers an exciting future in the cell therapy field, with the potential to overcome current obstacles to cancer clearance, for example in the treatment of solid tumours.

P059 / No. 88

Topic: AS05 Non-viral CAR/TCR engineering

HOW TO IMPROVE MRNA-BASED CAR-T CELL GENERATION AND FUNCTIONALITY? A LAB-SCALE COMPARISON

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Transient chimeric antigen receptor (CAR)-T cell generation is an attractive alternative to the currently approved CAR-T products generated by viral transduction. It offers the possibility to overcome safety concerns, and thereby, cost-intensive manufacturing processes. In several preclinical studies, transient CAR-T cells are effective, and clinical trials showed preliminary evidence of antitumor efficacy. However, there is a need to optimize the manufacturing process since mRNA-based CAR-T cells require the application of multiple doses for an effective antitumor response. For temporary modification of T cells, a CAR-encoding mRNA can be delivered as *in vitro* transcript (IVT). Compared to viral transduction, an *ex vivo* mRNA-CAR-T cell production process requires different steps. After leukapheresis, T cell isolation, and activation, the unmodified patient-specific T cells are expanded first to achieve an appropriate cell number, followed by transfection, quality controls, and reinfusion into the patient shortly after transfection with CAR-mRNA. Due to the transient character of CAR-expression after mRNA transfection, high CAR-T cell viability and functionality are more important than for virally transduced CAR-T cells that keep the CAR expression after each cell division cycle. Therefore, T cell activation and expansion prior to transfection are key parameters for a high-quality mRNA-CAR-T cell product. Here, we present an optimized lab-scale process for mRNA-CAR-T cell generation using mRNA electroporation and show that the optimal composition of the culture medium significantly impacts T cell viability, expansion, and CAR-T functionality. We defined critical criteria for upscaling workflows and process development in a closed system for high-quality mRNA-based CAR-T cell products.

P060 / No. 147

Topic: AS05 Non-viral CAR/TCR engineering

ISOLATION AND CHARACTERIZATION OF NEOEPITOPE-SPECIFIC TCRs FOR T CELL THERAPY IN GASTROINTESTINAL CANCER

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TCR-engineered T cells for adoptive cell therapy represent a major promise in cancer immunotherapy, in particular for aggressive tumor types with limited therapeutic options like gastrointestinal cancers. Neoantigens arising from frequently occurring frameshift-mutations are interesting immunological targets as they provide tumor specificity and hence a safer profile. In addition, the derived neoepitopes could be potentially shared among different mutations and individuals. We developed a platform for the isolation of extremely rare CD8 naïve neoepitope-specific T cells from peripheral blood of healthy donors via single-cell sorting on combinatorial pMHC multimer staining. As proof-of-principle, we targeted a HLA-A*02:01-restricted neoepitope deriving from frameshift mutations in the tumor suppressor gene Ring Finger Protein 43 (RNF43). After re-expression of the identified TCRs via CRISPR-Cas9-mediated orthotopic TCR replacement, we identified functional TCRs with sufficient epitope sensitivity and cytotoxicity. Expanding on this, we extracted from the COSMICS database the 7 most frequent

mutations in tumor suppressor genes in gastrointestinal cancers, for which we predicted 10 HLA-A*02:01 and 27 HLA-B*07:02-restricted neopeptides. By exploiting the principle of UV peptide exchange, we loaded those neopeptides on the relevant HLA and used the generated pMHCs for the isolation of novel TCRs. We currently work on combining the established platform with an initial functional screening approach to estimate TCR avidity for preselection of the most promising TCR candidates worth of re-expression and in-depth characterization. In summary, we aim at mapping the frameshift-derived neopeptide landscape of gastrointestinal cancers to set up a library of well-characterized and highly functional TCRs for therapeutic use.

P061 / No. 104

Topic: AS06 Universal donor cells & advanced TCR engineering

EARLY MEMORY-ENRICHED ALLOGENEIC CAR-T CELLS TARGETING NKG2D LIGANDS: A FURTHER STEP TOWARDS UNIVERSAL CANCER IMMUNOTHERAPY

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Chimeric antigen receptor (CAR) T cells have demonstrated impressive clinical results against hematological malignancies, but all commercialized CAR-T therapies are autologous and target a unique antigen, which involves some limitations such as manufacturing delays, high costs, and antigen escape relapses. Additionally, CAR-T production strategy is crucial for their clinical efficacy, since it determines the proportion of memory T cells in the manufactured product, which determines survival, expansion and long-term persistence of CAR-T cells *in vivo*. With the aim of developing a novel CAR-T cell product that overcome these challenges, we performed CRISPR/Cas9 knockout of *TRAC* and *B2M* genes to avoid the risk of graft-versus-host disease and immune rejection, respectively, and used an atypical NKG2D-CAR, which targets eight different ligands overexpressed in both solid and hematological tumors. Moreover, in order to enrich the stem cell memory (scm) T cell subset of our allogeneic NKG2D-CAR-T cell product, we tested different interleukin (IL) supplementations (IL-2, IL-7/15, and IL-7/15/21) during culture. As a result, we observed that the percentage of Tscm cells was significantly higher with IL-7/15 and IL-7/15/21 than with IL-2 supplementation. However, cell proliferation was greater with IL-2 and IL-7/15/21 than with IL-7/15. All the allogeneic NKG2D-CAR-T cells obtained, with the different supplementations, demonstrated antitumor activity against human cervical cancer HeLa cells and colorectal cancer HCT116 and HT29 cells. These results suggest that IL-7/15/21 supplementation is the most appropriate among the IL combinations assayed and that our allogeneic NKG2D-CAR-T cell product possesses *in vitro* antitumor efficacy against solid tumors.

P062 / No. 43

Topic: AS06 Universal donor cells & advanced TCR engineering

DESIGNER CAR TRANSMEMBRANE DOMAINS PROVIDE PREDICTABLY TUNABLE T CELL FUNCTIONAL POTENCY

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The transmembrane domains (TMDs) of chimeric antigen receptors (CARs) serve multiple functions. While they are primarily viewed as providing inert membrane anchors and physical links to intracellular signalling domains, their sequences also influence CAR expression levels, intracellular trafficking and oligomeric state. The latter is of particular significance, since the TMD sequences used in most current-generation clinical CAR constructs derive from naturally dimeric signalling proteins (CD28, CD8, CD4, membrane Ig) and consequently generate dimeric CAR structures. To interrogate the role of oligomeric structure in CAR function, we created *de novo*-designed TMD sequences that form computationally defined, experimentally validated and highly specific monomeric, dimeric, trimeric or tetrameric structures in the cell membrane. When incorporated into a standard HER2-directed, second-generation CD28-zeta CAR expressed in primary T cells, these TMDs provided stable surface expression and elicited both *in vivo* tumour control and *in vitro* cytokine secretion levels that scaled directly with the oligomeric state encoded by the designed TMD. Interestingly, all of our structurally programmed CARs (proCARs) elicit lower cytokine production than an otherwise identical reference CAR containing the CD28 TMD sequence. Building on other recent reports, we show that the CD28 TMD recruits endogenous T-cell CD28, generating much stronger co-stimulation than intended by design and accounting for a large part of the observed potency of CD28 TMD-tail CAR constructs. Our proCAR TMDs provide a new rational CAR design tool for precise and predictable tailoring of engineered T cell potency, and their modular nature provides broad and facile cross-compatibility with other CAR modifications.

P063 / No. 149

Topic: AS06 Universal donor cells & advanced TCR engineering

T-CELL RECEPTOR (TCR)-MODIFIED T-CELL DEVELOPMENT FOR THE RECOGNITION OF NY-ESO-1119-143 PEPTIDE PRESENTED ON HLA-DRB3*02:02

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Natural transgenic T-cell receptors (tTCR) represent an emergent type of cell therapy against cancer, recognizing peptides expressed both on the intracellular and extracellular level. Recognizing intracellular antigens constitutes a significant advantage in comparison to CAR therapy that can only recognize extracellular surface proteins, which are difficult to define as Tumor Associated Antigens (TAA), especially on solid tumors. However, it must be taken into consideration that TCR therapies against TAAs could potentially generate off-tumor on-target toxicity. In order to prevent this risk, this work is focused on NY-ESO-1, from the cancer/testis antigen family, expressed on several types of tumors but not relevantly on healthy tissues besides testicles. Several tTCR clinical trials targeting NY-ESO-1 have been performed, but all of them are focused on peptide presentation to class I HLA molecules. However, our work is focused on class II-presented peptides. Specifically, we are developing a TCR that can recognize NY-ESO-1 presented on HLA-DRB3*02:02 (DR52b), expressed approximately by half of the Caucasian population in a similar manner to HLA-A*02:01, for which many more studies have been performed. Other studies have determined that stimulation of T-cells from patients with NY-ESO-1 peptides triggers a CD4⁺ T-cell-specific immune response, restricted to HLA-DR52b and with a preserved TCR repertoire. It remains important to develop research enabling the generation of new TCR therapies that can recognize intracellular antigens presented by HLA-II, that can be useful to a high percentage of population and that can be applicable to several types of tumors.

P064 / No. 173

Topic: AS06 Universal donor cells & advanced TCR engineering

TCRVB MODIFICATIONS TO IMPROVE TCR EXPRESSION AND T CELL FUNCTION

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Introduction: T cell receptor (TCR) therapy is a promising treatment option for human malignancies and viral infections. Enhancing the introduced TCR expression and equipping T cells with an improved antigen-specific function remains a challenge. In this study we aimed to understand if the modification of TCRV β framework region amino acids could enhance TCR expression and antigen specific T cell function.

Methods: Bioinformatic and TCR structural analyses have been performed to identify candidate TCRV β residues. In vitro mutagenesis was employed to introduce the identified changes, followed by gene transfer to assess TCR expression levels by flow cytometry. CFSE labelled, relevant or irrelevant peptide pulsed antigen presenting T2 cells were used to measure the specific killing activities of T cells. Intracellular cytokine staining was performed to quantify the produced IL-2 and IFN- γ cytokines following the cognate antigen stimulation.

Results: Results with human Jurkat76 and primary T cells revealed that single amino acid changes on the TCRV β framework region increased TCR surface expression. Residue change

combinations further improved the TCR expression. Elevated IFN- γ and IL-2 cytokine release and boosted specific killing activity were observed with more than 100-fold increased antigen sensitivity.

Conclusion: We demonstrated that substitution of certain TCRV β framework region amino acids enhanced the TCR expression and the antigen-specific function of human T cells. Residue changes probably increased the quality of the introduced TCR chain proteins and more correct TCR α/β pairing occurred. This ultimately increased the TCR surface expression, which resulted in an improved antigen specific T cell function.

P065 / No. 140

Topic: AS06 Universal donor cells & advanced TCR engineering

THE INTEGRATED MULTI-OMIC CHARACTERIZATION OF “OFF-THE-SHELF” CD19-CAR-T CELLS ALLOWS THE IDENTIFICATION OF ENGINEERED CELLS ENDOWED WITH SUPERIOR ANTI-TUMOR FITNESS AND LOWER PRO-INFLAMMATORY ACTIVITY

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Background: The goal of this study is to optimize the manufacturing of “off-the-shelf” CD19-CAR-T cells utilizing umbilical cord blood (UCB) as source of T lymphocytes.

Methods: T cells were isolated from UCBs (N=15) through negative magnetic selection and upon activation *in vitro* using CD3 and CD28 mAbs. The T lymphocytes were then transduced with lentiviral vectors encoding for CD19-CD28z-or CD19-4-1BBz-CARs. Engineered T cells were also produced from the peripheral blood lymphocytes (PBL; N=5) as reference. The multidimensional phenotype analysis was utilized to assess the differentiation and activation status of the T cells. CD19-CAR-T cells were co-incubated or not with either CD19⁺ or CD19⁻ target cells to mimic the antigen-mediated engagement of the CARs, and then, multi-omics analyses, including metabolomics, transcriptomics, and *in vitro* functional assays, (Elispot, Luminescence) were performed.

Results: CD19-CAR T cells with early stage of differentiation (CD45RA⁺) co-expressing either ICOS or BTLA were observed in UCB- vs. PBL-CAR T cells (p<0.0002-<0.05). Distinct transcriptomic, metabolomic and functional profiles were also observed according to the source of T cells used for the CAR-T cells and to the antigen-specific stimulation of the lymphocytes. Statistically significant lower levels or no secretion of pro-inflammatory cytokines and growth factor by UCB- vs. PBMC-CAR-T cells was observed. These data suggest that UCB-CAR-T cells might be associated with lower risk of inducing *in vivo* cytokine release syndrome.

Conclusions: The multi-omics results, although their integration is still ongoing, allowed the deep profiling of UCB-CD19-CAR-T cells and to identify the cell products endowed with superior “fitness”.

P066 / No. 137

Topic: AS06 Universal donor cells & advanced TCR engineering

INCREASING THE PERSISTENCE AND EFFICIENCY OF CHIMERIC ANTIGEN RECEPTOR - NK CELLS

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Chimeric Antigen Receptor (CAR) T cell therapies have shown great clinical success in the treatment of hematologic malignancies. However, current CAR-T cell therapies are autologous, expensive in production, and may have severe side effects. Another immune cell type, Natural Killer (NK) cells, pose an attractive alternative cell type to generate CAR-modified cells. NK cell-based products can be generated from healthy donor cells and applied to unmatched patients in need. Especially for patients with T cell malignancies, that lack healthy T cells for CAR-T cell generation, the development of allogeneic cell therapy approaches is essential.

Here, we developed CAR-NK cells directed against T cell malignancies that show potent cytotoxicity against diverse cell lines and primary T cells in vitro. However, similar to CAR-T therapies, CAR-NK cells often lack in vivo persistence and proliferation, especially when solid tumors are targeted. To overcome this limitation, we screen a variety of CAR signaling domains and explore NK cell-specific costimulatory and activating domains in particular, to identify combinations of signaling adapters with the most beneficial effects for the functionality of the cell therapy product. The combination of NK cell-specific signaling and precise selection of CAR targets allows the best possible exploitation of the properties of this unique immune cell type.

P067 / No. 178

Topic: AS07 Beyond alpha-beta T cells

CHIMERIC ANTIGEN RECEPTOR DEVELOPMENT IN V Δ 1 T CELLS

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In cancer immunotherapy, chimeric antigen receptors (CARs) have demonstrated remarkable success in haematological malignancies but remain underutilised with limited efficacy in solid tumours. V δ 1 cells are unconventional T lymphocytes with innate tissue-resident properties and a high prevalence in tumour infil-

trates, suggesting a key therapeutic role as an allogeneic platform with tropism and prolonged effector function within a solid tumour context. Previously, we have developed a successful *ex vivo* V δ 1 expansion method using antibody-crosslinking TCR activation and IL-15 supplementation. Following expansion, we found that unmanipulated V δ 1 cells depended on IL-15 for continuous proliferation and effector function, an important finding for therapeutic development. However, we hypothesized that CAR-mediated signalling might overcome this cytokine dependence in the presence of antigenic stimulation. Testing a novel anti-B7-H3 CAR with CD28 and CD3 ζ signalling domains previously generated in our lab and shown to have minimal tonic signalling, we examined prolonged functional responses to LAN-1 tumour targets and plate-bound B7-H3 antigen in the presence and absence of IL-15. We observed that B7-H3-mediated CAR stimulation only partially rescued V δ 1 cell proliferation and tumour-specific cytotoxicity after 9 days without cytokine supplementation and determined that the CAR alone was not sufficient to overcome IL-15 dependence. Incorporating the intracellular IL-15 receptor IL-2/IL-15 receptor β (IL2R β) with a modified STAT recruiting domain into our CAR, we showed that it failed to recover V δ 1 responses compared to exogenous IL-15. Interestingly, in repeat challenge assays, CAR engagement with IL-15 was additive, encouraging future engineering to provide IL-15 signal for therapeutically effective V δ 1-CAR-T cells.

P068 / No. 69

Topic: AS07 Beyond alpha-beta T cells

FEEDER-FREE CULTURE SYSTEM FOR EX VIVO GENERATION OF LARGE NUMBERS OF NK AND CAR-NK CELLS FROM HEMATOPOIETIC STEM AND PROGENITOR CELLS

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Natural Killer (NK) cells are currently under clinical investigation as an attractive alternative for immunotherapies since they neither cause graft-versus-host disease nor induce cytokine release syndrome. However, the use of these cells is restrained by the limited availability of ex vivo clinical-grade methods for their expansion and genetic modification. We patented a 7-day feeder cell-free culture system for ex vivo generation of ProTcell™ from CD34+ cells. This culture system can be combined with lentiviral vector transduction, generating up to 70% of transduced ProTcell™. Here, we exploited the dual differentiation potential (T/NK) of ProTcell™ to develop an efficient method for producing large numbers of NK cells. ProTcell™ were cultured between 7 and 14 days in NK differentiation culture conditions (SCF/FLT3-L/IL-2/IL-7/IL-15). Large numbers (up to 10,000 NK cells per CD34+ cell seeded at day 0) of highly pure NK cells (>90% CD3⁺CD56⁺) were generated. These cells expressed activating receptors (NCRs, NKG2D, DNAM-1), NK transcription factors (Eomes, T-bet, ID2), and did not express inhibitory KIRs. They underwent degranulation and expressed IFN γ , TNF α , Perforin, and Granzyme B upon stimulation with K562 cells. When transduced with an anti-CD19 CAR lentiviral vector, efficient expression of the CAR led to the killing of NALM-6 cells efficiently (65% of cytotoxicity for transduced NK

as compared to 3.5% for non-transduced NK). Therefore, the unique combination of our short (7-day) ProTcell™ culture system and NK cell differentiation/expansion culture (7 to 14 days) provides a valuable and clinically applicable method to generate abundant pure NK and CAR NK cell populations.

P069 / No. 215

Topic: AS07 Beyond alpha-beta T cells

BABOON ENVELOPE PSEUDOTYPED LENTIVIRAL VECTOR AS AN EFFECTIVE TRANSDUCTION TOOL FOR ENGINEERING CAR Γ T CELLS

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LB: Industry Abstract Body: $\gamma\delta$ T cells represent a promising cell platform for allogeneic immune interventions. However, their expansion and engineering are challenging due to their low number in peripheral blood and a lack of highly efficient transduction methods. By expanding $V\gamma9\delta2$ T cells in feeder cell-free conditions, using a low concentrations of IL-2 and IL-15, Zoledronic acid and human serum, we obtained expansion folds over 1000 in 14 days of culture. The expression of activation markers was significantly increased throughout the expansion process. The $V\gamma9\delta2$ T cells exhibited mostly a memory phenotype, known to be beneficial for adoptive immune therapies. The purity of the cell product could be further improved by depleting $\alpha\beta$ -T cells with magnetic beads before expansion. We then compared different CD19 and CD33 CAR transduction conditions with Design of Experiment (DoE). We found that the BaEV pseudotype combined with Vectofusin-1™ outperformed VSV-G and resulted in the transduction of @85% of $V\gamma9\delta2$ T cells with a MOI as low as 0.5. CD19 and CD33 CAR $V\gamma9\delta2$ T cells demonstrated their functionality by killing their respective target cells better than untransduced $V\gamma9\delta2$ T cells in luciferase-based and Incucyte® assays. We report here a feeder cell-free expansion protocol for $V\gamma9\delta2$ T cells from human peripheral blood and successfully transduced these lymphocytes with different CAR constructs using our Vg9Vd2 T-cell transduction protocol. The CAR enhanced the natural $V\gamma9\delta2$ T-cell cytotoxicity, without altering their phenotype. This method is scalable for clinical production in a closed system such as the CliniMACS Prodigy®.

P070 / No. 101

Topic: AS07 Beyond alpha-beta T cells

B7H3 CAR V DELTA 2 GAMMA DELTA T CELLS ARMORED WITH A POTENT SECRETED IL15 AGONIST SHOW EFFECTIVE KILLING OF OSTEOSARCOMA PDXOS IN-VITRO

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Introduction Although chimeric antigen receptor (CAR) $\alpha\beta$ T cells have demonstrated a breakthrough success in hematological malignancies, the search for treating solid tumors and cell sources that allow allogeneic off-the-shelf therapy continues. B7H3 is a recently identified immune-checkpoint molecule and is homogeneously overexpressed in most pediatric solid tumors, despite limited expression in normal tissues. We combined a second-generation B7H3 CAR with a potent secreted IL15 agonist (stIL15), consisting of IL15 connected to the IL15R α sushi domain, performed lentiviral transductions of expanded $V\delta2$ $\gamma\delta$ T cells, and evaluated them with phenotypic and functional assays.

Methods CAR vector, consisting of B7H3 scFv, CD8a stalk and transmembrane domains, CD28 and CD3 ζ signaling domains with stIL15 was cloned into lentiviral plasmid backbone. Cryopreserved PBMCs from healthy donors were thawed, rested overnight, and stimulated with Zoledronate and IL2. After 2 days they were transduced with stIL15-B7H3CD28 ζ CAR lentivirus. Transduction efficiency, viability, purity, fold expansion, cytotoxicity, IL15 production, and activation&exhaustion phenotype were evaluated.

Results stIL15-B7H3CD28 ζ CAR- $V\delta2$ s showed effective secretion of stIL15 and target-specific killing against supT1 engineered to express B7H3. stIL15-B7H3CD28 ζ CAR- $V\delta2$ s showed comparable expansion quality with stIL15- $V\delta2$ s and better expansion than unmodified $V\delta2$ s. stIL15-B7H3CD28 ζ CAR- $V\delta2$ s showed superior cytotoxicity against osteosarcoma PDXOs that highly express B7H3 compared to stIL15- $V\delta2$ s.

Conclusion $\gamma\delta$ T cells are a potential allogeneic CAR chassis due to the absence of alloreactivity and provide innate cytotoxicity against tumors in addition to CAR-mediated killing. B7H3 CAR- $V\delta2$ s armored with stIL15 could be promising for allogeneic cell therapy for solid tumors and should be investigated further.

P071 / No. 93

Topic: AS07 Beyond alpha-beta T cells

METHOD COMPARISON OF TIME-RESOLVED CYTOTOXICITY DETERMINATIONS IN NK-92 CELL EXPANSION

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Natural killer (NK) cells play an increasingly important role in the field of personalized cellular therapies. The expansion of NK cells for use as Advanced Therapy Medicinal Products requires an understanding of the Critical Process Parameters (CPPs) in order to control the Critical Quality Attributes (CQA) that will be used to assess the final products quality. This CPP-CQA interaction is still widely unknown for NK cells, however, one of these CQAs is the cytotoxic potential of NK cells against target cells. Here, we demonstrate an experimental setup, where NK-92 cells were expanded in a pseudo-static format. Cytotoxicity of the expanded cells was monitored daily for 21 days by four different methods: (i) flow cytometric measurements targeting killing of target cells, (ii) lactate-dehydrogenase-release by effector cells, (iii) assay of Calcein release from the target cells, (iv) assay quantifying the production of Interferon- γ

by NK-92 cells. This is to our knowledge the first direct comparison of these commonly used methods to determine the cytotoxicity of NK cells. The results from our study will be beneficial for choosing the right method to determine cytotoxicity levels depending on the question researchers want to answer. Additionally, we correlated the findings to the growth rate and viability, IL-2 availability, changes of nutrient uptake including amino acids, production of metabolites. We could show changes of cytotoxicity depending on the situation of the cells in culture and suggest that cytotoxicity could be used as a prediction tool for the growth rate over the next days of cells in culture.

P072 / No. 57

Topic: AS08 *In vivo* T cell engineering

GENERATION OF IN VIVO CAR-T CELLS VIA NON-VIRAL DELIVERY VECTORS

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CAR-T cell therapies are a breakthrough in oncology and have recently shown tremendous promise by generating therapeutic efficacy against previously incurable haematological tumours. These therapies improve on current standards such as radiotherapy, chemotherapy and surgery, and are currently manufactured in an external biomanufacturing facility. As a result, CAR-T therapies are currently extremely costly, inconsistent and complex to deliver, and are only available to a minority of cancer patients. To overcome these challenges, we propose to manufacture CAR-T therapies directly **inside** the patients using a non-viral vector approach. These vectors are specifically designed to target T cells *in vivo*, and deliver the CAR transgene directly to T-cells to create therapeutically active CAR-T cells. With this approach, patients can be instantly dosed at the clinic, and T-cells don't require expert and expensive manipulation in factories. Instead, we harness the natural capability of our bodies to produce active and functional CAR-T cells. This presentation will detail our data to demonstrate the effectiveness of this approach and discuss our future plans. This work is done in collaboration with ImmTune Therapies Ltd, a UK based biotech developing *in vivo* cell and gene therapies.

P073 / No. 168

Topic: AS09 Targeting non-malignant diseases

GENOME INTEGRITY AND SCALABLE GMP-COMPLIANT MANUFACTURING OF HDR GENE EDITED CD4+ T CELLS FOR THE TREATMENT OF HYPER IGM 1

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Background We have been developing a homology driven recombination (HDR) gene editing strategy of CD4 T-cells using Cas9 and a viral donor template for the treatment of Hyper-IgM 1, a combined immunodeficiency caused by mutations in the X-linked CD40LG gene. Anticipating clinical translation we have developed a suitable manufacturing process and addressed genome integrity upon editing.

Methods CD4+ T cells were edited with Cas9 and adenovirus associated virus serotype 6 (AAV6) or integrase defective lentivirus vector (IDLV) carrying a corrective partial cDNA cassette and a functionally linked NGFR reporter, that could be exploited for the enrichment of edited cells.

Results We developed a scalable GMP-compliant large scale manufacturing process that enriches for edited cells, expands them, while maintaining a relevant T memory phenotype, with documented potency of edited cells. We designed robust ddPCR assays documenting the presence of large on-target deletions upon editing with AAV6 that were counter-selected culture. We validated the sensitivity of high coverage optical genome mapping (OGM), retrieving rearrangements of 7-300kb at 2.5-10% variant allele frequency. In IDLV-edited CD4 T-cells we found on-target integrations compatible with the presence of template trapping/concatemers, that increased with the proportion of edited cells, up to 21% variant allele frequency. These events did not compromise functionality, as assessed by expression of CD40LG, binding to CD40 and CD40-mediated signal transduction. No other recurring events were documented. In summary, on-target large deletions and integrations can be frequent and should be anticipated. High coverage optical mapping allows for unbiased genome integrity assessment in these settings.

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Topic: AS09 Targeting non-malignant diseases

THERAPEUTIC GENE EDITING OF T CELLS CORRECTS CTLA4 INSUFFICIENCY

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Background: Heterozygous mutations in *CTLA4* result in an inborn error of immunity (IEI) with a severe clinical phenotype. Autologous T cell gene therapy may offer a cure without the immunological complications of allogeneic stem cell transplantation.

Methods: We designed several homology directed repair (HDR) editing strategies that would correct the genetic defect. We first assessed correction of an individual point mutation. We then evaluated several universal strategies that enable correction of most disease-causing mutations with a single edit.

Results: Superior editing efficiencies were obtained with an intronic approach, and this strategy was then further evaluated. CTLA4 function and expression kinetics were assessed following editing using flow cytometry-based assays. Our approach resulted in regulated expression of CTLA-4 in CD4+ T cells, and functional studies demonstrated CD80 and CD86 transendocytosis levels comparable to wild type T cells. Gene editing of T cells isolated from three patients with CTLA-4 insufficiency restored CTLA-4 protein expression and rescued transendocytosis of CD80 and CD86 *in vitro*. Using a similar approach in murine cells, gene-corrected T cells from CTLA-4^{-/-} mice engrafted and prevented lymphoproliferation in an *in vivo* murine model of CTLA-4 insufficiency.

Conclusion: Together these data demonstrated that CTLA4 edited T cells survived *in vivo*, expressed CTLA4 and were able to control the clinical phenotype of CTLA4 insufficiency, providing a powerful proof-of-principle of our T cell gene therapy approach. A similar approach could be used in other IELs that are caused by multiple heterozygous mutations.

P075 / No. 92

Topic: AS09 Targeting non-malignant diseases

DEVELOPMENT OF A NOVEL ACE2 DECOY FOR BOTH SARS-COV-2 VARIANT NEUTRALIZATION AND INFECTED CELL ELIMINATION VIA UNMODIFIED OR CAR MODIFIED IMMUNE CELLS

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Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) caused a pandemic with millions of infections and deaths worldwide and devastating impact on global economy. Up to now, vaccines and monoclonal antibody (mAb) therapies lack to provide a long-lasting protection against rapidly evolving new emerging SARS-CoV-2 variants. Thus, novel therapeutic options are pressingly needed especially for immunocompromised patients and/or patients with high risk for developing a severe coronavirus disease 2019 (COVID-19). In that regard, we developed a novel immunotherapeutic drug based on the SARS-CoV-2 entry receptor angiotensin-converting enzyme 2 (ACE2). This ACE2 decoy potentially binds to the SARS-CoV-2 receptor binding domain (RBD), neutralizes SARS-CoV-2 as well as the Delta and Omicron variant and protects hamsters from a SARS-CoV-2 infection. To additionally use this ACE2 decoy for elimination of virus infected cells, we equipped it with an epitope tag. Thus, it can be applied as adapter molecule in the modular platform technologies UniMAB and UniCAR, which already demonstrated great success in the setting of malignant diseases. As adapter molecule the ACE2 decoy is able to efficiently recruit either universal chimeric antigen receptor (UniCAR) modified T cells or, in combination with an anti-peptide epitope-anti-CD3 bispecific Ab of the UniMAB system, unmodified T cells to efficiently kill SARS-CoV-2 RBD expressing human cells. Taken together, the ACE2 decoy represents a very promising immunotherapeutic drug for both SARS-CoV-2 variant neutralization and infected cell killing via the UniMAB and UniCAR system and might, therefore, clearly improve the treatment of COVID-19 patients.

P076 / No. 68

Topic: AS09 Targeting non-malignant diseases

CAGA-SPECIFIC CD8+ T CELLS CONTROLLING HELICOBACTER PYLORI FOR PREVENTION OF GASTRIC CANCER DEVELOPMENT

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Helicobacter pylori infection is the most significant risk-factor for gastric cancer development, making the bacteria a leading cause of cancer-related deaths worldwide. Its carcinogenic potential is primarily promoted by the injection of the oncogene CagA into host epithelial cells, where it activates multiple pro-oncogenic pathways. Although the strong immune response against *H. pylori* has been extensively researched, CD8⁺ T cells are not covered in current models of *H. pylori* mediated pathologies. Here, we spatiotemporally characterize gastric CD8⁺ T cell responses to *H. pylori* infection by flow cytometry, (single-cell) RNA sequencing, and ChipCytometry in mice and humans. We find that highly functional CD8⁺ tissue-resident memory T (T_{RM}) cells infiltrate the gastric mucosa shortly after infection and dominate over the gastric CD4⁺ T cell response during the early infection phase. Importantly, CD8⁺ T cell depletion results in increased *H. pylori* colonization levels. Our data suggests that pathogen control is mediated by cytotoxic T cell effector functions. These observations are highly depended on the ability of the infecting strain to deliver CagA. In fact, the majority of *H. pylori* induced gastric CD8⁺ T_{RM} cells show antigen specificity to CagA. We corroborate our findings of a CagA-dependent gastric CD8⁺ T_{RM} cell infiltration in *H. pylori* infected patients and confirm that multiple CagA-derived peptide:MHC-I epitopes induce CD8⁺ T cell responses in humans. Considering CagA's mode of action,

therapeutically induced or engineered and transferred cytotoxic CD8⁺ T cell clones targeting CagA-infected epithelium could eventually be exploited to prevent gastric cancer development.

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Topic: AS09 Targeting non-malignant diseases

PROTCELL™: EX-VIVO GENERATED T LYMPHOID PROGENITORS EXHIBIT THYMIC REGENERATIVE PROPERTIES

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Although allogeneic hematopoietic stem cell transplantation (HSCT) remains the gold-standard treatment in the context of immunodeficiency (congenic or acquired), the slow peripheral replenishment of T lymphocyte makes the patients at high-risk for opportunistic infections and relapse. Our team developed a clinical grade, large-scale, feeder-free process to manufacture *ex vivo* human early T cell precursors, designated ProTcell™. These progenitors seeded the thymus of a xenograft mouse model rapidly, enabling a polyclonal T cell repertoire replenishment faster than classical CD34⁺ hematopoietic stem and progenitor cells. Furthermore, we expect the infusion of allogeneic ProTcell™ not to cause Graft-versus-Host disease in patients as the recipient's thymic stromal microenvironment can tolerate thymocyte to an individual's self-antigens. An in-depth characterisation of ProTcell™ phenotype revealed the expression of markers known to be involved in thymic homing, including CXCR4 and CCR9, as well as molecules of crosstalk between thymocytes and thymic epithelial cells, such as RANKL. Following engraftment in NSG mice, ProTcell™ gave rise within a month to double positive (CD4⁺CD8⁺) thymocytes and CD3⁺TCRab⁺ CD4⁺ or CD8⁺ (i.e. single positive) T cells. Immunocompromised animals reconstituted with lymphoid progenitors had an enlarged thymus and displayed medullary islets (keratin 14⁺), morphological features characteristic for effective thymopoiesis and typically absent in un-injected NSG mice. These results highlighted the regenerative properties of these human T cell precursors. Altogether, these preclinical data unravel the combined potential of ProTcell™ for T cell reconstitution and thymic epithelium rejuvenation. ProTcell™ are currently tested in clinical trials to improve significantly patient prognosis after HSCT.

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Topic: AS09 Targeting non-malignant diseases

CAR AGAINST ALLERGIES

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Introduction Current treatment for IgE mediated allergies fails to offer a robust cure. **Aim:** To design Chimeric Antigen Receptor T cells (CAR-T) that will target and destroy IgE+B cells, and abolish IgE production.

Methods We designed three 2nd generation anti-IgE CARs, of whom one design targets a domain specific to membrane-IgE (mIgE-specific CAR) and the other two target a domain shared by mIgE and secreted IgE. CARs were expressed on human primary lymphocytes via Retroviral transduction. We tested the potency of the therapy on engineered NALM6 cell line expressing mIgE, and on primary human B cells isolated from tonsils, and induced to class switch to IgE+ by IL-4 and anti-CD40-Ab.

Results IgE-targeting CAR-T cells successfully eliminated IgE+ expressing NALM6 cells, but not wild type NALM-6. This targeted elimination was accompanied by increase of gamma interferon production by the effector cells. Further more, the addition of high amounts of free IgE to the culture medium, to recapitulate the condition as in an allergic patient's blood, did not affect activation of mIgE-specific CAR-Ts, whereas it dramatically impaired activation of the other two designs. Upon *in vitro* co-culture with tonsillar human B cells, anti-mIgE CAR-Ts abolished all IgE production, as measured by ELISA. In the near future we will examine the potency of our therapy to prevent and cure egg allergy, in an *in vivo* mouse model, using a transgenic allergy prone mouse with an IL4-R mutation.

Conclusion CAR targeting mIgE holds promise as a curative therapy for IgE mediated allergies.

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Topic: AS09 Targeting non-malignant diseases

ENGINEERING SARS-COV-2 SPECIFIC IMMUNOSUPPRESSIVE DRUG RESISTANT ARMORED (IDRA) T CELLS FOR CELL THERAPY IN ORGAN TRANSPLANTED PATIENTS

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Organ transplant recipients receive immunosuppressive drugs (ISDs) that impair their immune responses to infections and vaccines. Thus, this patient population is susceptible to develop severe COVID-19. We first characterized Spike-specific T cell response in kidney transplanted patients ($n=136$) treated with different ISDs and we observed that combination of tacrolimus (TAC), mycophenolate mofetil (MMF), and prednisone (Pred) treatment displayed suppression of mRNA vaccine-induced Spike-specific T cells. To compensate these defects, we engineered T cells specific for different SARS-CoV-2 epitopes and capable to escape, transiently, the immunosuppressive effect of TAC and MMF. T-cell receptors (TCRs) specific for 6 different epitopes located in Spike and Nucleocapsid of SARS-CoV-2 and restricted by 6 different HLA-Class I molecules were cloned from SARS-CoV-2-specific-CD8 T cells of vaccinated

and convalescent donors. We then concomitantly electroporated T cells with mRNAs coding for SARS-CoV-2 TCRs and mutated variants of calcineurin (CnB) and inosine-5'-monophosphate dehydrogenase (IMPDH). These engineered T cells recognize target cells both pulsed with synthetic peptides containing the different SARS-CoV-2 epitopes or targets that endogenously synthesized SARS-CoV-2 Spike and Nucleocapsid. Their reactivity is minimally affected by mutations occurring in Beta, Gamma, and Omicron variants. Importantly, the transient expression of SARS-CoV-2 TCRs and of CnB and IMPDH variants induced by the electroporated mRNAs on T cells, limit the ability of SARS-CoV-2 TCR-redirection T cells to escape the drugs immunosuppressive effect only for about 72 hour, providing an intrinsic safety feature for potential adoptive T cell therapy in transplanted organs patients with severe or persistent Covid-19.

P080 / No. 134

Topic: AS09 Targeting non-malignant diseases

FCγRIIA-SPECIFIC DARPINS DISPLAYED ON VIRAL VECTORS FOR HIV GENE THERAPY

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FcγRIIA (CD32a) was recently identified as potential marker of T lymphocytes latently infected with HIV-1. The use of FcγRIIA as a marker has not been without controversy due to technical challenges complicated by trogocytosis and a lack of antibodies distinguishing between the closely related isoforms of FcγRII. To generate high-affinity binders specific for FcγRIIA, libraries of designed ankyrin repeat proteins (DARPin) were screened for binding to its extracellular domains by ribosomal display. The identified DARPins bound FcγRIIA with no detectable binding for FcγRIIB. Their affinities for FcγRIIA were in the low nanomolar range and discrimination from FcγRIIB was based on a single amino acid residue. When DARPin F11 was displayed on lentiviral and AAV vectors, we observed highly selective gene delivery into FcγRIIA+ T lymphocytes. This held true also in presence of a large surplus of off-target cells positive for FcγRIIB. On primary cells from healthy donors, between 0.03% - 1.26% (PBMC) and 0.14% - 3.46% (isolated CD4⁺ T cells) were transduced. Vector particles intravenously injected into NSG mice transplanted with SupT1-CD32a target cells showed transduction rates of up to 60%. Finally, F11-AAV transferring provirus directed endonuclease diminished HIV replication. The selected DARPins demonstrated the existence of FcγRIIA+ T cells, and, when displayed on viral vectors, serve as promising novel tool for *in vivo* HIV gene therapy.

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Topic: AS09 Targeting non-malignant diseases

ENGINEERED T REGULATORY CELLS AS A TREATMENT OPTION FOR MULTIPLE SCLEROSIS

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T regulatory cells (Tregs) have an important role in maintaining self-tolerance. Human Tregs express FoxP3, a transcriptional factor, which is considered the master regulator in Treg development and function. Pre-clinical murine models have demonstrated that antigen specific Tregs are more efficacious at controlling autoimmune diseases than their polyclonal counterparts. Here we have developed an antigen specific Treg therapy for multiple sclerosis (MS). A lentiviral vector was designed encoding two therapeutic genes; a T cell receptor (TCR) specific for human MBP (myelin basic protein - associated with multiple sclerosis) and exogenous FoxP3. The FoxP3 was introduced to prevent the conversion of Tregs into antigen specific effector T cells. Human engineered Tregs (TCR+/FoxP3+) expressed high levels of the TCR and displayed greater FoxP3 expression when compared to non-modified Tregs. Importantly, all TCR positive Tregs were FoxP3 positive, therefore introducing a safety switch to prevent conversion into T effector cells. Additionally, compared to polyclonal Tregs, TCR+/FoxP3+ Tregs showed antigen-specific suppression of IL2 production and were superior in ripping the co-stimulatory molecule, CD80, from APCs. Adoptive transfer of TCR+/FoxP3+ Tregs into HLA-DR transgenic mice resulted in long-term persistence (followed up to 6 months) with stable FoxP3 and TCR expression. In contrast, adoptive transfer of Tregs only expressing the MBP-TCR, resulted in a population of TCR+/FoxP3- cells which displayed antigen-specific effector function. These data show a promising strategy to generate a long-lived MS cellular therapy using autologous Tregs with redirected antigen specificity, and are engineered with exogenous FoxP3 to have a 'locked' Treg phenotype.

P082 / No. 111

Topic: AS09 Targeting non-malignant diseases

DEVELOPMENT OF A SARS-COV-2 CAR-T CELL THERAPY

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SARS-CoV-2 is a coronavirus known for causing the disease Covid-19. At the beginning of the pandemic SARS-CoV-2 had a high virulence, causing a huge number of severe cases and deaths. Although after the initiation of worldwide vaccination the severity of the cases decreased, there are still several subsets of the population, such as immunocompromised or immunosuppressed individuals, that are at risk of developing severe symptomatology. In order to tackle the lack of medical options for treating these individuals, who cannot eliminate the virus with their own immune system, we decided to develop a Chimeric Antigen Receptor (CAR) T cell therapy directed against the S protein of SARS-CoV-2. We have generated CAR-T cells with two different single chain variable fragments (scFv) that recognize the receptor binding domain of the Spike protein. We have tested these CAR-T in vitro, with different cell line models to test their specificity. The CAR-T were only capable of recognizing and mediate a cytotoxic response against in-house cell line models that expressed the spike protein on their surface.

Moreover, we did also test our CAR-T in cells directly infected with the virus, and they were also capable of mediating a specific response against the infected cells. Due to the evolution of the virus with time, new variants with mutations in the spike protein have appeared. We also have conducted affinity studies of our scFv for these new viral variants. Our results show that our scFv are capable of recognizing new viral variants such as Delta and Omicron.

P083 / No. 146

Topic: AS10 Other

IN-DEPTH CHARACTERIZATION OF THE IMMUNE MICROENVIRONMENT OF PATIENTS UNDERGOING CAR T CELL THERAPY

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Currently available molecular biomarkers and nonclinical models poorly predict chimeric antigen receptor (CAR) T cell mediated cytokine release syndrome (CRS) *in vitro*. To address this need, we conduct a multi centric research study where we analyze molecular and cellular features of the immune system of patients undergoing CAR T cell therapy with FDA/EMA approved CAR-T products, with already 100 patients enrolled. Here, we collect peripheral blood as well as serum at four time points: twice before and twice after CAR T cell infusion. Serum samples are subjected to multiplex cytokine analysis. Mononuclear cells isolated from whole blood are used for transcriptome analysis by next generation sequencing (NGS). Furthermore, we collect the patients' clinical and therapy follow-up data. Primary endpoint is the comparison of gene expression patterns in patients with severe vs. low grade CRS. Using flow cytometry, we monitor CAR T cells as well as conventional T cells, NK cells, B cells and dendritic cells during the first 14 days after infusion. Clinical parameters to distinguish CRS symptoms include fever, high levels of IL-6 and C-reactive protein. Samples of patients with clinical symptoms of CRS after CAR-T infusion are selected for NGS analyses for the characterizing of immune cell populations and their microenvironment before and after CAR T cell infusion. By correlating CRS development with treatment response we seek to identify biomarkers applicable to nonclinical test systems. This fuels the IMI2/EU project "immune safety avatar" (imSAVAR), where we already established an immune-related adverse outcome pathway for CAR-T mediated CRS.

P084 / No. 107

Topic: AS10 Other

RAPID AND EFFICIENT GENE EDITING OF PRIMARY HUMAN RESTING CD4 T CELLS ALLOWS UNPRECEDENTED FUNCTIONAL ANALYSES

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Resting CD4 T cells are major cell types that contribute to the HIV-1 reservoir. A detailed understanding of molecular mechanisms that underlie HIV persistence is critical to the development of an HIV cure. To study suspected genes enabling or restricting HIV infection and latency, primary cells must be manipulated without inducing activation or altering cell homeostasis. Until now, such efforts have been inefficient or non-physiological. Here, we report a method for rapid, efficient, activation-neutral gene editing of resting, polyclonal human CD4 T cells using optimized cell cultivation and nucleofection conditions of Cas9–guide RNA ribonucleoprotein complexes (RNPs). We established a workflow where resting CD4 T cells are freshly isolated from leukoreduction system chambers and nucleofected once with two target-prevalidated RNPs and then cultivated up to 6 weeks. This workflow allowed us to knockout (KO) up to six genes simultaneously at efficiencies of up to 99%. Notably, despite the simultaneous use of 12 gRNAs in the RNP complex, little or no off-targets effects were observed using both biased and unbiased approaches, despite extensive editing in the specific KO loci. We further demonstrate that the combination of specific RNPs and double-stranded DNA donor templates resulted in the knock in (KI) of a reporter fusion gene into different endogenous loci, enabling the study of the physiological interplay of cellular and viral components at single-cell resolution. In conclusion, applying these KO and KI protocols will yield important insights into the processes governing infection, latency, reactivation, and immune recognition of HIV-1.

P085 / No. 125

Topic: AS10 Other

DUAL-TARGETING CD19 AND NKG2D LIGANDS CAR T-CELLS ARE EFFECTIVE AGAINST CD19 POSITIVE AND CD19 NEGATIVE B CELL MALIGNANCIES

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Despite the impressive 70-90% complete remission observed in ALL patients treated with CD19 CAR T-cells, this complete response is short-lived and 30-60% of treated patients relapse within 1 year (next to 20% of ALL patients, over half of patients with DLBCL or CLL that fail to respond to CD19 CAR T-cell treatment). Relapse can be either CD19 positive or negative. The molecular mechanism that underlies the majority of CD19 negative disease can be explained by antigen loss, with the CD19 positive relapse remaining elusive. To tackle this short-lived efficacy, several dual CAR T-cells were developed many of whom are currently assessed clinically. NKG2D ligands (MICA, MICB and ULBP1-6) are stress-induced and thus expressed by virtually all cancer types. we developed different NKG2D/CD19 dual CAR T-cells, utilizing both tandem and bicistronic NKG2D-based CARs that encompass the extracellular domain of the natural NKG2D receptor fused to, or co-expressed with an anti-CD19 CAR. We show that the majority of CD19/NKG2DL dual CAR T candidates were able to secrete cytokines, proliferate and eliminate CD19 KO Nalm-6 cells *in vitro*. Interestingly, some of these dual CAR T cells display an even higher level of *in vitro* functionality against WT Nalm-6 cells, showing

the potential of this approach against CD19 positive cancer cells as well. This NKG2D-based dual CAR technology can also be applied to eliminate solid tumors that often display antigen expression heterogeneity. We are currently developing several NKG2D-based dual CAR T-cells against diverse solid cancers.

P086 / No. 188

Topic: AS10 Other

GETTING OUT OF THE MOUSE HOLE: OPTIMIZING MURINE CAR T CELL GENERATION TOWARDS IMPROVED CAR T CELL FUNCTION

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Chimeric antigen receptor (CAR) T cells are successfully used to treat a variety of hematologic malignancies. However, for half the patients therapy fails and resistance mechanisms are poorly understood. To study the tumor microenvironment (TME) of aggressive lymphoma treated with CAR T, we developed an immunocompetent model growing in distinct TME of various lymphoid organs (Gsottberger et al.).

To enhance generation of murine CAR T cells for use in immunocompetent models, we developed a guide for optimized production.

To optimize each step of production, transfection of packaging cell lines, optimization of the CAR vector and of the CAR codon usage, an ideal T cell activation before transduction and an expansion that achieves optimal CART cell phenotypes were evaluated *in vitro*.

The packaging cell line Plat-E was identified as a suitable producer of murine retroviruses. Further, codon optimization increased CAR expression levels. More cost-efficient T cell activation with anti-CD3-coating in combination with soluble anti-CD28 results in similarly well activated T cells than anti-CD3/anti-CD28 magnetic beads. With this well working protocol on production of murine CAR T cells, the effects of CAR-modulating small molecules on phenotype and function of CAR T cells will be determined to complement ongoing questions from clinical trials in a suited, immune competent model system.

Facilitating murine CAR T cell generation with standardized protocols, our findings may pave the way for urgently needed immunocompetent mouse models with the long-term goal of enabling mechanistic understanding of CAR T cell therapy failure within distinct TMEs.

P087 / No. 122

Topic: AS10 Other

PHOSPHOPROTEOMIC ANALYSIS OF SECOND GENERATION CHIMERIC ANTIGEN RECEPTOR SIGNALLING FOR THE TREATMENT OF GLIOBLASTOMA

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Chimeric Antigen Receptors (CARs) targeting of CD19 or BCMA proteins in late-stage haematological adult and paediatric cancers have shown significant increases in patient survival, leading to FDA approval and integration into clinical care. The FDA approved CARs are all second-generation and contain either CD28 or CD137 signalling domains. A previous study by Stan Riddell's group comparing CD28 and CD137 signalling using phosphoproteomic analysis, reported minimal differences between the two CARs due to endogenous CD28 recruitment. It remains to be determined if co-stimulation domains, such as CD27, CD134 or CD278, have conserved signalling in a CAR and thus the variation in phenotype observed between CARs is a measure of structural variations rather than signalling variations. Determining the mechanism in phenotypic variation is important to determine if efforts should be focused on different signalling components or structural variations to improve CAR design to enhance efficacy in cancers such as glioblastoma, where CARs are presently less effective. To examine differential signalling pathways we constructed a standardised CAR library (CD3 ζ , CD27CD3 ζ , CD28CD3 ζ , CD134CD3 ζ , CD137CD3 ζ , CD278CD3 ζ) varying only the signalling domain. All six CARs were validated for antigen dependent killing and cytokine production before kinetic samples were generated for phosphoproteomic analysis. Our analysis shows that each CAR has a unique signalling program with only ~20% significantly changed phosphoproteins shared between CARs. We performed a kinase substrate enrichment analysis to determine potential rational drug combinations and show that co-stimulation domain choice is an important consideration when developing combination therapies for cancers such as glioblastoma.

P088 / No. 110

Topic: AS10 Other

CONTROLLING GLIOBLASTOMA WITH CAR T CELLS TARGETING AN ALTERNATIVELY SPLICED DOMAIN D OF TENASCIN C

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Glioblastoma (GBM) is a highly aggressive and incurable primary brain tumor, with poor therapeutic advances made in the past decades. Therefore, the need for developing new therapies to improve patient outcomes is crucial. Chimeric antigen receptor (CAR) T cell therapy has shown breakthrough success in the treatment of hematological cancer. However, it showed limited efficacy in clinical studies for GBM, most likely due to tumor heterogeneity, antigen loss, and lack of cancer-restricted antigens. Targeting the cancer-specific alternatively spliced domain D of the extracellular matrix protein Tenascin C (TNC) could help overcome these limitations. In GBM, only the alternatively spliced TNC isoform is extensively expressed by cancer cells and adheres to the cell surface. Here, we have developed a 2nd generation CAR using the publicly available R6N scFv clone with a CD28z costimulatory domain. TNC-CAR T cells could successfully bind to soluble TNC, triggering a dose-dependent activation phenotype, by looking at CD25 and CD69 by flow cytometry, of TNC-CAR T cells when cocultured with purified

TNC protein or supernatants from TNC-expressing GBM patient-derived cells. Furthermore, TNC-CAR T cells efficiently killed naturally TNC-secreting patient-derived GBM cell lines *in vitro*. Development of murine TNC-CAR T cells and the assessment of this therapy in both immunocompromised and immunocompetent mice models are ongoing. Altogether, these preliminary results suggest that targeting the tumor-specific TNC splice variant D with CAR T cells is feasible, and further pre-clinical development of this strategy is needed to better understand its therapeutic impact for the improvement of GBM patient outcomes.

P089 / No. 211

Topic: AS10 Other

LIVECYTE: ANALYSIS OF T-CELL KILLING INTERACTION KINETICS USING LABEL FREE IMAGING OF T-CELLS

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LB: Industry Abstract Body: T-cell based immunotherapy is an exciting emerging therapy in the fight against cancer, with *in vitro* T-cell killing assays being the first step in validating the effectiveness of new therapies. Conventional assays either depend upon time intensive manual tracking, or use of fluorescence imaging of T-cells, which are often primary cells, and highly sensitive to phototoxicity as a result. In addition, the resulting metrics from fluorescence assays only give population-based metrics of cell death and interaction time, with no scope for cell interaction kinetics, leaving a large gap in knowledge on why certain treatments are more efficacious than others.

In this experiment the kinetic cytometer, Liveocyte, was able to use its single cell tracking capabilities to derive T-cell:Target cell kinetics, such as number of T-cell visits, total and average interaction time, as well the number of T-cell attached at death, and the contact time of final T-cell interaction. Additional investigation also elucidated morphologies of both cell subtypes, included with conventional metrics such as cell death, and total cell count. This was performed with entirely unlabelled T-cells, using Quantitative Phase Imaging (QPI), a label free imaging technique to segment every cell, and fluorescence to categorise between Target cells, T-cells, and apoptotic cells.

Liveocyte's detail of analysis over multiple, large ROIs, with label free imaging of T-cells allowed a greater depth of analysis, without the need for labelling and potentially altering the behaviour of the effector T-cells. Overall, this demonstrates Liveocyte's effectiveness as a hugely powerful tool for immunotherapy researchers.

P090 / No. 221

WITHDRAWN

P091 / No. 158

Topic: AS10 Other

RAG2^{-/-}γC^{-/-} MICE HUMANIZED WITH CD34+ HEMATOPOIETIC STEM CELLS ARE A SUITABLE TOOL TO SCREEN FOR FUNCTIONAL EWING SARCOMA-SPECIFIC T CELLS IN VIVO

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Successful immunotherapy is hampered by an ineffective T cell repertoire against tumor antigens. Here we implement a humanized mouse system to scan for allorestricted CD8+T cells against Ewing's sarcoma (EwS) associated antigens. Rag2^{-/-}γc^{-/-} mice were irradiated (3.5 Gy) at birth and transplanted into the liver with 1.2x10⁵ HLA-A*0201⁻ human cord blood-derived hematopoietic stem cells. At the age of ≥6 weeks, mice were i.v. vaccinated with EwS peptides pulsed on HLA-A*0201⁺ dendritic cells (DC) or received either no humanization, no DC vaccination or DCs vaccination without peptide load. After three vaccinations with 2x10⁶ DCs, all mice received 2x10⁶ A673 EwS cells s.c. At day 17 mice were analyzed for lymphatic organs, tumor burden and antigen-specific allorestricted HLA-A*0201⁻ CD8⁺T cells. Mice regularly showed human CD4⁺/CD8⁺/CD4⁺CD8⁺T cell reconstitution in thymi and T and B cell reconstitution in spleens. Two mice showed lymph nodes with presence of human T cells. 65% of humanized mice showed peripheral reconstitution with >10% human CD45⁺ cells. Tumor size between study group mice vs. respective controls was significant (p<0.05). The degree of peripheral reconstitution was not associated with tumor size. EwS peptide multimer stained T cells were detected in lymph nodes of the only study group mouse without tumor burden. Respective T cells specifically recognized peptide-pulsed target cells and HLA-A2⁺/peptide⁺ EwS cells. Immunization of humanized mice with peptide-antigen loaded DC induced an allorestricted T cell response capable to suppress EwS growth. This tool allows screening for antigens presented by different tumor entities to identify functional antigen-specific T cells.

P092 / No. 44

Topic: AS10 Other

ENHANCING ANTITUMOR IMMUNITY WITH IMMUNOTHERAPY: A COMBINATION OF NOVEL BISPECIFIC T-CELL ENGAGERS TARGETING COLORECTAL CANCER

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Bispecific T-cell engagers (BiTEs) represent a powerful tool in supporting natural immunity of patients against hematologic malignancies. However, their use in the treatment of solid tumors is still challenging. Here we propose a novel strategy to enhance the efficacy of this therapeutic approach by using a BiTE binding the costimulatory receptor CD28 in combination with a CD3-based BiTE. We designed CD3- and CD28- BiTEs, highly specific for a novel tumor-associated antigen recently identified on primary colorectal cancer stem cells. The sequences encoding for the cDNA of the BiTEs were cloned in lentiviral vectors and HEK293T human kidney cells were infected to obtain stable clones producing both engagers. The BiTEs were purified using the FPLC AKTA pure system and tested on primary human colorectal cancer cells expressing luciferase (UM11-LUC). The activity of the BiTEs was assessed upon co-culture of UM11-LUC and T lymphocytes in the presence of the

CD3-BiTE alone or in combination with the CD28-BiTE. The specific killing activity was evaluated by a luminescence-based assay and the cytokine production was evaluated by ELISA. Furthermore, T cell activation was investigated by flow cytometry. We found that the CD28-BiTE strongly promoted T-cell activation, proliferation and cytokine secretion when coupled with CD3-BiTE, leading to an enhanced ability of lymphocytes to kill target cells *in vitro*. These results suggest that our approach of combining BiTEs that activate different receptors on T cells may be a valid tool to fight solid tumors. This hypothesis will now be tested *in vivo*.

P093 / No. 108

Topic: AS10 Other

NEW GMP CLEAN ROOM SOLUTIONS AT FINNISH RED CROSS BLOOD SERVICE (FRCBS) FOR PRODUCTION OF ADVANCED CELL THERAPIES

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FRCBS is the nationwide blood service provider in Finland that holds a central position between donors and patients and manages the entire blood product chain from donor selection and donation to quality-assured manufacturing and transport to hospitals. Novel cell therapies are a central part of our strategy. We have over 20 years of experience in stem cell and immune cell research, and together with clinicians, researchers, and partners, we develop novel cell therapy products. FRCBS Advanced Cell Therapy Centre (ACTC) was established in 2012 and over 100 patients have been treated with locally manufactured bone marrow mesenchymal stromal cell and autologous keratinocyte advanced therapy medicinal products (ATMPs) under hospital exemption. We also produce TCRα/β-CD19 depleted and CD34-enriched haploidentical stem cell transplants for pediatric patients of Helsinki University Central Hospital. In 2022, we moved into new facilities with custom-build 300m² clean rooms containing separate rooms for different product types, including a GMO/ATMP production and quality control premises. We use closed systems (Grade A) in Grade D clean room background. We have set up CAR-T production processes utilizing closed, automated systems such as CliniMACS[®] Prodigy platform and have applied a Bioquell QUBE M-2 isolator (Bioquell Ltd.) for handling the open phases of GMO/ATMP processes. Furthermore, we have set up a variety of quality control and characterization analyses for CAR-T cells and have the capacity for controlled rate freezing and storage. At ACTC, we have excellent capabilities to provide contract manufacturing services for ATMP production from early development to clinical manufacturing.

P094 / No. 109

Topic: AS10 Other

TARGETING SENESCENT CELLS IN PROSTATE CANCER USING CAR T CELL THERAPY

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Prostate cancer (PCa) is the second most commonly diagnosed cancer and the sixth leading cause of death in men worldwide. Standard of care for the treatment of PCa is chemotherapy, radiotherapy, and androgen-deprivation therapy (ADT), however, a significant fraction of patients develops an aggressive metastatic castration-resistant form of PCa (mCRPC). Cellular senescence contributes to the development and aggressiveness of mCRPC. As cell therapy with chimeric antigen receptor (CAR) T cells has shown promise in various malignancies, we aim to develop CAR T cells for PCa, specifically targeting senescent cells. Two target antigens, PSMA and CD112, were discovered to be upregulated on senescent PCa cells. Novel PSMA- or CD112-specific scFvs were generated from a human phage-display library screen, and cloned in a lentiviral vector to generate monospecific 4-1BB/CD3 ζ CARs. Monospecific anti-PSMA and anti-CD112 CAR T cells efficiently and specifically lysed PSMA- or CD112-expressing human PCa cells *in vitro*. In addition, as an indicator of successful T cell activation, monospecific CAR T cells secreted high levels of IFN γ upon co-culture with PSMA⁺- or CD112⁺ PCa cells, respectively. To prevent potential on-target/off-tumor effects due to expression of CD112 in various normal tissues, we designed a combinatorial CAR approach, restricting CAR T cell activation to cells expressing both antigens. Combinatorial CAR T cells were evaluated to preferentially lyse PSMA⁺/CD112⁺ human PCa cells *in vitro*; high levels of IFN γ were detected upon co-culture with PSMA⁺/CD112⁺ PCa cells. These combinatorial CAR T cells are now being tested *in vivo* in relevant mouse models.

P095 / No. 213

Topic: AS10 Other

IMMUNOTHERAPY TARGETING MUTANT NUCLEOPHOSMIN-1 ON ACUTE MYELOID LEUKEMIA

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LB: Academic Abstract Body: Acute myeloid leukemia (AML) is a malignant disease of the bone marrow. In 30-35% of AML, 4 base pair insertion occurs in the nucleophosmin-1 gene creating an 11 amino acid alternative reading frame (dNPM1). We previously demonstrated that CLAVEEVSL and AVEEVSLRK are dNPM1 peptides presented on AML by HLA-A*02:01 and HLA-A*11:01, respectively. For CLAVEEVSL,

we isolated a T cell receptor (TCR) and demonstrated that this TCR can be used to redirect T cells to target AML *in vitro* as well as in immunodeficient NSG mice engrafted with OCI-AML3, which is a dNPM1 positive AML cell line. Safety and efficacy of dNPM1-A2 TCR-T-cells will soon be evaluated in a phase I/II trial. Here, we searched for specific T cells using peptide-HLA tetramers for AVEEVSLRK in HLA-A*11:01. Two T-cell clones were isolated from an HLA-A*11:01 positive (6.F11) and negative (26.2.D6) individual. Both clones reacted against AML *in vitro*, but 26.2.D6 outperformed 6F11. Safety analysis did not reveal any reactivity against healthy hematopoietic cell types. We also cloned both dNPM1-A11 TCRs and investigated reactivity of TCR-T cells against AML. Similar as the original clones, dNPM1-A11 TCR-T cells were reactive against AML *in vitro* and the 26.2.D6 TCR outperformed the 6F11 TCR. In AML engrafted NSG mice, however, TCR-T cells with the 26.2.D6 TCR induced a similar anti-tumor response as dNPM1-A2 TCR-T cells, while no anti-tumor reactivity was observed for 6F11 TCR-T cells. In conclusion, AVEEVSLRK is an HLA-A*11:01 neoantigen on dNPM1 AML that could serve as a target for TCR gene therapy.

P096 / No. 161

Topic: AS10 Other

THE GENOMIC AND IMMUNOLOGICAL CHARACTERIZATION OF COLORECTAL AND BREAST CANCER STEM CELLS: IMPLICATIONS FOR CANCER IMMUNOTHERAPY

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Background: The aim of this study is to identify the molecular mechanisms regulating the immunological properties of cancer stem cells (CSCs) isolated from solid tumors.

Methods: Colorectal cancer (CRC; N=15) and breast cancer (BC; N=21) cell lines, including differentiated tumor cells and CSCs, and, for BC cell lines, selected *in vitro* for radioresistance or invasiveness were used for this study. The expression profile of HLA and of the antigen processing machinery (APM) molecules was assessed through flow cytometry. The transcriptomics (total RNA sequencing, Illumina) and microRNA (miRNAs; nCounter-Nanostring) characterization of these cell lines was assessed.

Results: Differential miRNAs, transcriptomic and methylation profiles (p<0.01 or 0.05) were identified in either CRC or BC cells with stemness properties vs. differentiated cells, and in different subtypes of cells. The differentially expressed miRNAs

include regulators of immunological functions, such as miRNA299-3p, miRNA4833p, miRNA15ap, miRNA3625p and miRNA196a-5p. These miRNAs regulate the expression of MHC and APM molecules, IL-4 and IL-13 and immune checkpoint (PD-L1, CTLA-4). The functional validation of the role of the aforementioned miRNAs is ongoing through the modulation (either their silencing or over-expression) of their expression in the cell lines. The ability of CSCs to elicit *in vitro* antigen-specific T cell responses correlated with the pattern of the expression of the miRNAs and the associated MHC and immunoregulatory molecules.

Conclusions: These investigations contribute to understand the mechanisms regulating the susceptibility of CSCs to T cell responses and possible interventions that could increase the efficacy of adoptive cell therapy for solid tumors.

P097 / No. 182

Topic: AS10 Other

TCRCLASS: A NOVEL COMPUTATIONAL APPROACH FOR IDENTIFYING T CELL RECEPTOR SPECIFICITY AND PHENOTYPE IN SINGLE CELL RESOLUTION

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Recent advances in immune cell profiling in single cell resolution allow for antigen-specific T Cell Receptors identification and sequencing via highly specific barcoded multimers. Here we present a novel approach to identifying and profiling T Cell Receptor specificities *in-silico* and assess their diversity and phenotype in an integrated and interactive web-based tool. This method can be applied to viral as well as cancer specific epitopes using TCR sequencing data and single cell immune profiling assays. Our method implements metrics of sequence similarity, clonal expansion, gene usage, and defines a novel metric for repertoire diversity based on graph-based network modularity optimization. We use published antigen and epitope-specific TCR sequencing data of cancer neopeptides presented on MHC-I in mice to extract and engineer CDR3 sequence-based features and train a machine learning classifier for TCR specificity prediction. Additionally, our method integrates single cell RNA-Seq functional analysis for each tested epitope with its corresponding specificity and repertoire features.

P098 / No. 66

Topic: AS10 Other

CHARACTERIZATION OF CAR T CELL PRODUCTS FOR FUNCTIONALITY AND FITNESS IN REGARD OF MANUFACTURING PROCESS ADJUSTMENTS

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Intro: Personalized immunotherapies with genetically modified autologous T cells have proven clinical efficacy and tre-

mendous potential in several medical fields. A standardized and reliable manufacturing of these cellular products is challenging as it requires meeting critical quality attributes for Drug Products (DPs), especially with respect to the shelf-life of DPs in fresh formulation. Critical material parameters (CMPs), such as human AB (hAB) serum, influence the reproducibility of manufacturing CAR T cell products and impact the cell characteristics at different levels. Thus, understanding how these CMPs influence the CAR T cell manufacturing and final DP properties such as stability will help to optimize manufacturing processes.

Methods: Using the CliniMACS Prodigy instrument, we performed experiments varying the supplementation of hAB serum during the manufacturing process of CAR T cells and compared cellular composition as well as growth kinetics. Additionally, different flow-based assays were used to address fitness and functionality of DPs in fresh formulation, even upon storage. Furthermore, we used in-process controls (IPCs) during manufacturing and samples from final DPs to characterize cellular fitness on the transcriptional level by single-cell sequencing.

Results and Conclusion: We identified hAB serum as a CMP for CAR T cell manufacturing. The duration of hAB medium supplementation during the manufacturing process significantly impacts cell characteristics, especially the shelf-life of fresh DPs. Prolonged supplementation improved cellular fitness for extended storage, even for patient-derived material. In summary, our results demonstrate the impact of CMPs on CAR T cell manufacturing and enable continuous improvement of DP quality.

P099 / No. 204

Topic: AS10 Other

AUTOLOGOUS ANTI-GD2 CAR-T CELLS EFFICIENTLY TARGET PRIMARY HUMAN GLIOBLASTOMA

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LB: Academic Abstract Body: Glioblastoma multiforme (GBM) is a deadly tumour showing negligible response both to chemo- and radiotherapy. CAR-T cells targeting specific brain tumour antigens represent a promising therapeutic strategy for this challenging malignancy. Disialoganglioside GD2 is a known antigen associated to neuroectoderm-derived cancers, with limited expression on normal cells. Notably, we have recently shown a high level of expression of GD2 in GBM patient-derived cells and demonstrated a strong antitumour activity of anti-GD2 CAR-T cells in both *in vitro* and *in vivo* allogeneic settings, although associated with allogeneic background (Prapa M. NPJ Precision Oncology 2021). CAR-T cell-based technology has been mainly conceived as an autologous approach; however, standard treatments and corticosteroids are known to impair the functionality of lymphocytes, potentially compromising the manufacturing of autologous CAR-T cells. Thus, to further challenge our approach, we generated pre-clinical *in vitro* models of autologous anti-GD2 CAR-T cells against 2D and 3D spheroid cultures of GBM primary cells. We isolated T lymphocytes from frozen PBMCs of three patients and we retrovirally transduced them to express a second-generation GD2 CAR. We then tested the killing effect of anti-GD2 CAR-T cells against the matched *in vitro* 2D and 3D spheroid assays. Autologous anti-GD2 CAR-T cells showed a remarkable anticancer effect with clusters of activated cytotoxic lymphocytes, while autologous GFP T cells did not significantly affect tumour viability, confirming that the CAR introduction drives a specific anti-glioblastoma effect in this setting. Collectively, these data confirm this approach as a potential novel therapeutic option for this incurable brain cancer.

P100 / No. 154

Topic: AS10 Other

ENHANCING EFFECTOR FUNCTION OF TUMOUR INFILTRATING T CELLS IN KIDNEY CANCER

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Clear-cell Renal cell carcinoma (ccRCC) is known as a hot tumour which means that it is heavily infiltrated by immune cells, which includes a high frequency of T cells. However, cancer cells have mechanisms to avoid T cell detection and killing. Over the decades, several promising immunotherapies have been developed such as immune checkpoint inhibitors (ICI), but only a minority of patients with metastatic RCC have a positive response to the drugs and in some cases the treatment with these checkpoint inhibitors even leads to the progression of the tumour. The aim of this project is to define defects of tumour-infiltrating helper and cytotoxic T cells in renal cancer patients and test whether genetic engineering can restore T cell effector function. Isolated lymphocytes from tumour kidney, non-tumour kidney, and blood of patients with renal cancer were stained with 21 antibodies and analysed using a Cytex Aurora flow cytometer. In addition, single cell RNA expression and single cell TCR sequencing was performed in CD4 and CD8 T cell subsets. There was enrichment of CD8 T cells in tumour compared to adjacent normal kidney tissue. Tumour infiltrating T cells had substantial TCR clonal expansion in both CD4 and CD8 T cells. Distinct RNA expression profiles were found in individual cells of expanded CD8 T cell clones, indicating phenotypic heterogeneity of the members of a T cell

clone. The data suggest antigen-driven expansion of CD4 and CD8 T cells in patients with RCC, which may impact on disease prognosis and response to ICI.

P101 / No. 85

Topic: AS10 Other

DISSECTING PHENOTYPICAL AND FUNCTIONAL DIFFERENCES BETWEEN REGULATORY T CELLS FROM ADULT PERIPHERAL BLOOD, UMBILICAL CORD BLOOD AND PEDIATRIC THYMUS

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FOXP3+ Regulatory T cells (Tregs) represent 4-7% of the CD4+ T-cell population and significantly contribute towards tolerance induction and immune homeostasis. Due to their immunosuppressive properties, Tregs possess a great potential to prevent autoimmune diseases, transplant rejection and graft-versus-host disease (GvHD). However, the first completed Treg clinical trials have shown varying *in vivo* persistence and clinical efficacy, presumably linked to differences in the sources of Tregs, manufacturing processes and phenotype of the final infused product. The aim of this study is the in-depth flow-cytometric characterization of the FOXP3+ Treg compartment in adult peripheral blood, umbilical cord blood and pediatric thymus, routinely discarded during pediatric heart surgery. A comparative 37 Treg marker analysis including recently published Treg lineage and subtype markers was performed between the three cell sources, evaluating Treg phenotype, activation status and subset distribution. In addition, we investigated phenotype stability, exhaustion and function after *ex-vivo* expansion, in inflammatory conditions and after cryopreservation in liquid nitrogen. The functional stability of the Tregs was also correlated to the methylation status of the Treg-specific demethylated region (TSDR) in FOXP3. We concluded that all three Treg sources are suitable for therapeutic applications. However, our findings argue for a careful consideration of the starting material based on indications, availability of HLA matched donors, planned genetic modifications and intended dosage. Moreover, the recently published exceptional expansion capacity and proposed low immunogenicity of thymus Treg may offer the possibility to develop an “off-the-shelf” allogeneic Treg cell therapy.

P102 / No. 87

Topic: AS10 Other

SINGLE-CELL CRISPR SCREENS IN PRIMARY HUMAN T CELLS IDENTIFY REGULATORS OF TH2 CELL SKEWING

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CRISPR screens have become the primary discovery engine in modern biology. At Myllia, we combine CRISPR screening with single-cell RNA sequencing in primary T cells, leveraging two transformative technologies to enable genetic screening for complex phenotypes. We utilize pooled CRISPR screens to map the impact of thousands of genetic perturbations at single-cell resolution which allows to identify novel drug targets or to elucidate unknown mechanisms of actions of drugs. Primary human T cells are key players in autoimmunity and other inflammatory diseases, but also represent attractive targets for immunotherapy of cancer. To enable the discovery of novel targets, we built a workflow that utilizes CD4⁺ T cells from peripheral blood and allows functional genomic screens. Upon activation, naïve CD4⁺ T cells proliferate and differentiate into specific Th1, Th2, or Th17 cell subsets. Here, we present data of a pooled CRISPR screen for regulators of T cell fates in which we aimed to identify genes whose knockout boosts or attenuates the ability of primary naïve CD4⁺ T cells to become Th2 cells. In our screen, the different T cell subsets could be captured using curated transcriptomic signatures. Importantly, several gene KOs introduced in a pooled fashion using CRISPR/Cas9 accumulated in distinct subpopulations, suggesting that these genes regulate the differentiation of naïve T cells into the various T helper cell subsets. Overall, our pooled CRISPR screening platform enables to decipher primary T cell plasticity and identifies genes that could serve as drug targets in autoimmunity, inflammation and immuno-oncology.

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Topic: AS10 Other

COMBINATION OF CD4+ AND CD8+ T CELLS IN T CELL THERAPY OF HBV INFECTION ENHANCES VIRUS CONTROL IN VIVO BY INCREASING IFN- γ AND TNF- α SECRETION

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T cell therapy represents a promising therapeutic approach to treat chronic hepatitis B virus (HBV) infection and HBV-associated hepatocellular carcinoma. To date, most T cell therapies have focused on CD8⁺ T cells as primary effector cells. We here investigate whether redirected CD4⁺ T cells support CD8⁺ TCR-T cells. Both CD8⁺ and CD4⁺ T cells engrafted with HBV-specific MHC I-restricted and MHC II-restricted TCRs, respectively, secreted IFN- γ and TNF- α upon encounter with target antigen and killed HBV-positive target cells in cell culture. Furthermore, the transfer of MHC I-restricted TCRs into a humanized mouse model infected with HBV caused a significant decline of virological markers with limited injury of the liver. We subsequently investigated the therapeutic potential of MHC II-restricted HBV core-specific CD4⁺ T cells and MHC I-restricted HBV surface antigen-specific CD8⁺ T cells alone and in combination in a mouse model of chronic HBV infection. While all groups were able to control HBV infection by lowering HBeAg and HBsAg, in the combination group, virological markers declined more pronounced by 2 to 3 logs. Better clearing of HBV antigens was accompanied by increased IFN- γ

and TNF- α secretion in CD4⁺ and CD8⁺ T cells from the liver and spleen. Despite higher cytokine levels, the liver damage was limited with alanine aminotransferase levels peaking between days 15 and 22 and normalizing again thereafter. Based on these data, we hypothesize a positive helper effect of CD4⁺ T cells by stimulating CD8⁺ cells and endogenous cytokine secretion.

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Topic: AS10 Other

IDENTIFICATION OF 13 T-CELL RECEPTORS FROM SARS-COV-2-SPECIFIC CD8+ T-CELL CLONES

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SARS-CoV-2 seems to enter the human system without triggering a strong early innate immune response, which would normally lead to a subsequent priming of an adaptive immune response. In order to investigate T-cell mediated cytokine secretion in SARS-CoV-2 infection, we isolated a library of MHC I-restricted T-cell receptors from SARS-CoV-2-specific CD8⁺ T-cell clones. 21 different SARS-CoV-2-specific peptides derived from structural (spike, envelope, membrane, nucleocapsid) and non-structural proteins (nsp3, nsp7, nsp8, ORF3a) were used to stimulate PBMCs of HLA-A2⁺ donors with either resolved SARS-CoV-2 infection or vaccinated twice against SARS-CoV-2. Following two weeks of in vitro expansion, the cells were restimulated with peptide-pulsed T2 cells. Positively stimulated CD8⁺ cells identified by IFN γ -secretion were single-cell sorted by flow cytometry and clonally expanded. Subsequently, the T-cell receptor sequences of 13 SARS-CoV-2-specific T-cell clones were identified, codon-optimized, cloned into a retroviral vector and transduced into T-cells from healthy donors for further characterization. The identified T-cell receptors are specific for four different SARS-CoV-2 epitopes from nsp8, ORF3a and spike protein. In order to show the recognition of naturally processed peptides, human cell lines HepG2 and A549 stably expressing the respective SARS-CoV-2 proteins were generated using the PiggyBac transposon system. In addition, TCR-transduced T-cells are co-cultivated with SARS-CoV-2 infected cells to evaluate T cell killing and the antiviral effect of T cell cytokines.

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Topic: AS10 Other

MASS SPECTROMETRY-BASED IMMUNOPEPTIDOMICS APPROACH REVEALS IMMUNOGENICITY OF A POTENTIAL NEOANTIGEN IN THE MULTIPLE MYELOMA CELL LINE JJN3

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Multiple myeloma (MM) is an incurable plasma cell neoplasm that accounts for ten percent of all hematological malignancies. The approval of the first chimeric antigen receptor (CAR) T cell therapy for relapsed or refractory MM in 2021 as well as the emerging platform of bispecific antibodies demonstrate the potential effectiveness of immunotherapeutic approaches for the treatment of MM patients. Approaches using T cell receptor (TCR)-transgenic T cells enable targeting of tumor cells that present mutated peptides (neoantigens) from intracellular proteins via the major histocompatibility complex (MHC). To identify these tumor-specific neoantigens and neoantigen-specific TCRs in MM, we examined the immunopeptidome of MM cell lines using a mass spectrometry (MS)-based im-

munopeptidomics approach. This approach is based on the immunoprecipitation of MHC-restricted peptides followed by MS analysis. Mutated peptides were then identified with pFind, MSFragger, and MaxQuant, using a personalized database derived from whole exome and RNA sequencing data. After the successful establishment and optimization of the immunoprecipitation, we were able to identify four potential neoantigens in the cell line JJN3 that were derived from two DNA and two RNA variants. Immunogenicity testing revealed reactivity of HLA-matched allogeneic T cells to one of the DNA-derived neoantigen candidates. We thereby demonstrated the feasibility of identifying potential neoantigens in a MM cell line with our MS-based immunopeptidomics approach. As a next aim, we will analyze the immunopeptidome of primary MM tumor tissue aiming towards a better understanding of druggable immunogenic mutations in MM.