American Society of Gene + Cell Therapy

Spotlight on Immuno-Oncology Abstracts

ORAL ABSTRACTS

The Program Committee has chosen 6 top-notch abstracts to represent each category

Category: B Cell Malignancies & Beyond
When: Tuesday, August 1 | 9:50 AM - 10:05 AM
Title: High Safety and Efficacy of CRISPR-Based Non-Viral PD-1 Locus Specific Integrated Anti-CD19 CAR-T Cells (BRL-201) in Treating Relapsed or Refractory Non-Hodgkin's Lymphoma: First-in-Human Phase I Study

Category: Novel CAR Designs and Approaches
When: Tuesday, August 1 | 11:55 AM - 12:10 PM
Title: Microbial Metabolites Guide the Engineering and Epigenetic-Metabolic Reprogramming of CAR T Cells for the Treatment of Solid Tumors

Category: TCR-Based Cancer Immunotherapy
When: Tuesday, August 1 | 2:40 PM - 2:55 PM
Title: Identification of Peptides Derived from Alternative Splicing for TCR-T Cell Therapies and Cancer Vaccines

Category: Direct In Vivo Delivery
When: Tuesday, August 1 | 4:45 PM - 5:00 PM
Title: Targeted in Vivo Generation of CAR T and NK Cells Utilizing an Engineered Lentiviral Vector Platform

Category: Genome & Epigenome Editing for Cancer Immunotherapy
When: Wednesday, August 2 | 9:40 AM - 9:55 AM
Title: CD47 Enhances CAR T Survival in NSG Recipients and Allogeneic Mice

Category: Beyond T-Cells
When: Wednesday, August 2 | 11:45 AM - 12:00 PM
Title: CD70 Knockout Provides Increased NK Cell Proliferation In Vivo

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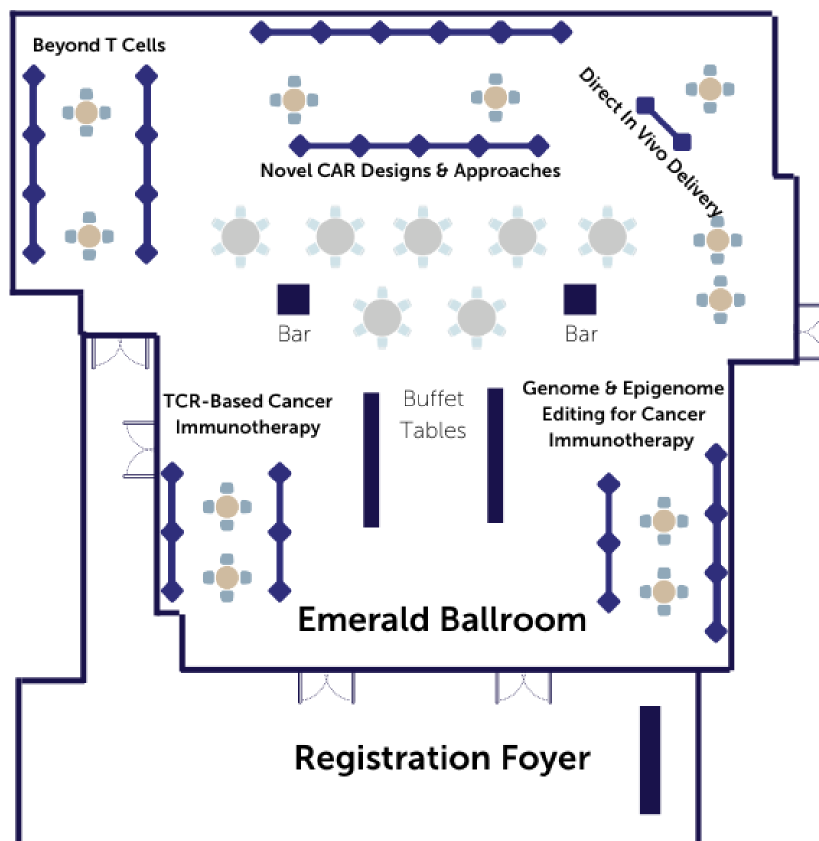
Spotlight on Immuno-Oncology Abstracts

POSTER SESSION

Posters will be set up by category in the Emerald Ballroom throughout the conference

Join us for a special poster viewing reception detailed below

Details: Tuesday, August 1 | 5:00 PM - 6:30 PM
Emerald Ballroom



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ORAL ABSTRACTS

1. High Safety and Efficacy of CRISPR-Based non-viral PD-1 Locus Specific Integrated Anti-CD19 CAR-T Cells (BRL-201) in Treating Relapsed or Refractory non-Hodgkin's Lymphoma: First-in-Human Phase I Study

Biao Zheng, MD[1], Yongxian Hu[2], Jiqin Zhang[1], Mingming Zhang[2], Wei Li[1], Wenjun Wu[2], Jiazhen Cui[2], Guoqing Wei[2], Bing Du[1], Mingyao Liu[1], He Huang[2]
[1]BRL Medicine Inc., [2]The First Affiliated Hospital, Zhejiang

BACKGROUND :
CAR-T cell products are manufactured traditionally by using virus, which leads to the potential tumorigenesis risk and makes CAR-T cell therapy expensive and time-consuming. We developed a novel approach to generate non-viral, genome-specific integrated CAR-T cells through CRISPR/Cas9, thereby breaking through both virus usage and random integration simultaneously. Here, we update the newest follow up data of of non-viral PD1 locus specifically integrated anti-CD19 CAR-T cells (BRL-201) in patients with relapsed/refractory (r/r) Non-Hodgkin's lymphoma (NCT04213469).
***METHODS*:**
This phase I trial evaluates BRL-201 in adult patients with r/r B-NHL. Adult patients with r/r B-NHL underwent leukapheresis and a lymphodepletion chemotherapy with cyclophosphamide (500mg/m^2, D -3 to -2) and fludarabine (30mg/m^2, D -4 to -2) before BRL-201 infusion. Dose escalation are based on 3+3 escalation rule, including three cohorts: 2×10^6/kg, 4×10^6/kg, 6×10^6/kg. Besides, 3 subjects received non-standard infusion doses at 0.56~0.8 × 10^6/kg. The primary endpoint was the incidence of dose-limiting toxicities (DLT). The secondary endpoint was the proportion of patients achieving an objective response at 3 months as per investigator's assessment.
RESULTS:
Between May 3, 2020 and August 10, 2021, 25 patients with r/r B-NHL were enrolled and 21 received BRL-201 with a median age of 56 years (34-70) and a median of 4 (1-9) prior lines of therapy. Among all the treated patients, 17 patients (93.8%) were diagnosed with disease stage III or IV, and 13 patients (81.3%) were assessed with high-intermediate to high risk according to IPI or aaIPI score assessment. Two patients had undergone autologous hematopoietic stem cell transplantation (HSCT) and one patient had a history of primary refractory disease. Of 17 patients with pretreatment tumor samples PD-L1 expression detection, 4 (23.5%) had > 50% PD-L1 expression, 13 (76.5%) had ≤50% PD-L1 expression.
As of May 17, 2023, the median follow- up was 29.0 months (21.5-36.2 m). All of 21 (100%) patients had an objective response to BRL-201 and 18 (85.7%) patients had a complete response (CR) as best response (Fig. 1). 7 patients achieved and maintained CR at data cut-off date, among these, 3 patients had PD-L1 expression > 50%, 1 patient was negative and 3 unknown. The median duration of response (DOR) for all 21 patients was 15.1 months (95%CI：5.9, NA) (Fig. 2a). The median progression free survival (PFS) was 20.8 (95%CI：8.2, NA) months (Fig. 2b), while the estimated median overall survival (OS) was not reached, 12-month OS rate was 76.2% (95% CI: 60%, 96.8%) (Fig. 2c). No grade 3-4 CRS and ICANS were observed. 14 patients (66.7%) experienced grade 1-2 cytokine release syndrome (CRS) and only one patient received tocilizumab. 4 patients (19.0%) experienced grade 1-2 immune effector cell-associated neurotoxicity syndrome (ICANS). No new AEs/SAEs were observed during the last follow up.
***CONCLUSIONS*:**
This is a first-in-human study of a novel type of non-viral genome specific targeted CAR-T cells in treating r/r B-NHL. As a two-in-one approach without using virus, the manufacturing procedure is simplified, with shortened preparation time, reduced production expenses. Low incidence and mild grade of CRS/ICANS preliminarily showed the high safety of BRL-201. The efficacy of BRL-201 in r/r B-NHL subjects was more advantageous than the marketed products, with a median PFS of 20.8 months and 12-month OS rate of 76.2%.

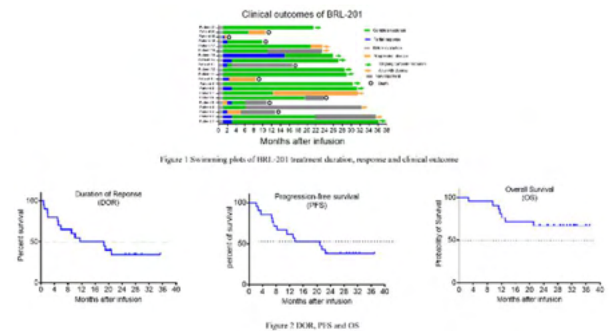


Figure 1 Swimming plots of BRL-201 treatment duration, response and clinical outcome

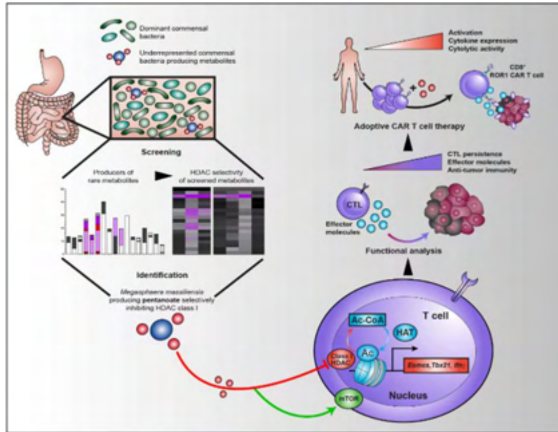
Figure 2 DOR, PFS and OS

2. Microbial Metabolites Guide the Engineering and Epigenetic-Metabolic Reprogramming of CAR T Cells for the Treatment of Solid Tumors

Sarah Staudt, M Sc[1], Kai Ziegler-Martin, M Sc[1], Nazdar Adil-Gholam, B Sc[1], Imke Mulder[2], Hermann Einsele[1], Alexander Visekruna[3], Michael Hudecek, MD[1], Maik Luu, PhD[1]
[1]University Hospital Würzburg, [2]4D Pharma PLC, [3]Philipps-University Marburg

Background:
The intestinal microbiota has been identified as a crucial factor in influencing the efficacy of cancer immunotherapy. Gut microbes, such as *Akkermansia muciniphila*, are able to improve immune checkpoint inhibition, thereby enhancing immune cell-mediated anti-tumor responses. Notably, the impact of soluble microbial molecules and metabolites such as short-chain fatty acids (SCFAs) on the outcome of cellular cancer immunotherapy remains poorly understood. Identifying their molecular modes of action can inspire the development of next-generation T cell therapies.
Methods:
In this study, we compared 14 abundant bacterial species representing proportionally the most common phyla in the human intestine and performed GC-MS analysis to assess their SCFAs production profile. Intracellular histone modification and phospho-protein stainings in combination with metabolic flux assays were used to analyse the phenotype of T cells which were differentiated in vitro in presence of SCFAs and other epigenetic or metabolic modifiers. The functional characterization of pharmacologically or genetically reprogrammed antigen-specific T cells and engineered chimeric antigen-receptor (CAR) T cells was performed by cytotoxicity assays and adoptive transfer experiments in syngeneic solid tumor models.
Results:
Here, we identified the SCFA pentanoate as a metabolite produced by the low-abundant commensal *Megasphaera massiliensis* and analysed its effect on CD4$^+$ and CD8$^+$ cytotoxic T
lymphocyte (CTL)-mediated anti-tumor immunity. We could show that the enhanced cytotoxic capacity of CTLs was achieved by SCFA-mediated inhibition of class I histone deacetylases (HDACs) promoting production of effector molecules such as perforin, granzyme B, TNF-a and IFN-g. Pentanoate treatment led to histone hyperacetylation at activating chromatin loci and the promoter regions of master transcription factors in T cells highlighting that epigenetic modifications are involved in inducing the CTL-associated phenotype.
Importantly, while the SCFA treatment of antigen-specific CTLs and CD8$^+$ chimeric-antigen receptor (CAR) T cells augmented their capacity to reduce tumor progression in syngeneic solid tumor models, modulation with conventional HDAC class I inhibitors failed to mimic SCFA administration to the same effect. We identified that pentanoate additionally induced the metabolic reprogramming of T cells towards elevated mitochondrial activity. By pharmacologic enhancement of oxidative phosphorylation in HDAC class I-deficient CAR T cells, we

demonstrated a synergistic link between epigenetic and metabolic reprogramming as underlying mechanism of SCFA-mediated improvement of CAR T cell function.
Conclusion:
Collectively, our work demonstrates that SCFAs are unique commensal-derived modifiers of epigenetic and metabolic pathways with therapeutic potential in the context of adoptive cancer immunotherapy. Our data provide new insights into the microbiome-CAR T cell interface and the molecular mechanisms that we can learn from to improve the efficacy of engineered CAR T cell products.



3. Identification of Peptides Derived from Alternative Splicing for TCR-T Cell Therapies and Cancer Vaccines

Elise Wilcox, PhD, Alex Chen, Chenwei Lin, PhD, Lisa Jones, Philip Gafken, PhD, Roland Strong, PhD, Stanley Lee, PhD, Aude Chapuis, MD
Fred Hutch Cancer Center

T cells genetically modified to express a high affinity T cell receptor (TCR) have considerable potential for the treatment of cancer, however identifying immunogenic, tumor-specific targets remains a challenge. We have developed a pipeline to find cancer-associated peptides presented across HLA types. We applied our technology to the discovery of neoepitopes from alternative splicing events that are prevalent across multiple high-risk myeloid neoplasms. We then used computational analysis to select promising mutant-derived peptides and tested them for their ability to induce tumor-specific killing. This work aims to create a robust method to discover novel HLA/peptide pairs and extend the reach of immunotherapies.
Recent transcriptomic analysis has shown that splicing mutations are frequently found across many different cancer types and can be drivers of tumorigenesis. We focused on mutations in SRSF2 (Serine and arginine Rich Splicing Factor 2), a protein involved in pre-mRNA splicing, which are prevalent across multiple types of high-risk myeloid neoplasms. Heterozygous mutations in SRSF2 (e.g. P95H) are found in 20-30% of patients with myelodysplastic syndromes (MDS) and ~50% of chronic myelomonocytic leukemia patients. Experiments with cell lines modified to express a SFSR2-P95H/+ mutation showed mis-regulation of hundreds of splicing events potentially exposing a large pool of unique, tumor-associated peptides that could be used to develop effective treatments.
To identify presented peptides, HLA-specific single chain dimers (SCDs), which have the transmembrane domain replaced with a His-Tag allowing for secretion of the peptide/HLA in the supernatant, are transduced into wild type and mutant cells lines for downstream mass-spectrometry analysis. We used this versatile platform, ARTEMIS, to discover HLA-specific peptides resulting from alternative splicing events induced by a mutation in SRSF2 in three different HLAs (HLA-A2, A11, and A24). These HLAs have unique binding preferences and together cover 71% of phenotype of the US. With SCDs, each individual HLA can be interrogated separately without the complex result deconvolution required by traditional immunoprecipitation methods. Extensive validation studies have shown agreement between ARTEMIS, traditional IP/MS approaches, and NetMHCpan predictions.
Using ARTEMIS, we identified thousands of peptides exclusive to mutant P95H/+ K562s cells for HLA-A11 and A24. Next, computational analysis of RNA sequencing data from wild type and mutant cells narrowed the list of peptides to those which resulted from alternate splicing events, against which T cell lines can be developed. To validate the identified peptides, we screened for T cell lines against these shared peptide/HLA pairs and assessed their ability to recognize and kill cell lines carrying the SRSF2 mutation. These techniques can easily be adapted for other cell lines and mutations associated with RNA or protein processing including SF3B1, U2AF1 and DDX41. This work demonstrates a robust method to discover novel HLA/peptide pairs and extend the reach of engineered TCR-T cell therapies.

4. Targeted *In Vivo* Generation of CAR T and NK Cells Utilizing an Engineered Lentiviral Vector Platform

James Andorko, PhD, Ronnie Russell, Bruce Schnepp, Ramesh Singh, Debasish Boral, Thomas O'Malley, Leticia Kuri-Cervantes, Muneeswara Medi, Philip Johnson, MD
Interius BioTherapeutics

CAR T cell therapies have revolutionized the treatment of B cell malignancies. *Ex vivo* CAR manufacturing is complex, costly, and cumbersome, prompting efforts that support shorter manufacturing and allogeneic or "universal donor" strategies. Recent data also show that CAR T product efficacy is correlated with reduced *ex vivo* manipulation of cells. Here, we have designed a system to transduce effector cells *inside* the body to generate autologous CAR cells, circumventing *ex vivo* cell manipulation, avoiding patient conditioning chemotherapy, and providing an "off-the-shelf" therapy for B cell malignancies.
Our product (INT2104) is an intravenously administered lentiviral vector encoding an anti-CD20 CAR transgene (Figure 1). INT2104 was rationally designed with an engineered fusogen and a novel binder to provide targeted transduction of $CD7^+$ T and NK cells. The CAR20 molecule encoded by INT2104 is constructed of fully human domains, including an anti-CD20 scFv which is cross-reactive to both human and NHP B cells, enabling studies in both humanized mouse and NHP models.
Incubating INT2104 with activated primary human PBMCs *in vitro* confirmed that T cells, including $CD4^+$ and $CD8^+$ subsets, and NK cells were specifically transduced. No B cell transduction was seen across a wide range of MOIs when INT2104 was exposed to B cell tumor lines and primary PBMCs isolated from patients with B cell malignancies. INT2104-treated PBMCs were cocultured with B cell tumor targets and resulted in dose-dependent killing, confirming CAR functionality.
In vivo evaluation of INT2104 has been conducted using humanized mouse and NHP models (Figure 2). Delivery of INT2104 to CD34-engrafted NSG mice via tail vein injection resulted in B cell depletion within 7 days, with CAR^+ cells detectable in blood coincident with B cell ablation. INT2104 administration to mice bearing an established B cell tumor also resulted in B cell aplasia, with complete tumor ablation seen in all treated mice across a 15-fold range in dosing, including a dose matching the proposed FIH dose in TU/kg.
Over 20 cynomolgus macaques have received intravenous vector infusions, most at the highest anticipated human dose. 15 of 16 NHPs receiving a human dose of CAR vector experienced at least a 75% decline in absolute numbers of circulating B cells in the weeks following vector infusion. As expected, immune responses (mediated by T and B cells) to non-macaque sequences in the CAR are associated with eventual B cell rebound in most NHPs although three animals had B cell

depletion beyond 30 days. Biodistribution, immunogenicity, along with safety and treatment tolerability has been assessed in these experiments. No vector toxicity has been observed.
Taken together, these data suggest that intravenous delivery of INT2104 will be both safe and effective, supporting plans to continue development and transition into the clinic.

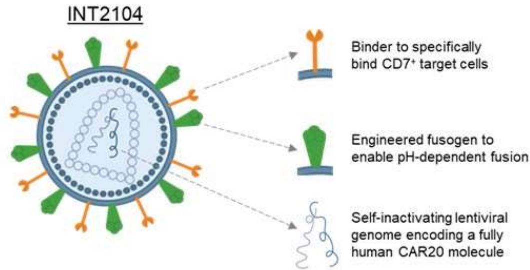


Figure 1. Visual of Product INT2104.

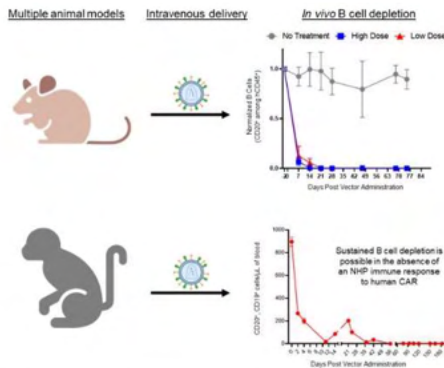


Figure 2. *In vivo* delivery of IN2104 results in CAR$^+$ cell generation and B cell depletion with no toxicity.

5. CD47 Enhances CAR T Survival in NSG Recipients and Allogeneic Mice

Xiaomeng Hu[1], Karl Manner[1], Kathy White[1], Corie Gattis[1], Priscilla Ngo[1], Elaine Chu[1], Chi Young[1], Frank Wells[1], Ronald Basco[1], Annabelle Friera[1], Divy Kangeyan[1], Pascal Beauchesne[1], Tobias Deuse[2], Terry Fry[1], Aaron Foster[1], Sonja Schrepfer[1]
[1]Sana Biotechnology, [2]UCSF

Off-the-shelf CAR T cells may offer advantages over autologous strategies, including ease of manufacturing, improved quality control, and the ability to avoid malignant contamination and T cell dysfunction due to generating a final product from healthy T cells. While TCR editing can effectively prevent graft-versus-host reactions, the significant host-versus-graft immune response against histoincompatible T cells prevents the expansion and persistence of allogeneic CAR T cells and mitigates the efficacy of this approach. A major challenge is that, while HLA deletion can result in adaptive immune evasion, innate reactivity is enhanced. CD47 overexpression can block both NK cell and macrophage killing (J Exp Med (2021) 218 (3): e20200839). While T cells express CD47, we demonstrate here that CD47 expression above endogenous levels is important for immune evasion. We describe here the engineering of human immune evasive CAR T cells building on our previously described hypoimmune technology (Nat Biotechnol 2019;37(3):252-258 and Proc Natl Acad Sci U S A 2021;118(28):e2022091118). The goal is to achieve improved rates of durable complete remissions by improving allogeneic CAR T cell persistence, since it has been shown that autologous CAR T cells have greater durability over years than allogeneic CAR T cells (N Engl J Med. 2021;384(7):673-674).
Human T cells from healthy donors were obtained by leukapheresis. To generate hypoimmune (HIP) CD19 CAR T cells, gene editing was used to eliminate HLA-I/II and TCR expression and lentiviral transduction was used to express CD47 and CD19 CAR containing a 4-1BB costimulatory domain. Control CD19 CAR T cells were unmanipulated, i.e., unedited, except for lentiviral transduction used to express CD19 CAR. For 3 month persistence studies, allogeneic SGM3 humanized mice were injected with 1´10^6 Luc$^+$ Nalm6 cells and received 7´10^6 unmodified CD19 CAR T cells or HIP CD19 CAR T cells. In the mice treated with either unmodified CD19 CAR T cells and HIP CD19 CAR T cells, tumor control was initially rapidly achieved. However, unmodified CD19 CAR T cells were eventually rejected by the host and the loss of these cells resulted in re-growth of tumor. By contrast, in HIP CD19 CAR T injected mice, tumor control was maintained throughout the study, including following a rechallenge at day 83 with Nalm6 cells without further administration of HIP CD19 CAR T cell. Flow cytometry at endpoint from bone marrow and spleen confirmed persistence and enrichment of HIP CD19 CAR T cells.
HIP CD19 CAR T cleared Nalm6 leukemic cells in NSG mice across a range of tumor cell:CAR T cell ratios and tumor re-challenge studies highlighted that CD47 overexpression seems to be critical for CAR T survival in vivo.
Thus, hypoimmune engineering seem to provide universal CAR T cells for the treatment of cancer and autoimmune patients with the potential to persist without immunosuppression and CD47 overexpression might be a “sine qua non” for successful adoptive T cell therapies.

6. CD70 Knockout Provides Increased NK Cell Proliferation *In Vivo*

Joshua Krueger, Walker Lahr, Erin Stelljes, Nicholas Slipek, Jae Woong Chang, PhD, Ethan Niemeyer, Joseph Skeate, PhD, Beau Webber, PhD, Branden Moriarity, PhD
University of Minnesota

Immunotherapy with chimeric antigen receptor (CAR) expressing NK cells engineered via viral vectors has shown exceptional efficacy against hematologic cancers in clinical trials. This method, however, is limited in cargo capacity, carries the risk of insertional mutagenesis, and large-scale manufacturing for clinical use is cost-prohibitive. Even with optimal engineering, CAR NK cells lack robust in vivo expansion requisite for tumor control. Furthermore, as research into the tumor microenvironment continues to reveal an ever growing and complex interactome between effector and target cells, there is a need for more advanced approaches that effectively implement simultaneous CAR knockin and targeted gene knockout of immune checkpoints or other cellular targets.
Notably, NK cells have increased survivability in the presence of IL15, but in unpublished data we have also observed that CD70 knockout, initially done for reducing fratricide while generating CD70 CAR NK, also causes increased expansion in vivo. To this end, we engineered an anti-CD19 CAR NK armored with soluble IL15 via DNA transposition using a hyperactive TcBuster transposase and concurrently knocking out CD70 using Cas9 in one electroporation. This was accomplished by delivering a construct in a nanoplasmid-TcBuster containing a second generation CD19-targeting CAR, selection epitope (RQR8), and soluble IL15 (CD19-RQR8-IL15) (3.4 kB) along with a Cas9 mRNA and sgRNA targeting CD70 (Figure 1a). Post RQR8 bead selection, we achieved >95% CAR+ cells (Figure 1b) which also maintained >75% CD70 KO (Figure 1c).
These armored CAR NKs with CD70 KO had superior tumor clearance over armored CAR NKs without CD70 KO in vivo (Figure 2a/b). All mice received a single dose of 5E6 cells of their designated therapy. Additionally, a large-scale expansion of the armored CAR NK with CD70 KO was noted as compared to all non-armored therapies (Figure

2c). This enhanced expansion also came with increased levels of serum IL15 (Figure 2d). The mice which exhibited robust expansion of NK cells showed signs of systemic toxicity. We hypothesized that this was due to toxic levels of IL15 or overcrowding of NK cells in the circulating blood. Future directions aim to precisely control the IL15 levels in order to maintain therapeutic NK counts without adverse effects.

Figure 1:

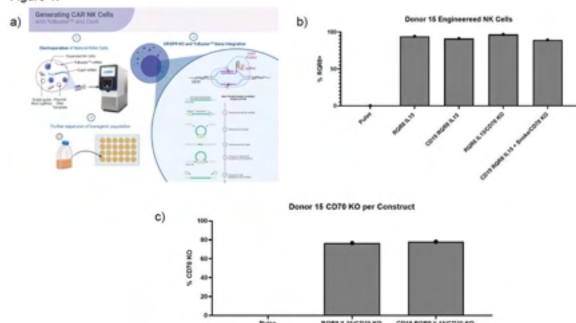
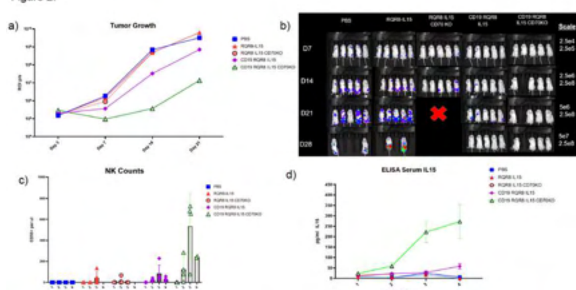


Figure 2:



POSTER SESSION

TCR-BASED CANCER IMMUNOTHERAPY

7. A Novel Approach for Neoantigen Discovery from Splicing Factor Mutated Cancers

Teryn Mitchell, Abdullah Ali, Siddhartha Mukherjee, Ai Yamamoto
Columbia University

Mutations in splicing factor (SF) genes can cause alternative splicing/mis-splicing of pre-mRNA, leading to aberrant transcripts. These alternative/mis-splicing events include: a. constitutive splicing, b. mutually exclusive exons, c. cassette alternative exons, d. alternative 3' splice site, e. alternative 5' splice site, and f. intron retention. Recurrent SF mutations are frequently found in splicing factor genes including SF3B1, SRSF2, U2AF1, and ZRSR2. These mutations are more common in hematologic malignancies compared to solid cancers. Mutations in the SF3B1 gene happen to be found in up to 80% of patients with a subtype of Myelodysplastic Syndromes (MDS) characterized by ring sideroblasts. Other cancers with recurrent mutations in SF genes include uveal melanoma (15-29% in SF3B1), CLL (6-26% in SF3B1), CMML (28-47% in SRSF2; 8-17% in U2AF1), lung cancer (3% in U2AF1), and breast cancer (1-4% in SF3B1). As exemplified in our prior work, disease-causing mutations in SF3B1 (the most common mutation) alter splicing by disrupting interaction with a protein called SUGP1. Essentially, mutations in the SF3B1 splicing factor gene are the most common/important in MDS, leading to cryptic 3' ss and upstream BP, which leads to an RNA gain of function, ~200-1000 mis-spliced transcripts, and intron retention of ~18-20 extra nucleotides. From here, the intron retention results in 2 aberrant transcript events, which are 1. in-frame transcripts (e.g. those without a stop codon) that are translated/some proteins are degraded. In the second event, out-of-frame transcripts are degraded via NMD, but a fraction are translated. Somatic mutations in splicing factor genes (e.g. SF3B1 K700E) from these two events result in degraded proteins that may be presented as neoepitopes by the MHC Class I antigen presentation complex to T-cells. From here, one might imagine it possible to establish an immunotherapy target by isolating and identifying potential neoepitopes presented by the HLA complex in SF3B1-mutated AML cells. Next, one could isolate, engineer, and validate a TCR therapy. Therefore, we designed a novel approach in which we purified HLA-presented peptides using a tandem affinity purification method from cells engineered to express the mutant or wildtype SF protein. To identify the peptide sequence, we ultimately searched the mass spectra against a custom de novo assembled transcriptome generated from the RNAseq dataset obtained from the same cells expressing mutant or wildtype SF protein. Ultimately, we found a total of 1596 peptides with 615 unique to the mutant, which may prove to be novel immunotherapeutic targets for AML.

8. Mechanistic Study and Fine Tuning of CD8ab-CD28 Chimeric Co-Receptor (CCR) for TCR-T Cell Function Enhancement

Tzu-Hao Tang, BS, Shihong Zhang, PhD, Yapeng Su, PhD, Philip Greenberg, MD, Thomas Schmitt, PhD, Aude Chapuis, MD
Fred Hutchinson Cancer Center

Costimulation is critical for T cell function and persistence, especially for adoptive cell therapies (ACT) for cancer, as the T cells need to function in the immunosuppressive tumor microenvironment. Our previous study has shown that compared to CD8ab without additional costimulation signaling domains, a chimeric co-receptor (CCR) consisted of the extracellular domain of CD8ab and intracellular domain of CD28 (CD8ab-CD28) can enhance CD4+ TCR-T cell cytokine production, proliferation, and ability to efficiently kill tumor cells both in vitro and in vivo. CD28 as a chimeric costimulatory signaling domain has been extensively studied and well understood in CAR T cells, but the mechanism of action of CD28 costimulation in the CCR TCR-T cells is unknown. This mechanistic study aims at optimizing the function of the CCR by altering the functional motifs of CD28 signaling domain, as well as unraveling the signaling cascade induced by the CCR.
We created 16 different combinations of null mutations of functional motifs on the CD28 intracellular domain, including LL, YMNM, PRRP and PYAP. CD4+ T cells were co-transduced with each of the 16 CCRs (CD8ab-CD28 and mutants) together with an HLA-A2 restricted MAGE-A1 specific TCR. Although cognate peptide stimulation and intracellular cytokine staining didn't show clear difference between CCR constructs with different patterns of CD28 mutations, in vitro serial killing assay by repeated challenging of the T cells with tumor cells every 2 to 3 days showed CD8ab-CD28 with all four domains mutated (CD8ab-CD28mut) outperformed other CCR mutants for inducing T cell proliferation and exhibited superior tumor control.
To explore both proximal and downstream CCR signaling pathways, calcium flux was performed to assess immediate TCR signaling activation upon antigen recognition for CD8ab, CD8ab-CD28 and CD8ab-CD28mut transduced CD4+ TCR-T cells. Upon cognate antigen stimulation, no difference in T cell activation dynamic and magnitude was observed, suggesting the CCR does not affect TCR triggering. Signaling pathways of protein phosphorylation downstream of the mutated motifs including pSLP-76, p-VAV, p-LCK, p-PLCy1, and p-ZAP70, as well as CD3 and CD28 downstream adapter protein interactions by immunoprecipitation are currently underway. Bulk RNA sequencing on CD8ab, CD8ab-CD28 and CD8ab-CD28mut transduced

CD4+ TCR-T cells showed clear difference in gene expression upon early (4 hours) and repetitive (13 days) cognate antigen stimulation. Indeed, CD8ab-CD28 and CD8ab-CD28mut had significantly prolonged expression of genes associated with proliferation, survival and effector functions compared to CD8ab. Gene set enrichment analysis (GSEA) revealed different signaling cascades activation patterns with the addition and mutation of CD28 signaling domain compared to the wild type CD8ab co-receptor.
Our study provides a deep dive into the mechanism of CD28 costimulatory signaling in the CCR plus TCR-T cell settings to reveal how functional motif mutations contribute to the signaling pathway. The goal is to identify a chimeric molecule that will further optimize the T cell signaling cascade for a better TCR T cell efficacy.

9. Advancing Adoptive T Cell Therapy Against Cancer Through the Development of Dual Costimulatory Receptors

Ryma Toumi, PhD, Edison Chiu, Aitong Ruan, Sofia Nguyen, Simonne Guenette, Cody Clayhold, Aayushi Soni, Shannon Oda, PhD
Seattle Children's Research Institute

Adoptive cell therapy (ACT) utilizing genetically-modified T cells has revolutionized cancer treatment, enhancing patient immune response against tumors and providing potential long-term protection against tumor recurrence. However, the success of immunotherapies, particularly against solid tumors, has been limited by inhibitory tumor microenvironments (TMEs). Clinically targeting the CD40 can effectively enhance the antitumor function of multiple immune cell types, including in "immune desert/excluded" tumors. In contrast to antibodies that block inhibitory checkpoint receptor signaling and "remove a brake" from the immune system, agonist monoclonal antibodies targeting tumor necrosis family receptor (TNFRs) (e.g. 4-1BB, CD40, OX40) can "push the gas pedal," invoking an anticancer immunotherapeutic response by delivering costimulatory signals. Combined with chemotherapy, agonistic anti-CD40 activation of dendritic cells (DCs) drove T cell-mediated anti-cancer immunity in syngeneic mouse models, but monoclonal antibody toxicities have limited enthusiasm. We developed CD40L Dual Costimulatory Receptors (DCR) that combine a costimulatory TNFR ligand ectodomain (CD40L) with a costimulatory endodomain (e.g. CD40, 4-1BB). Costimulatory signals transmitted through cell surface receptors can initiate gene expression programs that address multiple issues in the TMEs by lowering the threshold of activation, metabolic reprogramming, and reducing exhaustion. We hypothesized that the co-expression of CD40L-based DCR would improve the function and longevity of T cell receptor T (TCR-T) cells, also promoting the recruitment and activation of endogenous immune cells in the TME. With comprehensive *in vitro* testing, we confirmed the successful expression of CD40L DCRs on the surface of TCR-T cells engineered to target tumors. We showed that the CD40L DCR/TCR-T cells killed the tumors more effectively than TCR-only T cells. Of note, CD40 signaling can enhance the antitumor functions of other immune cells, including by licensing DCs to activate antitumor T cells with increased expression of major histocompatibility complex (MHC) molecules and costimulatory ligands, such as CD80. We documented the *in vitro* ability of CD40L-DCRs to induce maturation of DCs generated from the THP-1 monocyte cell line, with increased expression of MHC and CD80 molecules. Our results support the high translational potential of our CD40L DCR in the development of innovative ACT strategies, enhancing the function of immune cells and opening doors to novel therapeutic interventions against a wide range of cancers.

10. CD8αβ-CD28 Chimeric Co-Receptor (CCR) Enhances Anti-Tumor Function of TCR-T Cells

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[1]Fred Hutchinson Cancer Center, [2]University of Washington

Inclusion of functional CD4+ T cells along with CD8+ in adoptive cellular therapies (ACT) products show improved efficacy compared to CD8+ T alone. HLA class I restricted TCRs targeting self-antigens identified from human CD8+ T cells mostly require the CD8 coreceptor stabilization to initiate downstream signal. Therefore, to functionally engage TCR transduced CD4+ T cells, the CD8ab coreceptor co-transduction is needed. In addition to TCR signaling, T cells also require a signal 2 to remain viable and functional in an immunosuppressive tumor microenvironment. We hypothesized that the tumor controlling efficacy of TCR-T cells could be enhanced by not only incorporating functional CD4+ TCR-T cells but also engaging our novel chimeric co-receptors (CCRs) that provide a signal 2 downstream of TCR activation without the additional requirement of a ligand binding for signal transmission.
An HLA-A2 restricted, melanoma-associated antigen-1 (MAGE-A1)-specific, CD8 dependent TCR isolated from an HLA-A2+ healthy donor was used as our model TCR. We first evaluated the role of functional CD4+ TCR-T cells in TCR-T cell treatment and found that the inclusion of functional CD4+ TCR-T cells could enhance CD8+ TCR-T cell proliferation and cytotoxicity *in vitro*. However, unlike *in vitro* data, *in vivo* model treated with low dose TCR-T cells showed only limited improvement by mixed CD4+ and CD8+ T cells compared to CD8+ T cells alone, suggesting further enhancement of TCR-T cell function is plausible.
To incorporate costimulation signals into TCR-T cells, we designed CCRs that tether the extracellular and transmembrane domains of CD8ab and intracellular domains of different costimulatory molecules, including CD28, 41BB, ICOS, OX40 and GITR. These CCRs were co-transduced into CD4+ TCR-T cells to force CCR utilization and evaluated for their potential to enhance TCR-T cell function. Although the functional avidity of CCR+ CD4+ TCR-T cells was only marginally enhanced compared to CD8ab+ CD4+ TCR-T cells, CCR+ CD4+ TCR-T produced significantly more cytokines after one week of HLA-A2+MAGE-A1+ tumor cell exposure. Sustained cytotoxicity and proliferative capacity were further observed in CCR+ CD4+ TCR-T cells in a repeated tumor challenge assay, suggesting the CCR molecules provide prolonged signals for T cell survival and function.
Among the CCR constructs, CD8ab-CD28 CCR transduced TCR-T cells showed the most functional enhancement and were therefore assessed in HLA-A2+MAGE-A1+ cell line derived xenograft mouse models. Treatment of subcutaneously established melanoma tumors by CD8ab+ TCR-T cells slowed tumor growth compared to irrelevant TCR-T cells, CD8ab-CD28 CCR+ TCR-T cells showed rapid and effective tumor control, robustly infiltrated tumors and presented a less exhausted phenotype.
Our study demonstrates functionally enhanced CD4+ TCR-T cells can effectively improve TCR-T cell function both *in vitro* and *in vivo*. We plan to evaluate the mechanism of action of the most effective CD8ab-CD28 CCR by more in-depth transcriptomic and proteomic analyses and explore possibilities to further improve its function. Meanwhile, safety evaluation of the CCR will also be performed. The addition of our novel CCR in TCR-T cell therapies has great clinical potential to improve cancer treatment in the future.

BEYOND T-CELLS

11. Dysregulated Metabolite Secretion Facilitates a Treg-Mediated Suppressive Environment in the Solid Tumor

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Fred Hutchinson Cancer Center

The success of T cell immunotherapies in the solid tumor is impeded by multiple challenges including the secretion of metabolic byproducts from the tumor, which generate a hostile and immunosuppressive tumor microenvironment (TME). Dysregulated tumor metabolism results in the accumulation of metabolites that are exported into the interstitial space in the TME, where they act as signaling molecules promoting an immunosuppressive landscape and inhibiting CD8+ effector T cell function, ultimately reducing anti-tumor responses to therapy. Additionally, metabolic pressure in the TME results from nutrient competition between the highly metabolic tumor and surrounding cells. Regulatory T cells (Tregs) can overcome this metabolic pressure and rewire their energetic needs to survive in the TME, but CD8+ effector T cells do not adapt and become non-functional. Tregs obstruct anti-tumor immunity by suppressing proliferation and activation of CD8+ T cells and CD4+ helper T cells in the TME. High tumor infiltration of Tregs is associated with poorer responses to immunotherapies and clinical outcomes in general. Therefore, there is an imminent clinical need for the development of more synergistic therapies that specifically target Treg accumulation and function in the TME to successfully overcome the barriers of infiltration and persistence of antigen-specific T cells in the TME. Multiple metabolites have been identified to mediate immunosuppression, including the mitochondrial metabolite succinate. High levels of succinate accumulate in hypoxic environments, such as that of the solid tumor, and can be secreted into the interstitial space, where it can bind to the cell surface succinate receptor (SUCNR1) and signal downstream to activate Ca2+-associated pathways. Tumor-derived succinate has been identified to promote the polarization of macrophages to an immunosuppressive phenotype in the TME and drive tumor progression. Moreover, increased expression of SUCNR1 in the solid tumor has been associated with poor-prognosis in several cancers. Given these findings, we hypothesized that succinate may act on other suppressive immune cells, such as Tregs, to drive the immunosuppressive environment of the TME. Consistent with this hypothesis, we treated healthy PBMCs with extracellular succinate and identified that Tregs (CD4+CD25+FoxP3+) could be induced after incubating CD4+ T cells with succinate for 5 days. Additionally, we demonstrated that succinate promotes the production of immunosuppressive cytokines, such as TGFb. We further determined that co-culture of CD4+ T cells with tumors modified by CRIPSR to over produce succinate led to enhanced FOXP3 expression, demonstrating that tumor-derived succinate can promote Tregs, either by induction or expansion, and propose this as a mechanism for suppression in the solid tumor. Furthermore, using a humanized mouse model, we identified that CD4+ T cells and Tregs preferentially infiltrate the H1299 lung tumor, and detected high levels of secreted succinate in the interstitial space. We further indicate this to be a barrier mechanism to the efficacy of infiltrating cellular therapies. Overall, our data demonstrates a novel mechanism of Treg induction driven by succinate, identifying an innovative molecular target for the development of superior combination therapies that can overcome the immunosuppressive barriers to solid tumor immunotherapy.

12. Cyto-Adhesive Micro-Platforms for Neutrophil-Based Immunotherapy for Solid Tumors

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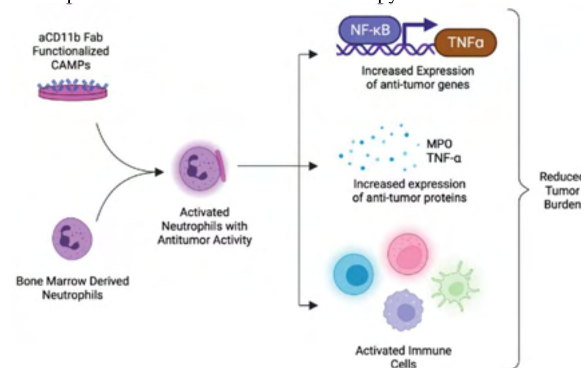
[1]Harvard University, [2]Cornell University

Neutrophils are the most abundant leukocytes in human blood circulation and are the first responders to the site of inflammation including tumors. Tumor-Associated Neutrophils (TANs) have the potential to exert an anti-tumor effect. However, they assume a pro-tumor phenotype in the immunosuppressive tumor microenvironment, thus contributing to a worsening prognosis. Here we report a material-based approach for activating neutrophils and thereby polarizing them towards the antitumor phenotype. We designed polymer micro-patches, termed 'Cyto-Adhesive Micro-Platforms (CAMPs),' that adhere to the surface of neutrophils without internalization due to their discoidal geometry. Attachment of CAMPs activated the anti-tumor phenotype of neutrophils as indicated by increased expression of N1 neutrophil-related genes, upregulation of N1 biomarkers (CD95 and CD54) and the release of TNFα and myeloperoxidase (MPO). Intravenously administered neutrophil-CAMPs accumulated in the spleen and tumor-draining lymph nodes and activated splenic Natural Killer (NK) and T cells and subsequently increased accumulation of dendritic cells and NK cells in the lymph nodes. Intravenous injection of CAMP-loaded neutrophils in 4T1-Luc and B16F10 tumor-bearing mice resulted in a robust systemic immune response, leading to a reduction in tumor burden, and subsequent improvement in survival rate. The combination of CAMP-activated neutrophils with anti-CTLA-4 checkpoint inhibitor antibody led to strong tumor-growth inhibition of B16F10 tumors, with complete regression achieved in 33.3% of treated mice. CAMP-loaded neutrophils represent a potent, scalable, and drug-free approach for neutrophil-based solid tumor immunotherapy.

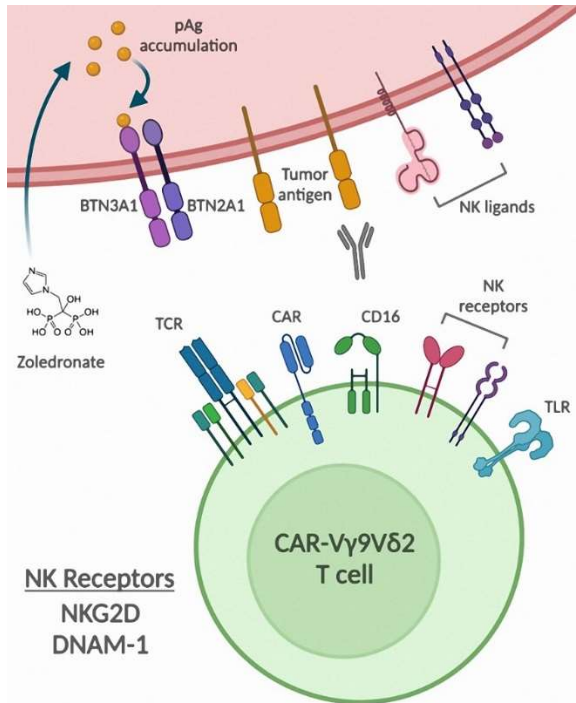


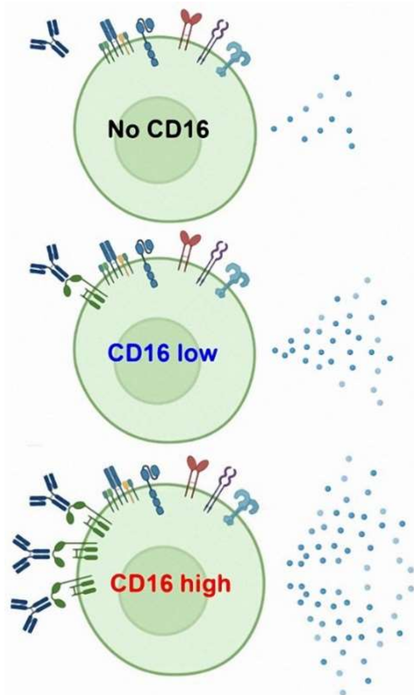
13. Unlocking the Potential of Allogeneic Vδ2 T Cells for Ovarian Cancer Therapy Through CD16 Biomarker Selection and CAR/IL-15 Engineering

Derek Lee, PhD[1], Zachary Dunn, PhD[2], Wenbin Guo[1], Pin Wang, PhD[2], Lili Yang, PhD[1]
[1]UCLA, [2]USC

Allogeneic Vγ9Vδ2 (Vδ2) T cells are attractive candidates in the development of cancer therapies due to their demonstrated safety in allogeneic settings and innate ability to fight tumors. However, the limited clinical success of Vδ2 T cell-based treatments may be attributed to donor variability, short-lived persistence, and tumor evasion. To address these limitations, we have generated Vδ2 T cells with improved properties. By utilizing CD16 as a donor selection biomarker, we have generated Vδ2 T cells with high levels of cytotoxicity and potent

antibody-dependent cellular cytotoxicity (ADCC), and RNA sequencing characterization supports the increased effector function of Vδ2 T cells obtained from CD16 high (CD16Hi) donors. Further improvement was achieved through chimeric antigen receptor (CAR) and IL-15 engineering techniques. Preclinical studies in two ovarian cancer models showed that engineered CD16Hi Vδ2 T cells are both effective and safe, targeting tumors through multiple pathways, exhibiting long-term persistence in vivo, and not causing GvHD. These findings support the potential of engineered CD16Hi Vδ2 T cells as a viable cancer therapy option.

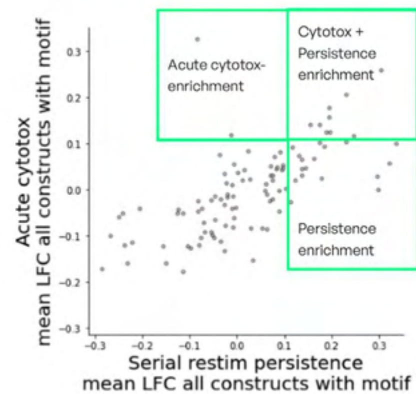




14. High Throughput Screening of TGFb Switch Receptors in NK Cells

Rebekah Turk, Brian Belmont, PhD, Lan Guo, PhD, Matt Stone, Aye Chen, Maddie Williams, Bryce Daines, PhD, Max Darnell, PhD
Modulus Therapeutics

Natural killer cells offer a promising treatment strategy for cancer, yet overcoming the inhibitory tumor microenvironment, one element of which is the presence of inhibitory cytokines, remains a significant challenge. An attractive strategy for functional rescue in the presence of inhibitory cytokines is the use of switch receptors, surface-expressed chimeric receptors that convert external inhibitory signals into intracellular activation signals. TGFb is an inhibitory cytokine present in some microenvironments and, although the concept of using switch receptors to drive activation in the presence TGFb has been validated, the exploration of endodomain choice has thus far been very shallow, presenting an opportunity to optimize signaling driven by switch receptors.
To identify optimal endodomain sequences for TGFb switch receptors, we developed an in vitro screening platform based on key functional attributes of NK cells, acute cytotoxicity and persistence. We designed and synthesized a switch receptor library comprising 11,000 endodomains drawn from pairwise combinations of co-stimulatory domains. Through two pooled screening experiments, we identified multiple signaling domains that improved both the acute cytotoxicity and persistence of NK cells in the presence of TGFb. Arrayed validation of the top hits demonstrated signaling domains exhibiting superior cytotoxicity and persistence in the presence of TGFb than 4-1BB.
Top hits in these screens included co-stimulatory domains known to enhance proliferation, cytotoxicity, and tumor clearance in animal models and commonly used in cell therapies. Notably, we frequently observed these domains in novel combinations or in combination with endodomains not presently used in immunotherapies. Our results suggest that switch receptor screens unlock potent strategies for enhancing fitness of cell therapies in the tumor microenvironment.



15. Polymer Micropatches as Natural Killer Cell Engagers for Tumor Therapy

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The intrinsic ability of Natural Killer (NK) cells to recognize and kill tumor cells has generated great interest in the adoptive transfer of NK cells as a cell therapy against cancer. However, their efficacy is still limited by tumor mediated suppression leading to NK cell deactivation or exhaustion. Efforts to enhance NK cell efficacy have manifested into CAR-NK cells and combination therapy with supporting cytokines or

checkpoint blockade. Still, there are no approved NK cell-based therapies till date. An NK cell targeted activating platform that can synergize with existing clinical approaches has the potential to accelerate the translation of adoptive NK cell therapy. Here, we report a material-based approach to activate NK cells. Specifically, we describe the use of polymeric micropatches as a platform for sustained, targeted activation of NK cells, referred to as Microparticles as Cell Engagers (MACE). Poly(lactide-co-glycolic) acid (PLGA) micropatches, 4-8 microns in diameter and surface-modified with NK cell receptor targeting antibodies, exhibited strong adhesion to NK cells and induced their activation without the need of co-administered cytokines. The activation induced by MACE was greater than that induced by free antibody as well as nanoparticles, attesting to the unique role of MACE geometry in the activation of NK cells. MACE-bound NK cells remained viable, exhibited trans-endothelial migration and anti-tumor activity *in vitro*. The activation induced by MACE synergized with cytokine pre-activation, a common strategy used to expand NK cells for clinical applications. Additionally, MACE-bound NK cells activated T cells, macrophages and dendritic cells *in vitro*. Adoptive transfer of NK-MACE also demonstrated superior anti-tumor efficacy in a mouse melanoma lung metastasis model compared to unmodified NK cells. Overall, MACE offers a simple, scalable and effective way of activating NK cells and represents an attractive platform to improve the efficacy of NK cell therapy.

16. Co-Operative Interaction Between Natural Killer Cells and Dendritic Cells Play a Role in Anti-Tumor Activity in Cervical Cancer

Sharel Figueredo, Anushka Dikshit, Jessica Rossol-Allison
Bio-Techne

The bi-directional cross talk between natural killer (NK) cells and dendritic cells (DCs) play a role in anti-tumor activity. The NK cells can respond to cytokines or chemokines produced by DCs thus resulting in activation and a promoting a strong adaptive immune response with sustainable cytotoxic response. These factors are presented by DCs upon binding to their specific receptors and can affect NK cell optimal activation and stimulate priming of protective NK cell response while the NK cells can also stimulate DC survival and recruitment. Being able to characterize the NK cell interactions with DCs in the tumor microenvironment (TME) can lead to improved efficacy of immunotherapies. Here we show that NK cells within the cervical cancer TME recruit specific DC cell subsets called conventional type 1 DCs (cDC1s) by secreting chemokines such as CCL5 and XCL1. However, detection of these small number of cells is challenging within the heterogenous and complex TME. Using a highly sensitive and specific RNAscope Multiplex Fluorescence *in situ* hybridization (ISH) assay we were able to spatially interrogate the TME and detect the recruitment of the specialized immune cells and quantitate the heterogenous expression of the secreted factors.

NOVEL CAR DESIGNS & APPROACHES

17. WITHDRAWN

18. ROR2-Specific CAR-T Cells Elicit Potent Antitumor Efficacy in Preclinical Models of Multiple Myeloma and Clear Cell Renal Cell Carcinoma

Justus Weber, PhD, Fabian Freitag, Peter Spieler, Charis Kalogirou, MD, Mathias Rosenfeldt, MD, Martin Kortüm, MD, Herrmann Einsele, MD, Christoph Rader, PhD, Thomas Nerreter, PhD, Michael Hudecek, MD
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Chimeric antigen receptor (CAR)-modified T cell therapy has shown profound clinical efficacy for the treatment of hematological malignancies. To broaden the therapeutic applicability of CAR-T cells, there is a need to identify novel tumor-associated antigens and the concomitant development of cell products. Here we were interested in evaluating the receptor tyrosine kinase ROR2, an oncofetal antigen, which has been reported to be overexpressed on the cell surface of various hematological and solid tumors, as a novel target for CAR-T cell therapy.
To assess the overall potential of ROR2 as a target for CAR-T cell therapy, RNAseq analysis of patient samples obtained from the TCGA and COMPASS datasets was performed. Our data revealed ROR2 to be overexpressed in considerable subsets (> 40% of cases) of 10 out of 27 tumor entities as compared to matched healthy tissues. Multiple myeloma (MM) and clear cell renal cell carcinoma (ccRCC) were selected as representative tumor entities based on their uniform ROR2 overexpression and correlation with disease specific survival, respectively. To validate our *in silico* findings, the expression of ROR2 was confirmed in representative patient cohorts of MM and ccRCC using flow cytometry, qPCR and immunohistochemistry. Our data reveal ROR2 to be expressed in 80% of MM and 100% of ccRCC samples.
Next, 2nd generation ROR2-specific CAR-T cells (ROR2-CART) were generated and their anti-tumor reactivity was analyzed *in vitro* and *in vivo*. Our data show potent anti-tumor reactivity - including antigen-specific tumor cell lysis, cytokine secretion and proliferation - of ROR2-CART in response to ROR2-positive MM and RCC cell lines but not ROR2^{KO} cells. Employing NSG xenografts models of MM and ccRCC, we observed in both cases a significant increase in median overall survival upon treatment with ROR2-CART ($p=0.0039$ and $p=0.0035$, respectively). While ROR2-CART elicited a curative response in 25% of mice in our MM model, only partial responses were obtained in ccRCC. In consequence, we explored advanced cell engineering and pharmacological approaches to augment the anti-tumor efficacy of ROR2-CART. Our data show that both over-expression of cJUN and the utilization of tyrosine kinase inhibitors significantly improve the anti-tumor efficacy of ROR2 CAR-T both *in vitro* and *in vivo*.
Furthermore, we were interested in assessing the safety of targeting ROR2 using preclinical models. Our data show that mice represent a relevant toxicity model to study the safety of ROR2-CART and revealed cross-reactivity ROR2-CART with murine ROR2. However, we observed no signs of dose-limiting toxicity or tissue damage as determined by histological analyses, when tumor-free mice were inoculated with up to $4x10^8$ ROR2-CART per kg.
Collectively, these data show that ROR2 is a relevant target in various hematological and solid tumors, which can be addressed effectively using ROR2-CART without severe adverse effects in preclinical models.

19. OutSmart™ IL-2/15 Safely Stimulates CAR T Proliferation and Persistence in the Tumor while Limiting Regulatory T Cell Activation

Howell Moffett, Brian Weitzner, Thaddeus Davenport, Leah Tait, Laura Baker, John Crowl, Willimark Obenza, Bob Bader, Bradley Hammerson, Robin Kirkpatrick, Paul Sample, Lesley Jones, Erik Hermans, Robert Langan, Kevin Haworth, Scott Boyken, Aaron Foster, Marc Lajoie
OutPace Bio

Introduction: Effective treatment with chimeric antigen receptor (CAR) T cells against solid tumors requires local production of immune-modulating cytokines to promote CAR T cell expansion and stimulate endogenous anti-tumor immune responses within the tumor microenvironment (TME). OutSmart™ IL-2/15 comprises a computationally designed IL-2 under the control of a T cell activation-induced promoter so that it preferentially stimulates the CAR T cells that produce it in the tumor as well as nearby bystander $CD8^+$ T cells and NK cells, while minimally activating immune-suppressive regulatory T cells (Tregs).
Methods and Results: Wild-type (wt) IL-2 was modified using Rosetta to eliminate IL-2Rα binding while retaining native IL-2Rβγ binding interfaces. Of 10,355 designs, 38 were prioritized based on in silico analysis and evaluated for IL-2R binding as recombinant proteins. Consistent with the design criteria, none of the designs bound IL-2Rα, while 33 (87%) retained IL-2/15Rβγ binding and signaling activity. pSTAT5 analysis showed wt IL-2 highly activated Treg cells ($CD4^+CD25^+FoxP3^+$), whereas novel IL-2 designs showed a >3-log reduction in Treg pSTAT5 levels. All designs stimulated $CD8^+$ T and NK cells at approximately 1 nM concentration. These designs were iteratively optimized to generate a version exhibiting improved thermostability and equivalent IL-2Rβγ binding compared to wt IL-2. To increase the potency against immune effector cells, but not Tregs, an anti-CD8α VHH was tethered to the modified IL-2 molecules to mimic high-affinity IL-2Rα binding and selectively activate $CD8^+$ T and NK cells. This appendage resulted in a dramatic increase in $CD8^+$ T cell activation (>2-logs) while retaining a reduced capacity to stimulate Tregs. OutSmart™ IL-2/15 gene expression was subsequently placed under the control of a T cell activation-dependent promoter and integrated into a lentiviral construct with a constitutively expressed ROR1-specific CAR. T cells transduced with a lentivirus comprising OutSmart™ IL-2/15 and a ROR1-targeting CAR showed inducible cytokine production following exposure to $ROR1^+$ H1975 lung carcinoma cells leading to enhanced CAR T cell proliferation and anti-tumor killing during repeat tumor challenge assays. Using immune-deficient mice (NSG) engrafted with s.c. H1975 tumors, OutSmart™ IL-2/15 enhanced tumor killing compared to ROR1 CAR only at low T cell doses (1 - 4e6 cells/animal) and showed similar efficacy to wt IL-2.
Conclusions: In summary, OutSmart™ IL-2/15 is a genetic module that localizes IL-2/15 signaling to the tumor and preferentially enhances effector function of $CD8^+$ CAR T cells, bystander $CD8^+$ T cells, and NK cells, but only minimally activates Treg cells due to the removal of the IL-2Rα binding interface. More generally, OutSmart™ protein design methods and control technologies can be applied to create other designed cytokines for oncology and beyond.

20. Saliogase-Based Gene Coding Technology, a Non-Viral Genome Engineering Platform for Efficient CAR-T Generation for Cancer Immunotherapy

Francisco Navarro, PhD, Zafira Castaño, Joshua LaMora, Javier García-Vilas, Hugues Bernard, Jonathan Labonne, Oleg Iartchouk, Omid Harandi
SalioGen Therapeutics

Adoptive Cell Therapies (ACT) using genetically engineered T cells expressing a chimeric antigen receptor, CAR T cells, have achieved great success in treating hematological malignances. Currently, there are six FDA-approved CAR T cell therapies for the treatment of relapse/refractory B-cell leukemia, lymphoma, and multiple myeloma. Common to all these therapies is the use of retroviral or lentiviral vectors for CAR T engineering, which although can provide high transduction efficiencies have limited genetic cargo capacity, are submitted to strict regulatory demands due to oncogenicity safety concerns and are associated with high manufacturing cost and complexity. Therefore, there is an urgent need for developing non-viral methods for engineering primary human T cells to overcome the limitations of current viral-based methods. SalioGen's gene coding technology is based on the use of a mammalian genome engineering enzyme called Saliogase. This enzyme seamlessly inserts new DNA into the host genome without causing double strand DNA breaks or relying on the host DNA repair machinery. Here, we show that our Saliogase-based EDIT technology efficiently engineered primary human T cells. The DNA cargo and mRNA Saliogase are delivered together to T cells by electroporation (EP). After extensive optimization of all EP parameters, we were able to consistently achieve high efficiency of transgene delivery with high viability, which ultimately resulted in high genomic integration efficiency and sustained transgene expression. To demonstrate the potential of our EDIT platform for engineering human primary T cells, we set out to generate CD19-specific CAR T cells using Saliogase and evaluate their efficacy *ex vivo* and *in vivo*. Using our optimized EP protocol, we were able to consistently achieve 55-60% CD19-CAR stable expression. Saliogase-generated CD19-CAR T cells had a balanced CD4:CD8 ratio, a favorable memory T cell immunophenotype with a high proportion of Tscm and Tcm cells, and very low expression of exhaustion markers. Importantly, Saliogase-generated CD19-CAR T cells demonstrated high cytotoxicity ability and high release of GZMB and pro-inflammatory cytokines (IFNg and TNFa) against CD19-expressing leukemia and lymphoma cells *ex vivo* and were able to abrogate tumor growth *in vivo* in a xenograft animal model. Furthermore, transgene genomic integration site analysis, by S-EPTS/LM PCR, of Saliogase-engineered CD19-CAR T cells demonstrated a good safety profile with high prevalence of integration events in intergenic and intronic regions and low copy number per genome. Overall, our results demonstrate that our non-viral Saliogase-based gene coding platform can be successfully used to generate highly functional CAR T cells with an optimal immunophenotype and safety profile. In addition, our platform can be readily adapted to cGMP clinical-scale manufacturing, with the potential of substantially reducing manufacturing time, cost, and complexity, and thus providing an optimal alternative to the use of viral vectors for engineering T cells for cancer immunotherapy.

21. Riboswitch-Regulated Chimeric Antigen Receptor (RiboCAR) Enhances CAR-T Cell Anti-Cancer Efficacy

Xuecui Guo, George Wang, Zhaojing Zhong, Zhikai Zhang, Samuel Waksal, Alexandria Forbes
MeiraGTx

Chimeric antigen receptor (CAR)-T cell therapy is a promising therapy against cancer. However, the uncontrolled CAR expression causes severe CAR-T cell-associated toxicity and CAR-T cell exhaustion, limiting the success of this living drug. Here, we present the development of RiboCAR, a mammalian synthetic riboswitch-regulated CAR expression via small molecule inducer. Unlike previously reported regulatable CAR platforms that utilize viral protease or chemical-induced protein dimerization, RiboCAR contains an RNA ON riboswitch in the coding sequence of a CAR transgene, in which the aptamer functions as a sensor for a specific novel small molecule inducer. The expression level of the CAR gene with the riboswitch completely depends on the presence of the riboswitch inducer, with undetectable CAR in the absence of the small molecule and significant CAR expression that is higher than constitutively active CAR upon

maximal small molecule induction. The induced CAR expression diminished after withdrawal of the small molecule inducer. Further, CAR expression is titratable in response to the levels of the small molecule inducer. Consistent with small molecule induced expression of the CAR molecule, CAR triggered-activation of CAR-T cells is also controlled by the small molecule inducer. More importantly, T cells with RiboCAR showed delayed exhaustion during expansion in the absence of small molecule inducer and enhanced target cell-stimulated T cell activation and anti-cancer cytotoxicity in the presence of small molecule inducer, when compared with T cells constitutively expressing CAR. With a bioavailable small molecule inducer, the RiboCAR-T cell activity can be precisely tuned and "remotely" controlled *in vivo*, thus improving the efficacy and safety of CAR-T cell therapy.

22. Detecting Engineered CARs/TCRs for Immunotherapy and Evaluating Safety and Efficacy in Preclinical Models.

Sharel Figueredo
Bio-Techne

Development of successful CAR and TCR T-cell immunotherapy approaches to treat solid tumors require advancement in areas such as antigen recognition, on-target / on-tumor trafficking and their ability to recruit immune cells at the tumor site. The challenge here is the inability to differentiate CAR T-cells from infiltrating T-cells in vivo and the low level of CAR antigen and gene expression. Here, we demonstrate a novel, highly sensitive RNAscope in situ hybridization (ISH) assay to address these challenges. With RNAscope ISH, we can detect unique regions in the CAR vector mRNA with to specifically detect CAR or TCR T-cell trafficking in tissue with spatial context. In this study, we have evaluated the efficacy of anti-BCMA CAR T-cell therapy using RNAscope ISH where we simultaneously visualize and quantify CAR T-cell trafficking and activation by target antigen engagement. We show recruitment of T-cells and macrophages and assess the immune landscape with RNA and protein co-detection. We also show that the high sensitivity of the assay provides detection of low levels of target expression necessary to avoid adverse events resulting from "on-target /off-tumor" toxicity necessary for preclinical safety assessment of anti-BCMA CAR T-cell therapy.

23. Leveraging Lentivirus Biology to Achieve Dual Car Expression

Diana Vedenova, Ryan White, Aaron Lampano, Garrett Zipp, Vandana Chaturvedi, Adam Johnson, Neal Van Hoeven, Semih Tareen
Sana Biotechnology

CD19-directed CAR T cell therapies have delivered promising clinical response rates, however, relapse due to antigen escape remains a challenge. As a result, some strategies have relied on utilizing a second antigen such as CD22 and co-expressing two CAR constructs. Although feasible, achieving dual expression of CAR constructs is not trivial. Molecular approaches for the expression of two transgenes present limitations related to construct size, unwanted sequences, and differences in expression. Similarly, methods that rely on simultaneous or sequential transductions with multiple lentiviral vectors (LVs) are complicated from a CMC, safety (vector copy number and insertion patterns), and cost of goods perspective. Therefore, using CD19- or CD22-targeted CAR constructs as a model, we developed a method that expresses two CAR constructs in T cells in a simpler way. Leveraging LV biology, where lentiviral derived vector particles package two RNA genomes, we produced a heterogeneous pool of LV through co-transfection of two transfer plasmids that each express a unique CAR. Using this LV pool, we generated a population of transduced CAR T cells that express either CD19-directed CAR, CD22-directed CAR, or both. In mouse models, these heterologous CAR T cells cleared the tumor challenge as effectively as T cells that were transduced sequentially. Moreover, by tuning the ratio of the two transfer plasmids we demonstrated the ability to control expression in T cells in favor of one CAR over the other. This co-transfection method allows for the generation of dual CAR T cells following a single transduction step from a heterogeneous LV pool. In conclusion, this elegant method has the potential to overcome limitations of biological, CMC, safety concerns posed by alternative approaches used to generate dual CAR T cells.

24. Aberrant Cell Surface Phosphatidylserine 'Eat-Me' Signal Identifies Acute Myeloid Leukemia (AML) Blasts Across Multiple Subtypes for Engineered T Cell Targeting

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Introduction: Development of engineered T cell therapies for Acute Myeloid Leukemia (AML), a clinically and biologically heterogenous disease, has proven difficult in part due to the identification of suitable target antigens. Phosphatidylserine (PS), a lipid moiety recognized by phagocytic cells as an eat me signal, has previously been shown to be aberrantly upregulated on acute promyelocytic (APL) blasts, a subset of AML. We evaluated a panel of primary bone marrow samples and peripheral blood from AML patients to quantify cell surface PS on AML blasts. Additionally, we tested PS-directed engineered T cells (CER-T cells) and quantified T cell activation, cytotoxicity, proliferation, and cytokine secretion against AML cell lines.
Results: We screened a preliminary panel of 15 primary, treatment-naïve and 2 on-therapy AML bone marrow samples by flow cytometry: (n= 5 adverse, n= 5 intermediate, n= 1 APL, n = 1 familial, n = 5 N/A). We observed both high percent (58.4 % ± 24.2) and gMFI of cell surface PS on a range of AML bone marrow samples. The median MFI of tertiles 1-3 was: T1 n=5, gMFI = 4042; T2 n=6, gMFI = 1799; T3 n=6, gMFI = 535. Of note, the 2 on-therapy samples showed the highest percent and gMFI of cell surface PS, with a patient receiving 5-azacytidine showing 1.8 fold PS gMFI over median. The second patient receiving TKI therapy showed 3.3 fold PS gMFI over median. Healthy donor samples had much lower cell surface PS, with a mean gMFI of 536. Circulating AML leukemic blasts were also evaluated for cell surface PS and showed high concordance with BM blasts, with high levels of cell surface PS compared to healthy donor PBMCs. Given these high levels of cell surface PS in both PBMC and blasts of a diverse sample of AML patients we next tested the function of TIM-4 CER T cells specific to PS against the Kasumi-1 AML cell line and observed >90% cytotoxicity as well as induction of T cell proliferation, and IFN-γ secretion after co-culture at E:T ratios as low as 1:1. Cell surface PS on the AML cell lines Kasumi-1 and MV-4-11 AML cells could be further induced by treatment with 5-Azacytidine. Pretreatment of Kasumi-1 cells with low dose 5-Azacytidine did not affect target cell growth, but moderately improved CER T cytotoxicity, cytokine secretion, and proliferation after co-culture, suggesting this strategy might augment CER T cell activity in vivo.
Conclusions: These data indicate that aberrant cell surface phosphatidylserine on AML blasts could potentially render them susceptible to therapeutic engineered T cell targeting. Our findings reveal significantly elevated levels of cell surface PS in a variety of treatment-naïve AML subtypes, as compared to healthy donor bone marrow. This is further corroborated by experimental data using AML cell lines exhibiting levels of cell surface PS (Kasumi-1 gMFI = 1921, MV-4-11 gMFI = 548) comparable to lower expressing primary samples. Additionally, AML cell lines could be eliminated in vitro by co-culturing with PS-targeting CER T cells. Addition of 5-Azacytidine led to increased PS cell surface density, resulting in modest enhancement of CER T cell functionality in vitro. Together these data suggest that TIM-4-specific CER T cells may be a novel way to treat genetically diverse subtypes of AML.

25. Regulation of CD19 CAR-T Cell Activation Based on an Engineered Downstream Transcription Factor

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CD19 CAR T- cells present a therapeutic option for various malignant diseases based on their ability to recognize the selected tumour surface markers, triggering immune cell activation and cytokine production resulting in killing cancerous cells. Efficient tumour cell destruction by CAR-T may cause serious side effects so their activity needs to be carefully controlled. Several attempts were made to influence the CAR T cell proliferation and their activation by adding T cell growth factors, incorporating cytokine expressing cassettes in T cells etc., however this approach of regulating T cells activity with no external control can again lead to non-optimal therapeutic effects. Different improvements were made also by designing synthetic receptors or small molecule-inducible systems etc., which influence regulated expansion and survival of CAR T cells.
To regulate CD19 CAR-T cells, we developed a regulatory system for therapeutic effect of CD19 CAR-T cells, a unique mechanism to control T cell activation and proliferation based on the engineered NFAT2 artificial transcription factor. A key transcription factor in T cells, NFAT that result in IL2 production, was truncated by deletion of its own activation domain and joined with domains of different heterodimerization systems. The interaction counterparts were fused to a transcriptional activator or repressor domains, resulting in formation of an engineered artificial transcription factors with external control. Chemical regulators were used to either transiently trigger engineered T cell proliferation or suppress CAR-mediated activation when desired or to enhance activation of CAR T cells upon engagement of cancer cells, shown also *in vivo*. Additionally, an efficient sensor to monitor activated CD19 CAR-T cells *in vivo* was introduced. This implementation in CAR-T cell regulation offers an efficient way for on-demand external control of CAR-T cell activity to improve their safety.

DIRECT *IN VIVO* DELIVERY

26. Adenovirus Mediated HSP70 Combined with GM-CSF Enhances HPV E6/E7 Antigen Immunotherapy for Cervical Cancer

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Abstract: The treatment of cervical cancer targeting HPV proteins E6 and E7 has always been a research hotspot. Our research utilized a combination of adenovirus vector mediated immune enhancer HSP70 and GM-CSF to enhance the immunogenicity of E6/E7 to achieve better treatment outcome for cervical cancer, which then led to the development of BBM-C101 adenovirus products, providing a new approach for immunotherapy of cervical cancer.
Introduction: In recent years, with the application of various immune activating factors, the immunotherapy of tumors has developed rapidly. Cervical cancer is one of the most common gynecological malignancies and poses a serious threat to women's health1. Persistent HPV infection is the main cause of most cervical cancers2, with E6 and E7 being the main oncogenic genes of HPV3. The prevention and treatment of E6 and E7 targets has always been a focus of research4.
In order to enhance the efficacy of therapeutic vaccines targeting E6/E7 for cervical cancer, we used a combination of HSP70 from mycobacterium tuberculosis5 and human GM-CSF, and used adenovirus vectors to mediate the expression of GM-CSF and HPV antigens fused with HSP70, in order to enhance the efficacy of immunotherapy for cervical cancer. The research results showed that the combination of HPS70 and GM-CSF could better promote the treatment of HPV positive tumors, induced stronger T cell immunity, and had a good synergistic effect. After multiple efficacy studies, the BBM-C101 adenovirus product was ultimately developed and is being further explored down its clinical path.
Discussion: Our drug has therapeutic and immunostimulatory effects, which could provide a novel treatment for HPV positive cervical cancer patients. However, due to the limitations of animal models related to immunotherapy for cervical cancer, current models in the field may not fully simulate the real state and environment of human cervical cancer. Therefore, we are currently conducting more in-depth research in the hope of providing more pharmacological support for clinical practice. In addition, the overall toxicological evaluation of viral drugs and immunotherapy drugs also requires a comprehensive consideration, which is also important for ensuring clinical safety. This is also our ongoing exploration.
Conclusions: Our research confirmed that the combination of HSP70 and GM-CSF has a better synergistic effect on enhancing the efficacy of drugs targeting E6-E7 antigen, and therefore we have developed the BBM-C101 product. This product demonstrated good efficacy in classic HPV16 positive TC-1 cell transplantation tumor models and had good immune effects on the production of specific T cells targeting E6/E7. This had positive implications for the treatment of HPV positive tumors, prevention of recurrence, and even prevention of HPV related cancer.

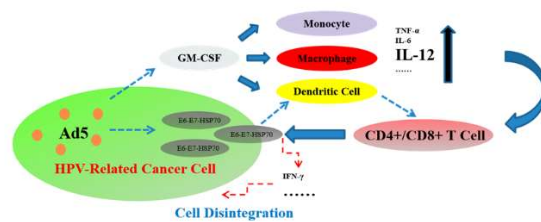

Figure 2. Treatment Mechanism Diagram of **BBM-C101**

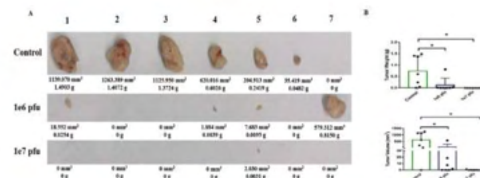

Figure 5. The endpoint tumor volume and weight of the first batch of **BBM-C101** on short-term TC-1 tumor bearing mice treatment experiment

27. A Platform of IL27 Fusion Immunokines for Targeted Gene Therapy Applications

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In the field of skeletal malignancies, a promising approach involves utilizing osteoimmunology to explore immune-stimulatory agents that can simultaneously combat tumor growth and promote bone repair. We propose that Interleukin-27 (IL-27), a cytokine, holds great potential as a biologic candidate to rebalance the interaction between prostate tumor cells and bone cells. To investigate this, we have developed IL-27 fusion immunokines for targeted gene delivery applications. In our studies, we have demonstrated the proof of principle for a platform of immunokines including Nanoluc-27, which involves fusing a short, secreted luciferase (Nanoluc) with IL-27. Through in vitro experiments, we have shown the efficacy of Nanoluc-27 in reducing prostate tumor growth and rebalancing bone cell proliferation and differentiation. The fusion cytokine, detectable in conditioned media, exhibits higher bioactivity

than the wild-type cytokine in various cell systems. This innovative Nanoluc-27 cytokine serves as a novel and flexible design immunokine, which allows for the incorporation of targeting domains, bioactivity modifying domains, enhancing bioactivity beyond that of the wild-type cytokine and rendering it more effective against prostate tumors and in promoting bone repair in syngeneic models with relevance to bone-metastatic prostate cancer. Furthermore, this approach has the potential for application of targeted Nanoluc-27 forms across different cell types, as we have shown in vitro and in vivo, characterizing the platform further with bioinformatics analyses to examine the potential and mechanisms for Nanoluc-27 priming of the tumor-bone microenvironment to chemotherapeutics and other types of immune therapy (anti-PDL1) with impact on rebalancing the immune system and bone tissue homeostasis.

GENOME & EPIGENOME EDITING FOR CANCER IMMUNOTHERAPY

28. Multiplex Epigenome Editing Enables Multi-Faceted Functional Modulation of CAR T Cells in Solid Tumor Setting

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Tune Therapeutics

Therapeutic clinical application of adoptive T cell therapies has yielded modest results in solid tumors, despite clearly convincing outcomes in heme malignancies. Anti-tumor T cells in the solid tumor are subject to multiple mechanisms that lead to dysfunction, including intrinsic exhaustion driven by high antigen load and suppressive signals from the tumor microenvironment that dampen the effector activity of T cells. The redundant nature of immunoregulatory mechanisms underscores the need to apply multi-faceted engineering strategies to adequately arm adoptive T cell therapies for effective durable responses. Translational insights from immuno-oncology, as well as basic T cell biology, suggest several key categorical pathways to engage in order to achieve optimal T cell activity in the tumor. These include homeostatic cytokines, molecular checkpoints, and regulators of epigenetic architecture that sustains T cell multifunctionality and replicative potential, among others.
Using enzymatically-dead Cas9 (dCas) linked to protein domains that activate or inhibit gene transcription via recruitment of molecular complexes, we are able to target multiple molecular pathways to enhance T cell activity without the deleterious effects of multiple DNA cuts and transgene insertions. Because this approach does not involve DNA breaks, but rather recruits endogenous cell factors that can achieve heritable control of genes, we refer to this as epigenome editing, or epi-editing. The dCas complexes for simultaneous epi-editing of functional gene networks were delivered by electroporation during ex vivo expansion of T cells bearing Her2-specific Chimeric Antigen Receptors (CAR). When co-cultured with Her2-expressing tumor target cells, the multiplex epi-edited CAR T cells produced higher levels of effector cytokines (IL-2, TNF-a and IFN-g) and sustained proliferation over multiple rounds of serial target killing. This effect was additive or synergistic compared to single-plex epi-editing, and enhanced T cell function in immunosuppressive conditions that mimic solid tumor biology. These results demonstrate a multiplexing platform capability that can be extended to clinically-applicable cell therapy with impactful translational benefit.

29. Unveiling Genomic Rearrangements in Engineered iPSC Lines through Optical Genome Mapping

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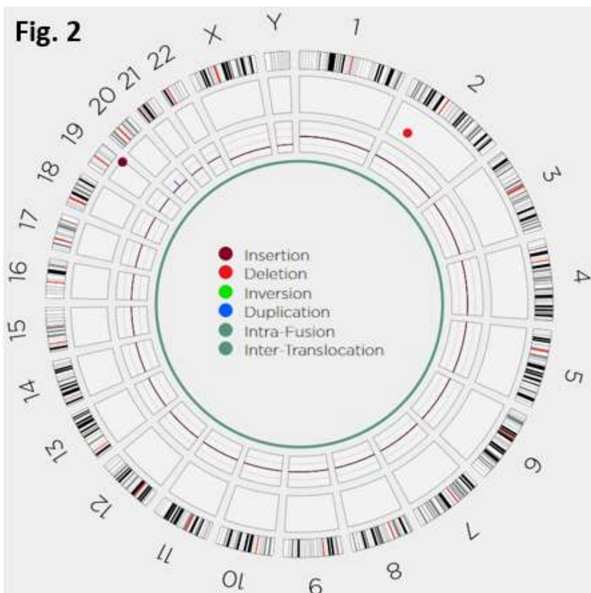
In this study, we present the application of optical genome mapping (OGM) to observe the genetic changes caused by different technologies in various engineered cells related to immune oncology, derived from human induced pluripotent stem cells (iPSCs). OGM enables an unbiased and comprehensive analysis of the entire genome, facilitating the detection of both intended and unintended effects. By consistently achieving adequate coverage, even rare variants occurring at a frequency of approximately 1% can be identified, allowing for the detection of infrequent or clonal events. Thereby, OGM surpasses next-generation sequencing (NGS) technologies and conventional cytogenetic techniques in detecting structural variants of all classes, achieving a resolution of 500 base pairs (bp) and a quantification of variant allele frequency (VAF) down to 5%. Additionally, this technology can be used to assess the ‘genetic fitness’ of the original iPSC lines and perform a ‘dual analysis’ by comparing the genetic alterations introduced through engineering with the parental cells, as demonstrated in this study. Based on our initial findings, we have verified the genetic suitability of several wildtype parental iPSC lines derived from distinct sources, including umbilical cord blood and fibroblasts. This confirmation allows for their utilization in subsequent gene-modification experiments.
Moreover, we conducted a comparative analysis, known as dual analysis, between the parental iPSCs and those that underwent genetic editing using various techniques, such as transposons, lentivirus and CRISPR/Cas9 safe harbor locus insertion at the AAVS1 site in 19q13. Figure 1 shows a Circos plot, of such a dual analysis, revealing the presence of multiple distinct genomic structural variants in the lentivirally transduced iPSCs that were not found in the parental line. Specifically, we detected a high number of insertions in each clonal line (ranging from 13 to 35), surpassing the desired or necessary number. Indeed, in the sample shown (Fig. 1) we also detected deletions, duplications, and a translocation. In contrast, the application of CRISPR/Cas9 for safe harbor locus insertion demonstrated a highly precise and accurate outcome, with only a single insertion of the target sequence in the intended locus observed (Fig. 2). With this methodology only one other unique SV, a deletion, was detected. In conclusion, this pilot study highlights the valuable application of OGM to provide a detailed view of the genetic changes caused by different genome editing technologies in engineered cells derived from human iPSCs, and underscores the importance of careful evaluation and selection of gene-editing techniques for their safety and efficacy in therapeutic applications.



30. Discovery of a Novel Anticancer T Cell Origin in NSCLC as an Effective Solid Tumour Immunotherapy by Single-Cell RNA-Sequencing

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T cell-based immunotherapies are effective for blood cancers, but low efficiency in solid tumours with largely unclear mechanisms. Single-cell RNA-sequencing (scRNA-seq), a leading-edge technology, can retrospectively capture the dynamics and development of immune cells at the transcriptome level, allowing us to better understand the uniqueness of the solid tumour microenvironment. Surprisingly, by scRNA-seq analysis, we discovered a novel T cell subset in non-small-cell lung carcinoma (NSCLC) patient biopsies, which is absent in leukaemia and positively associated with better patient survival of various solid cancer types. Advanced bioinformatics and antibody-mediated cell type depletion experimentally confirmed this novel T cell type is developed from an unconventional and unreported origin in vivo and in vitro. Adoptive transfer of the purified novel T cells markedly inhibited the progression of syngeneic lung cancer LLC and melanoma B16F10 in vivo and in vitro, highlighting their potential as an immunotherapeutic tool. Mechanistically, we identified a conserved pathway for blocking their development in tumour, which can be targeted by pharmaceutical and genetically inhibition for enhancing their abundance under cancer conditions in vivo. Most importantly, genetic engineering successfully mass-produced this novel cell type from human peripheral blood which dramatically blocked the growth of human NSCLC in vivo, representing a potentially effective immunotherapeutic strategy for solid tumours.

31. Single-Cell RNA Sequencing Discovers a Novel Regulator for MDSC-Dependent Immunosuppression in NSCLC

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Myeloid-derived suppressor cells (MDSCs) are important for promoting immunosuppression and cancer progression in solid tumor microenvironment associated with primary resistance against T cell-based immunotherapies. Better understanding of their regulatory mechanisms may help identifying new therapeutic strategies for solid cancer in clinic. By 10X single cell RNA-sequencing (scRNA-seq) analysis, we observed the hyperactivation of TGF-beta/Smad signaling in the tumor-associated MDSCs in human, where the Smad+ve MDSCs are associated with a poorer overall survival of non-small cell lung cancer (NSCLC) patients. Interesting, by using syngeneic Lewis Lung Carcinoma (LLC) mouse model, we observed a dramatic reduction of MDSCs in the LLC-tumor of mice lacking a Smad protein (Smad-KO) compared to their wild-type mice. Unexpectedly, advanced bioinformatic analysis uncovered the importance of that Smad protein in the developmental pathway and cell fate determination of MDSCs under cancer condition. Importantly, genetic deletion and pharmaceutical inhibition of the Smad protein in MDSCs dramatically blocked cancer progression of LLC-bearing mice due to the increment of anticancer immunity in tumor. Thus, we identified MDSC-specific Smad signaling as a novel therapeutic target for enhancing the efficiency of solid cancer immunotherapy in clinic.

32. The Enhancement of CRISPR/Cas9 Gene Editing Using Metformin

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The CRISPR/Cas9 technology is a revolutionary tool that can be used to edit the genome. Specifically, the genome of hematopoietic stem cells (HSCs) could be edited to produce immunotherapies. However, the efficiency of editing HSCs remains low. To overcome this hurdle, we set out to investigate the use of metformin, an FDA approved drug, to enhance gene modification. We assessed the effect of metformin on the growth of two hematopoietic cell lines: a myeloid-erythroid leukemic cell line (K562 cells) representative of the myeloid population and an immortalized T lymphocyte cell line (Jurkat cells) representative of the lymphoid population. No significant difference in growth patterns were observed in concentrations up to 10 mM metformin in both cell lines. We then assessed the ability of two different concentrations of metformin (0.001 mM or 1 mM), based on our observations, to enhance both (1) the cutting efficiency of Cas9 and (2) the targeting efficiency

with the use of a donor DNA repair template. The cutting efficiency of Cas9 was significantly enhanced in three of five guideRNAs following treatment with either concentration ($p = 0.0020$, $p = 0.0020$, and $p = 0.0185$). In addition, an enhancement in targeting was observed with the use of a GFP containing donor DNA repair template using fluorescent microscopy and flow cytometry ($p = 0.0361$). Overall, a two-fold increase in GFP expression was noted in cells treated with metformin. This suggests that metformin, an FDA approved drug, could be added to existing protocols to enhance CRISPR/Cas9 gene editing.