ORAL ABSTRACTS

CAR T Clinical Translation and Correlatives

1 Phase 1 Trial of Human Chimeric Antigen Receptor Modified T Cells (HuCART-meso) Administered in Combination with VCN-01 in Patients With Pancreatic and Serous Epithelial Ovarian Cancer

Janos Tanyi[1], Mark O'Hara[2], Elizabeth Hexner[3], Amy Marshall[4], Julie Jadlowsky[5], Olivia Farrelly[1], Carly Stein[4], Anne Chew[5], Emily Dowd[6], Vanessa Gonzalez[6], Joseph Fraietta[5], Gabriela Plesa[5], Neil Sheppard[7]*, Carl June[5]
[1]University of Pennsylvania, [2]Department of Hematology Oncology, Abramson Cancer Center, University of Pennsylvania, Philadelphia, PA, [3]Department of Hematology Oncology, Abramson Cancer Center, University of Pennsylvania, Philadelphia, [4]Center for Cellular Immunotherapies, University of Pennsylvania, Philadelphia, PA, [5]University of Pennsylvania, Philadelphia, PA, [6]University of Pennsylvania Perelman School of Medicine, [7]University of Pennsylvania Perelman School of Medicine, Philadelphia, PA
**Corresponding Author*

Background: We report interim results of a Phase I study from seven patients with epithelial ovarian- and pancreatic cancer treated with an intravenous infusion of VCN-01 oncolytic virus followed by autologous T cells transduced to express a chimeric antigen receptor directed against mesothelin (huCART-meso).

Methods: Seven patients received VCN-01 as a single IV infusion of either $3.3x10^{12}$ or $1x10^{13}$ vp on Day 0, followed by a single dose of $5x10^7$ huCART-meso cells on Day 14 via IV infusion. All seven patients were retreated with huCART-meso cells via IV infusion post-Month 2. VCN-01 is an oncolytic human adenovirus encoding PH20 hyaluronidase. HuCART-meso is a fully humanized autologous CAR-T cell. Both VCN-01 and huCART-meso are investigational agents and have previously been tested as monotherapies.

Results: The first three patients were enrolled in Cohort 1, and all received a dose of $3.3x10^{12}$ VCN-01 vp followed by $5x10^7$ huCART-meso cells and were re-treated with $5x10^7$ huCART-meso cells post-Month 2. In Cohort 2 two subjects were infused with a $1x10^{13}$ vp dose of VCN-01 followed by of $5x10^7$ huCART-meso cells. One subject experienced 2 events that met the definition of a DLT post-VCN-01 administration (AST increased, Grade 4 and ALT increased, Grade 4), which delayed huCART-meso infusion by 6 days. A third subject experienced a DLT qualifying event (hemophagocytic lymphohistiocytosis) post-VCN-01 administration which precluded huCART-meso administration. As a result of these DLTs, Cohort 2 was closed, and Cohort 1 was further expanded up to 6 subjects. There were no acute AEs related to the infusion and one grade 2 cytokine release syndrome experienced. Other AEs were mostly Grade 1 – 2 and anticipated from prior experience with VCN-01 and huCART-meso. Ongoing correlative studies showed huCART-meso T cells were detectable in the peripheral blood starting at 1 hour after infusion and expanded to a peak at Day 7-10 followed by a contraction phase and were detectable at low levels prior to re-treatment. VCN-01 was detectable at all post infusion timepoints assayed thus far to at least 150 days. Five of seven patients had measurable disease at baseline and were evaluable for response per RECIST 1.1. Response assessments are in progress as of the abstract submission deadline.

Conclusions: Regimen of IV delivery of 3.3×10^{12} vp VCN-01 followed 14 days later by 5×10^7 huCART-meso dose is acceptable and feasible. Repeat dosing with huCART-meso is also feasible. VCN-01 persistence suggests tumor infection and active replication. The huCART-meso cell expansion trended higher after VCN-01 as compared to monotherapy without lymphodepletion. RECIST1.1 responses will be detailed at the meeting.

2 Combining CAR T Cell Therapy with Targeted Proton Beam Radiotherapy

Ugur Uslu[1]*, Uri Amit[1], Ioannis Verginadis[1], Michele Kim[1], Seyyedeh Azar Oliaei Motlagh[1], Eric Diffenderfer[1], Charles-Antoine Assenmacher[2], Sandra Bicher[1], Sebastian Atoche[1], Edgar Ben-Josef[1], Regina Young[1], Carl June[1], Constantinos Koumenis[1]
[1]University of Pennsylvania, Philadelphia, PA, [2]Philadelphia, PA
**Corresponding Author*

To improve CAR T cell therapy in solid tumors, we explored the use of proton radiotherapy (RT) to modify the tumor microenvironment (TME) and stroma before administering CAR T cells. Proton RT, with its precise targeting ability, minimizes damage to surrounding healthy tissues compared to conventional photon RT. Using immunocompetent C57BL/6 mice and PDA7940b, a pancreatic ductal adenocarcinoma (PDAC) cell line, we tested proton RT alone, CAR T cells alone, and their combination. Untreated mice were used as control. Our analysis revealed that proton RT increased mesothelin expression of tumor cells and CAR T cell infiltration into tumors. Combined therapy showed superior tumor control and survival benefits over single treatments in both, orthotopic pancreatic and flank tumor models. Proton RT altered the TME by promoting antitumorigenic macrophages and reducing myeloid-derived suppressor cells, along with systemic immune activation indicated by elevated interferon-gamma levels in mouse serum. Further, abscopal effects were observed in a dual-flank tumor model. In accordance with this, higher levels of CAR T cells in peripheral blood and spleen were observed when mice were treated with proton RT in addition to CAR T cells. These promising results support advancing this combination approach to clinical trials.

3 WITHDRAWN

14 Engineering Allogeneic Cell Immunotherapies in a Single Intervention with the Innovative Pin-point™ Base Editing Platform

Shannon Hinsdale[1], Robert Blassberg[2], Bronwyn Joubert, Leigh-Anne Thomas[3], Olga Mielczarek[2], Jesse Stombaugh[2], Pablo Pérez-Durán[4], Kevin Hemphill[2], Immacolata Porreca
[1]Revvity, Lafayette, CO, [2]Revvity, UNITED KINGDOM, [3]Horizon Discovery Ltd, Cambridgeshire, United Kingdom, [4]Revvity, Cambridge, Cambridgeshire, United Kingdom

As our understanding of immune function regulators and effects of the tumor microenvironment on immune cells improves, increasingly more complex cell engineering is needed to improve the efficacy and durability of cellular immunotherapies. First generation genome engineering approaches utilizing CRISPR/Cas9 create double-strand DNA breaks (DSBs), which bring an increased risk of genomic rearrangements and cytotoxicity. When complex edits are required, such as for generating next-generation allogeneic cell therapies in T cells and iPSCs, the introduction of multiple DSBs amplifies the risk of genomic instability and greatly impacts cell health.

We have developed the RNA aptamer-mediated Pin-point base editing platform with the aim to facilitate complex cell therapy engineering in a safer manner than conventional DSB-based technologies. An implementation of the Pin-point modular platform consists of a nickase or nuclease deficient Cas enzyme, an extended guide RNA with an aptameric scaffold, and an aptamer binding protein fused to a deaminase[1]. Using aptamer-containing and aptamer-less guide RNAs, reagents are combined to introduce multiple knockouts by base editing, simultaneous to targeted insertion of cargo at endogenous loci in a single intervention with a single nickase[2]. A combination of mRNAs encoding the Rat APOBEC1 and SpCas9 nickase components and synthetic guide RNAs were transiently delivered for multiplex gene knockout and knock-in in a single intervention in clinically relevant primary human T cells and human iPSCs.

Flow cytometric analysis, NGS, and in vitro tumor cell killing assays confirmed functional protein knockout with efficient base editing and high purity at multiple targets including B2M, CD52, TRAC, and PDCD1, with successful integration of a CD19-CAR at the TRAC locus in T cells. Greater than 75% of protein knockout is achieved at each target, with a minimal impact on cell viability and with efficient tumour cell killing. Additionally, we engineered iPSCs for B2M and CIITA knockout in combination with human leukocyte antigen (HLA) E knock-in. Hypoimmunogenic iPSC clonal lines were easily and quickly derived from the population of cells that had undergone multiplexed base editing with targeted transgene integration. These clones retained genomic integrity, pluripotency, and ability to differentiate, and exhibited the expected reduced immune responses associated with their modified HLA phenotypes when differentiated to therapeutic cell products.

The modular and aptamer-dependent nature of the Pin-point base editing platform allows for complex genomic perturbations, including multiplex gene knockout and knock-in in a single intervention, with a wide range of uses across oncology and autoimmunity.

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4 Precision Biomaterials-Mediated Chronic Stimulation Sustains Supernumerary Expansion of Human CAR-T Cells In Vitro

Xiao Huang[1], Wendell Lim[2], Qizhi Tang[3], Tejal Desai[3]
[1]Drexel University, Philadelphia, PA, [2]University of California, San Francisco, San Francisco, CA, [3]University of California San Francisco, San Francisco, CA

Introduction Chimeric antigen receptor (CAR) T cell therapy has shown remarkable efficacy against B-cell malignancies, but challenges like relapse and limited durability persist. Chronic antigen exposure in cancer leads to divergent subsets: memory progenitors capable of expanding and differentiating into functional effectors upon antigen re-encounter, and exhausted T cells with restricted expansion and functionality. Effective priming of T cells into robust effectors and high-quality memory cells requires optimal levels of stimulatory and costimulatory signals. Synthetic biomaterials can be functionalized to mimic cell-cell crosstalk for T cell programming, allowing precise control over various chemical and physical parameters to engineer cell phenotype and dissect into fundamental mechanisms.

Methods In our prior work, we innovated a DNA scaffold-based approach on biomaterial surfaces that enables dense packing and precise ratio control of various stimulatory signals to effectively engage T cells. In this study, poly(lactic-co-glycolic) acid (PLGA) microparticles were functionalized with CAR-antigens and costimulatory ligands (CD28 agonist antibodies) at precisely controlled densities and ratios (precision biomaterials, PB) to stimulate human $CD8^+$ EGFR-CAR T cells *in vitro* (Fig. 1A).

Results We discovered that PB with an optimal 9:1 ratio of the two signals sustained T cell proliferation for over 100 days through continuous stimulations every 8-10 days, achieving cumulative expansions of ~10^{12}-10^{18} folds (Fig. 1B). Such supernumerary expansion is consistently achieved across multiple donors with both CD28- and 41BB-based 2nd-generation CAR constructs (Fig. 1B). In contrast, repeated stimulation by cancer cells (U87 line) resulted in a rapid loss of viability and stimulations by the gold standard CD3/28-Dynabeads led to significantly less overall expansion (Fig. 1C). Importantly, the massively expanded cells within ~80 days showed robust respiration capacity and potent

effector function against cancer targets (Fig. 2A-D). Interestingly, PB stimulated a gene expression program of sustained cell proliferation, restrained cell differentiation and cytokine signaling, telomere preservation, and enhanced mitochondrial respiration, diverging from U87-induced pathways that favor differentiation and cytotoxicity. Functional analysis revealed a temporary increase of telomerase activity in the first 1-2 rounds of stimulation by biomaterials, compared to U87 cells. The increase corresponded to stable telomere length within the first 30 days. Furthermore, single-cell RNA sequencing with TCR repertoire analysis revealed marked clonal enrichment of populations with characteristics of memory progenitors and durable effector-like state (Fig. 2E-K).

Conclusions These findings demonstrate that human peripheral blood harbors T cells capable of continuous supernumerary expansion while retaining effector potential. Importantly, precision biomaterials can induce decoupled proliferation versus differentiation signaling to support the continuous expansion of memory progenitors *in vitro*. These insights lay a crucial foundation for new cell engineering and manufacturing designs.

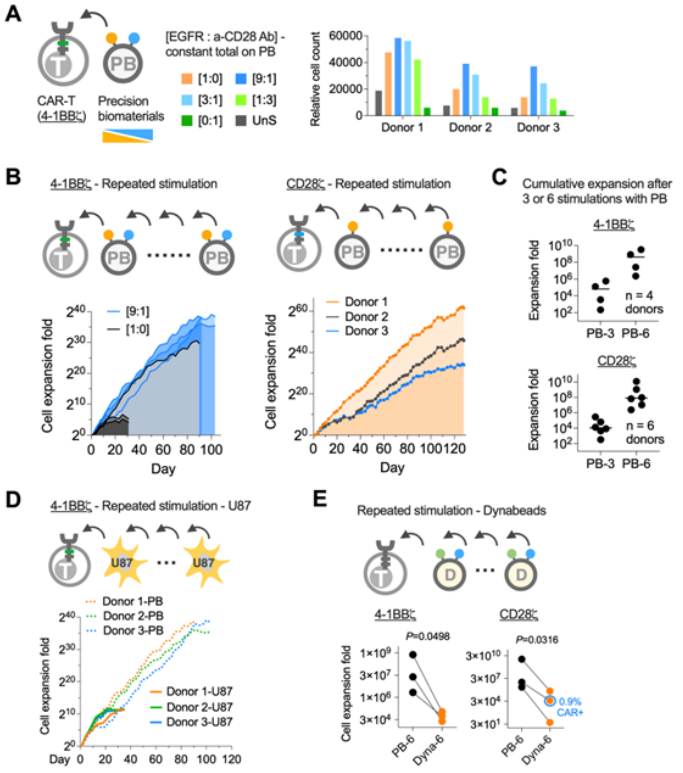

Figure 1 A-C) Supernumerary expansion of human a-EGFR CAR-T cells using precisely functionalized PLGA microparticles. **D,E**) Significantly less expansion and sustainability by cancer line (U87) or CD3/28-Dynabeads stimulations.

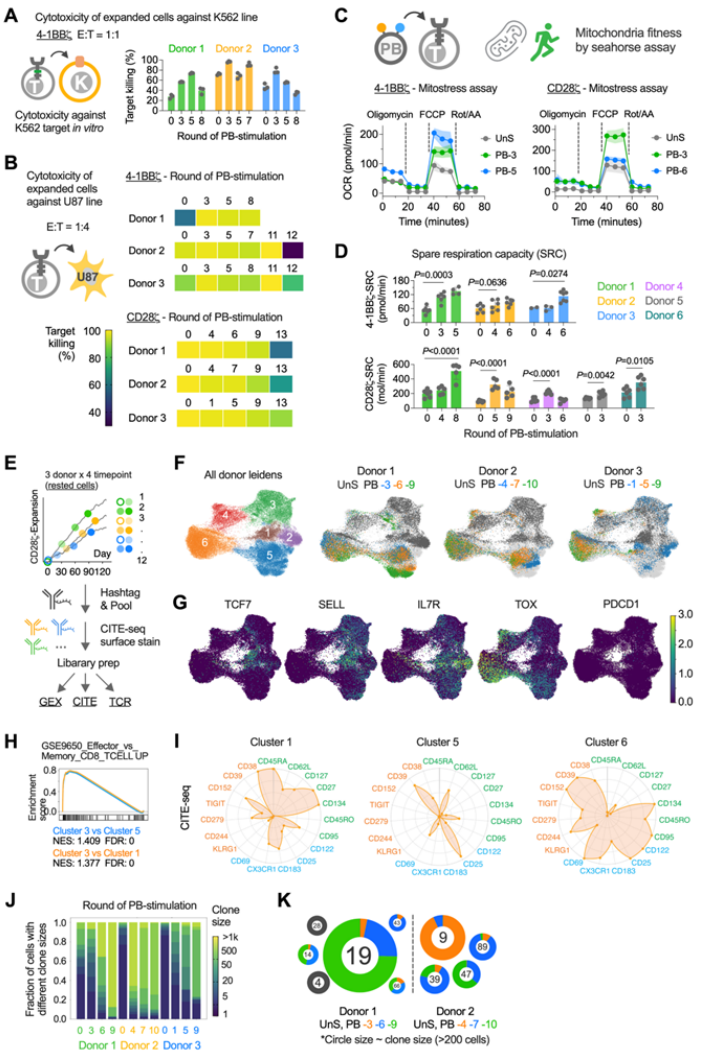

Figure 2 A,B) Potent cytotoxicity of massively expanded CAR-T cells along 9 to 11 rounds of biomaterial stimulations. **C,D**) Robust mitochondria fitness and respiration capacity of expanded cells up to 8-9 rounds of biomaterial stimulations. **E-I**) Single-cell RNA sequencing analysis identifies two distinct sub-populations, cluster 5 and 6, with characteristics of memory progenitors and durable effector state. **J,K**) TCR-based clonal tracking reveals enrichment of long-lived clones.

5 Single Cell Analysis of Longitudinal CSF from Glioblastoma Patients Post Intrathecally Delivered EGFR-IL13Rα2 CAR T Therapy

Nelson Freeburg[1], Daniel Chafamo[2], Lamia Lamrani[3], Kelly Hicks[4], Joshua Waterfall[5], Edward Wherry[4], Sebastian Amigorena[4], Joseph Fraietta[4], Stephen Bagley[2], Donald O'Rourke[6], Zev Binder[6]*, Cécile Alanio[7]*, **Dana Silverbush[8]***

[1]Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, [2]Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA, [3]Clinical Immunology Laboratory, Institut Curie, Paris, Paris, France, [4]University of Pennsylvania, Philadelphia, PA, [5]PSL Research University, Institut Curie Research Center, Paris, Paris, France, [6]University of Pennsylvania Perelman School of Medicine, [7]Institut Curie, Paris, Paris, France, [8]University of Pennsylvania School of Medicine, Philadelphia, PA
**Corresponding Author*

Glioblastoma (GBM) is the most common and lethal primary malignant brain tumor in adults, with a median survival of less than 15 months. The advent of chimeric antigen receptor (CAR) T cell therapy has emerged as a potential breakthrough in GBM

treatment, offering a targeted immunotherapeutic approach. We previously reported interim results from a phase I clinical trial demonstrating that intrathecal delivery of bivalent CAR T cells targeting the EGFR epitope 806 and IL13Rα2 led to reductions in enhancement and tumor size in all six patients with recurrent GBM. However, a subset of patients experience relapse after initial tumor regression. The molecular mechanisms underlying long-term efficacy remain unclear.

In this study, we conduct single-cell analysis of longitudinal pre- and post-treatment cerebrospinal fluid (CSF) and GBM samples, complemented with matching infusion product and blood. Using these unique settings and samples, we delineate the in-patient mechanisms of initial treatment response, subsequent immune evasion, and potential biomarkers predictive of therapeutic efficacy and decline.

Preliminary analysis of the CSF at Day 7 shows a mixture of CAR T cells, as well as abundant non-CAR and regulatory T cells. CSF-circulating CAR T cells have a distinct transcriptional profile compared to non-CAR T cells, with increased cytotoxicity and T cell exhaustion. Analysis of the evolution of these features longitudinally in the CSF is ongoing. Additionally, qPCR data performed on blood samples indicate delayed trafficking into the blood at Day 4. We are now exploring the characteristics of the clonotypes shared across the blood-brain barrier. Finally, we are further sorting CD45-negative tumor cells and CD45-positive hematopoietic cells from the tumors and performing single-cell transcriptomics on each. Our goal is to understand if local infusion of CAR T cells leads to infiltration of CAR T cells in the CSF and tumor, how this treatment modifies the tumor microenvironment and target characteristics, and how exposure to the tumor impacts intratumoral and circulating CAR T cells.

Novel Targets and Effector Cells

6 Tracking CARs; Cell Surface Barcoding Novel Multi-targeted CAR T Cell Therapies for High Grade Gliomas

Yasmin Nouri[1], Krishneel Prasad[1], **Ryan Cross[2]***, Misty Jenkins AO[3]*
[1]Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia, [2]Walter and Eliza Hall Institute of Medical Research Immunology Division, Parkville, Victoria, Australia, [3]Walter and Eliza Hall Institute, PARKVILLE, VIC, Australia
**Corresponding Author*

Paediatric and adult high-grade gliomas (HGG) comprise a rare and intractable subset of central nervous system cancers with an abysmal 5-20% survival rate. Maximal surgical resection (where possible) followed by concomitant adjuvant chemoradiotherapy is the standard of care for both adults and children, which has stagnated for two decades. Recent Phase I clinical trial reports using Chimeric Antigen Receptor (CAR) T cell therapy have demonstrated significant clinical efficacy in HGG. However, despite some patients' tumours completely vanishing within days, we must acknowledge that almost all patients' tumours eventually relapsed. Using cell surface mass spectrometry proteomics of primary HGG tumours to identify new CAR target we aim to tackle the inherent tumour heterogeneity to increase CAR T cell therapy efficacy to increasing patient survival.

Analysis of the top quartile cell surface proteins expressed in primary tumours resulted in the identification of 29 different target antigens. A library of 56 different antibodies were converted into single chain antibodies, including positive and negative controls, which were then screened in a pool of 112 second generation CARs in both Heavy-Light and Light-Heavy orientation. Top hits were validated *in vitro* and *in vivo*, before being combined with different ProCodes (cell surface flow cytometry barcodes) to facilitate tracking of CAR expression in multi-targeting experiments *in vitro* and *in vivo*. ProCode barcoding facilitates the tracking of up to 63 multi-CAR populations expressing one of six different targeted CARs. Each subpopulation of CAR combinations can be examined within the pool using high dimensional flow cytometry, facilitating the interrogation of optimal CAR combinations as well as examining CAR/antigen dynamics within in a single pool of cells through co-culture with different target cell populations.

The successful implementation of CAR T cell therapies in a solid tumour setting will require the development of novel CARs, as well as determining optimal combinations of these CARs in order to cover the inherent heterogeneity. The methodologies we outline here have led to significant biological insight into what is at the surface of a tumour cell, which has allowed us to identify hundreds of novel targets which can then be used to create new CAR T cell therapies. Recent CAR T cell clinical outcomes in brain cancer, both adult and paediatric, have clearly demonstrated that if the right target, eg GD2, or combination of targets, eg IL13Ra2+EGFR, is translated then these highly refractory tumours can be responsive to CAR T cell therapy.

7 The Augmentation of Smac Levels through an Armed Vesicular Stomatitis Virus Enhances Antitumor Immunity by Triggering PANoptosis

Yong Teng[1]*, Fanghui Chen[1], Liwei Lang[2], Ming Luo[3]
[1]Emory University School of Medicine, Atlanta, GA, [2]Augusta University, augusta, GA, [3]Georgia State University, Atlanta, GA
**Corresponding Author*

Oncolytic therapy represents an emerging approach in the fight against tumors, utilizing natural or genetically engineered oncolytic viruses (OVs) to specifically target and inhibit tumor growth. Despite documented instances of successful clinical outcomes, certain tumors exhibit resistance to OV infection. The protein second mitochondria-derived activator of caspases (Smac) plays a pivotal role in apoptosis by binding to inhibitor of apoptosis proteins, thus enabling the activation of caspases. We have observed a significant reduction in endogenous Smac levels in head and neck cancer (HNC) cells during vesicular stomatitis virus (VSV) infection. To overcome these challenges, we developed an armed VSV (VSV-S) by incorporating a transgene to express Smac/DIABLO during viral infection. Here, we report for the first time that elevation of Smac levels by VSV-S infection enhances the antitumor immune response in HNC by inducing tumor cell PANoptosis, a new mode of cell death combines the key features of pyroptosis, apoptosis, and necroptosis. Our mechanistic analysis further demonstrates that VSV-S induces HNC cell

PANoptosis through co-activation of caspase-1-GSDMD (pyroptosis), caspase-3 (apoptosis), and MLKL (necroptosis) signaling cascades. In a mouse model of orthotopic tongue tumors, VSV-S demonstrated superior potential to inhibit tumor growth and progression compared to VSV alone. It also facilitated enhanced recruitment and activation of T cells within the tumor microenvironment and reversed the immunosuppressive milieu, promoting T cell-mediated antitumor immunity through induction of PANoptosis. These groundbreaking findings not only reveal a novel mechanism underlying VSV-S-mediated antitumor efficacy, but also represent a potential therapeutic strategy to enhance the success of oncolytic therapy for cancer patients.

8 ADI-270: An Armored Allogeneic Anti-CD70 CAR γδ T cell Therapy Designed for Enhanced Potency and Persistence Against Multiple Oncology Indications

Shon Green[1]*, Gauri Lamture[2], Yvan Chanthery[3], Melinda Au[2], Alexander Teague[2], Morgan Smith-Boeck[2], Michael Salum[2], Jie Zhang[2], Yogendra Verma[2], Ramandeep Kaur[2], Smitha Gundurao[2], Amy Doan[2], Jyothi Sethuraman[2], Ngoc Hoang[3], Hayden Tessman[2], Philip Storm[2], Soojin Han[2], Swapna Panuganti Panuganti[2], Christopher Rold[3], Marissa Herrman[2], Meenakshi Bhat[4], Arun Bhat[2], Kevin Nishimoto[3], Blake Aftab[2]
[1]Adicet Therapeutics, Oakland, CA, [2]Adicet Therapeutics Inc, Redwood City, CA, [3]Adicet Therapeutics, Redwood City, CA, [4]Centre for Human Genetics
**Corresponding Author*

Background CD70 is a compelling target for CAR T cell therapy, given its elevated expression across multiple solid and hematologic malignancies, and with expression in normal tissues limited to activated lymphocytes. γδ T cells combine innate and adaptive anti-tumor immunity with a natural ability to traffic to tissues, including solid tumors. They also represent a preferred platform for allogeneic cell therapy as their TCR recognizes MHC-independent antigens, avoiding the risk of graft-versus-host disease without the need for gene editing. To build on these features, we developed ADI-270, an allogeneic γδ CAR T cell product engineered with a next-generation CAR to target CD70 using its natural receptor (CD27) and armored with a dominant negative TGFβ receptor (dnTGFβRII) that provides functional resilience to the immunosuppressive tumor microenvironment. ADI-270's design may also provide enhanced persistence through targeting of activated, CD70-expressing alloreactive host lymphocytes.

Methods Healthy donor PBMCs were used to activate, expand, and engineer Vδ1 T cells to express the armored anti-CD70 CAR construct. Functional characterization of ADI-270 was determined in vitro using a variety of cell culture assays, and the activity and safety of ADI-270 was also compared to multiple clinically relevant benchmarks representing scFV-based anti-CD70 αβ CAR T cell approaches. Human tumor xenografts in NSG mice were used to evaluate the in vivo efficacy and kinetics of ADI-270 activity against solid and hematological cancers.

Results ADI-270 exhibited potent in vitro cytotoxicity against a panel of cancer cell lines expressing varying levels of CD70. In contrast to αβ CAR T cell benchmarks, ADI-270 retained potent activity against low CD70 expressing cells and CD70+/- cancer cell mixtures, indicating an ability to extend function to cancer models with low and heterogenous antigen expression. Despite this increased potency, ADI-270 demonstrated a lower inflammatory cytokine profile when co-cultured with CD70+ renal cell carcinoma (ccRCC) A498 cells in the presence of macrophages and lower bystander killing of HUVEC cells as compared to αβ CAR T benchmarks. ADI-270 demonstrated improved persistence when compared to a CAR not targeting CD70 in co-culture with primed allogeneic T cells, suggesting ADI-270 has the potential for decreased host-versus-graft rejection. ADI-270 significantly inhibited tumor growth in CD70+ ccRCC (A498), T cell lymphoma (Hut78), Acute Myeloid Leukemia (THP-1) and Multiple Myeloma (MM1.S) human tumor xenograft models in NSG mice, some of which represent tumors with very low and heterogenous CD70 expression. In A498 tumor xenografts, robust ADI-270 tumor infiltration, proliferation, and effector function were observed as early as day 3, leading to complete eradication of CD70+ tumor cells.

Conclusion These results suggest that ADI-270 may exhibit superior potency and persistence against multiple cancers compared to relevant benchmarks. ADI-270 demonstrated innate anti-tumor immunity, potent CAR targeting, resilience to attenuation by TGFβ, reduced rejection by alloreactive host T cells, and favorable preclinical efficacy and safety characteristics compared to scFv-based CD70-targeting αβ CAR T cell benchmarks representing constructs in clinical development. Supported by these data, ADI-270 has potential to address multiple CD70+ cancers and initial clinical evaluation is proceeding in relapsed/refractory ccRCC.

9 Bioreducible LiPBAE miR-590-3p NanomiRs Inhibit Recurrent Glioblastoma Growth and Prolongs Survival

Hernando Lopez-Bertoni[1]*, Sophie Sall[2], Jordan Green[3], Stephany Tzeng[4], Kathryn Luly[5]
[1]Johns Hopkins School Of Medicine, Baltimore, MD, [2]Johns Hopkins University School of Medicine - Baltimore, MD, Baltimore, MD, [3]Johns Hopkins University, Baltimore, Maryland, [4]Johns Hopkins University, Baltimore, MD, [5]Johns Hopkins University School of Medicine, Baltimore, MD
**Corresponding Author*

Despite aggressive therapy consisting of surgery followed by radio/chemotherapy Glioblastoma (GBM) recurs in almost all patients and, currently, there are no proven therapies to treat recurrent GBM (rGBM). Recent developments in nanomedicine provide new and promising opportunities to develop new targeted therapeutics to treat brain tumors. In this study we combine bioinformatics, forward-thinking understanding of miRNA biology and cutting-edge nucleic acid delivery vehicles to advance targeted therapeutics for rGBM. Bioinformatic analysis of RNA sequencing from GSCs and clinical rGBM specimens identified TGF-beta receptor II (TGFBR2) signaling as a targetable pathway in rGBM. Mechanistically, we show that alterations in chromatin state driven by stem-cell driving events are conducive to a therapy-resistant state induced by TGFBR2. We show that blocking TGFBR2 via molecular and pharmacological approaches decreases the stem cell capacity, cell viability and re-sensitizes clinical rGBM isolates to temozolomide (TMZ) *in vitro*. miRNA-based

network analysis uncovered miR-590-3p as a tumor suppressor that efficiently simultaneously inhibits multiple oncogenic nodes downstream of TGFBR2 reducing self-renewal capacity of therapy-resistant GSCs. To translate these in vitro finding, we developed novel bioreducible Lipophilic poly(β-amino ester) nanoparticles (LiPBAEs) for *in vivo* miRNA delivery. Following direct intratumoral infusion, these nanomiRs efficiently distribute through the tumors and mir-590-3p nanomiRs inhibited the growth and extended survival of animals bearing orthotopic human rGBM xenografts, with apparent curative effects in 3 of 10 treated mice. These results show that miRNA-based targeted therapeutics provide new opportunities to treat rGBM and bypass the resistance that is developed to standard of care.

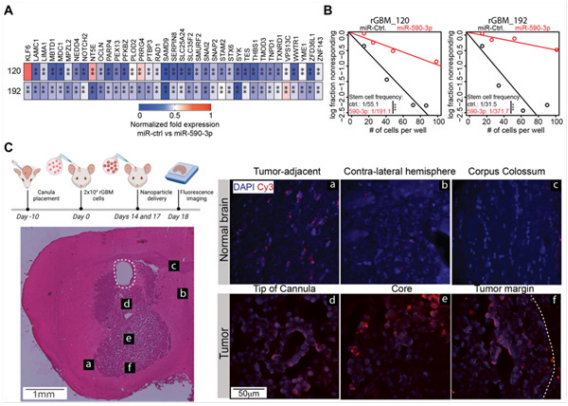

Figure 1: Polymer E49 is has low toxicity and effectively delivers miRNAs to GBM cells in vitro and in vivo. (A) qRT-PCR analysis showing expression of miR-590-3p targets 5 days after nano-miR transfections in rGBM clinical specimens. **(B)** ELDA assay to measure stem cell frequency 14 days after nano-miR transfection in rGBM clinical specimens. **(C)** Schematic showing in vivo nano-miR delivery using E49 nanoparticles containing fluorescently-tagged control miRNA (top left panel). H&E-stained section showing established orthotopic rGBM (bottom left panel). Dotted lines outline canula track and boxes mark regions used to capture fluorescence images. Student T-test was used to determine

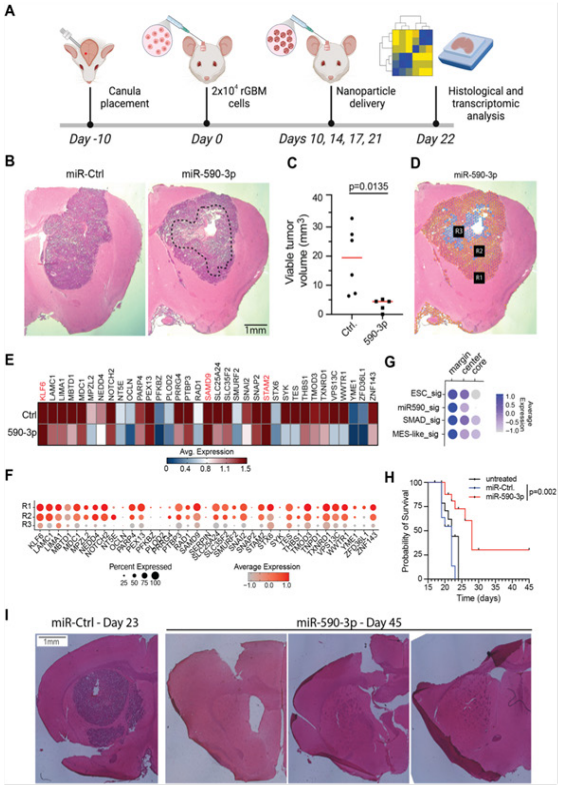

Figure 2: In vivo delivery of miR-590-3p inhibits rGBM growth an extends survival in orthotopic rGBM mouse models. (A) Schematic showing the in vivo delivery of miR-590-3p using E49 nanoparticles. **(B)** H&E-stained brain sections from mice bearing orthotopic rGBM 22 days after receiving nanoparticles loaded with control miRNA or miR-590-3p. Dotted line outlines necrotic region. **(C)** Viable tumor was measured from H&E-stained sections using computer assisted image analysis. **(E)** Transcriptomic analysis of miR-590-3p targets in mice that received control miRNA or miR-590-3p. **(F)** Transcriptomic analysis looking at expression of miR-590-3p targets in mice that received miR-590-3p nanoparticles. **(G)** GSEA from transcriptomic analysis of tumors treated with miR-590-3p looking at gene signatures associated with therapy resistance in GBM. **(H)** Kaplan-Meier survival curve comparing animals that received control miRNA, miR-590-3p or no treatment. Therapy in the survival study was initiated 7 days after tumor cell implantation. Survival was compared across arms using the log-rank test (N=10). **(I)** H&E-stained sections showing the last animal in the control group and the 3 long-term survivors. Statistical significance was calculated using unpaired, non-parametric, student T-test with Mann–Whitney post hoc test in panel **C** and **D.**

10 Tumor Associated Mitochondria Antigens (TAMAs) Vaccine Is Effective on Tumor and Tumor Associated Endothelial Cells: From the Mechanisms to the Clinical Validation

Francesca Costabile[1], Stefano Pierini[2], Renzo Perales[3], Adham Bear[3], Cameron Koch[1], Gerald Linette[4], Beatriz Carreno[5], Michael Lotze[6], Constantinos Koumenis[1], Andrea Facciabene[7]*
[1]University of Pennsylvania, Philadelphia, PA, [2]Carisma Therapeutics, Philadelphia, [3]Geneos Therapeutics, Philadelphia, PA, [4]Perelman School of Medicine, Philadelphia, PA, [5]University of Pennsylvania, [6]Hillman Cancer Center, Pittsburgh, PA, [7]Upenn: University of Pennsylvania
**Corresponding Author*

Background: A major challenge for immunotherapy is the scarcity of effective cancer-specific antigens. Identifying novel tumor-associated antigens (TAAs) is essential to advancing modern immunotherapy strategies. Altered energy metabolism, excessive reactive oxygen species production, and physical vicinity of the mitochondrial (mt)DNA to the oxidative phosphorylation chain are all factors associated with somatic mtDNA mutations in cancer. Somatic mutations in mtDNA, named by us as Tumor Associate Mitochondria Antigens (TAMAs), make mitochondria ideal candidates as a source for TAAs as they meet all the criteria for an ideal vaccine: they are immunogenic, specific to cancer, and functionally important.

Methods: Our group developed an effective antitumor dendritic cells (DCs)-based vaccine using mt lysates from renal cell carcinoma (RENCA), which contain immunogenic mtDNA mutations. We detected a higher expression of the Immune Checkpoint PD1/PD-L1 pathway upon vaccination in the tumor microenvironment hence we combined the DCs based TAMAs vaccine with ICI. We analyzed how the combination of the two treatments impacts the tumor growth, the tumor microenvironment and its immune system and vasculature.

Results: The combination of TAMAs vaccine and the ICI (Fig, 1A), improves the control of tumor progression compared to single therapy (Fig. 1B) and increases survival rate augmenting infiltration and reactivity of T cells (CD8+ IFNγ+) (Fig. 1C) and decreasing myeloid derived suppressor cells (CD11b++ GR1++) within the tumor. Interestingly, the combination of treatments favored tumor vessel normalization, which benefited tumor blood perfusion and decreased hypoxia. This consequently enhanced the infiltration of reactive T cells within the tumor, thereby boosting the efficacy of the TAMAs vaccine. To validate the translational relevance of the study, we demonstrated the immunogenicity of TAMAs in human samples: notably, 100% of the tested healthy donors' CD8+ T cells responded to at least three of the derived human TAMAs peptides. Furthermore, given the emerging importance of mitochondrial exchange between tumor cells and surrounding cells, including endothelial cells (ECs), we investigated the natural mt transfer from tumor cells to ECs. We demonstrated this transfer in murine (Fig. 2A) and human (Fig. 2B) models, as well as in tumor tissues from patients. Additionally, for the first time, we showed that ECs which have internalized tumor mitochondria, and hence TAMAs, are recognized by primed TAMAs T cells (Fig. 2C).

Conclusions: In conclusion, we validate the immunogenicity of TAMAs in human and unveil a new immunological mechanism of tumor vasculature pruning opening a new strategy that could improve cancer therapies targeting both cancer and ECs.

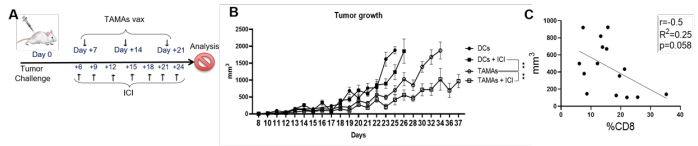

Figure 1. Administration of the TAMAs vaccine in combination with ICI contributes to control tumor progression due to T cells infiltration. A) Therapeutic treatment protocol that combines TAMAs vaccination with immune checkpoint inhibitor (ICI): mice were challenged s.c. with 10^6 RENCA cells and were vaccinated 1 week later with DCs pulsed with TAMAs or DCs alone (DCs) and with or without αPDL-1. **B)** RENCA tumor growth measurements shows enhancement of vaccine efficacy when in combination with ICI. **C)** Negative correlation between intratumor % of CD8+ T cells detected via flow cytometry and tumor sizes.

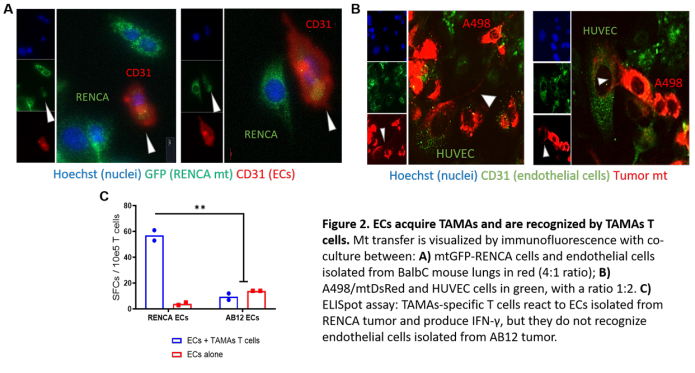

Figure 2. ECs acquire TAMAs and are recognized by TAMAs T cells. Mt transfer is visualized by immunofluorescence with co-culture between: **A)** mtGFP-RENCA cells and endothelial cells isolated from BalbC mouse lungs in red (4:1 ratio); **B)** A498/mtDsRed and HUVEC cells in green, with a ratio 1:2. **C)** ELISpot assay: TAMAs-specific T cells react to ECs isolated from RENCA tumor and produce IFN-γ, but they do not recognize endothelial cells isolated from AB12 tumor.

POSTER ABSTRACTS

11 Lentiviral Vector-based Polymeric Nanoparticles as Gene and Cell Therapy Product - A cross-functional Approach to Streamline and Control a Complex Manufacturing Process

Cecile Bauche[1], Céline Jaudoin[2], Julie BERGALET[3], Léa DANDAN[3], Sarra LESCHIUTTA, Alice COILLARD[3], Laurence SELLIER[3], Marion LHUAIRE[3], Eva MAUNICHY[3], Rachel PACHERIE[4], Frederic Mourlane, Renaud VAILLANT[1]
[1]Alaya.bio, Paris, Ville de Paris, France, [2]Alaya.bio, Antony, Paris, France, [3]Alaya.bio, PARIS, Paris, France, [4]PARIS, France

Intracellular delivery of nucleic acids to mammalian cells using non-viral gene delivery remains a challenge both *in vitro* and *in vivo*, with transfections often suffering from variable efficacy and limited stability. Moreover, physicochemical properties of nano-sized materials play a significant role on the *in vivo* fate of nanomaterials. Particle size, size distribution, global charge or shape are defined as the key parameters by health agencies for the manufacturing quality as well as the safety and efficacy of nano-sized particles.

Alaya.bio's platform is a unique technology that combines the versatility of cationic and shielding polymers as biodegradable and safe transfection material together with the gene transfer efficiency of lentiviral vectors (LVs). Here we report on the cross-functional approach we have implemented to control and optimize manufacturing process parameters of a complex nanoparticle-based therapeutic product.

Scalable and GMP-compatible bioprocesses have been engineered for the production of LVs lacking the VSV-G immunogenic protein. Process performance and repeatability have been demonstrated by verifying the integrity and functionality of viral particles (DLS, interferometry, sandwich ELISA, RT-qPCR, flow cytometry and qPCR). A portfolio of 40 families of cationic and shielding biodegradable and pH-responsive polymers (with diversified shape, MW and pKa) has been assembled and screened to formulate homogeneously sized LV-based nanoparticles (Dh<300 nm and PdI<0.3) that efficiently deliver *in vitro* transgenes to different types of human cells that are relevant for the development of gene and cell therapies.

Since many factors influence the coating of LVs by polymers such as active and inactive ingredient concentration, buffer composition and pH, mixing and incubation time, we took advantage of high throughput DLS and *in vitro* transduction assays to optimize the formulation development. Coating of the best functional nanoparticles has been scaled-up and tested for compatibility with microfluidics-based formulation in order to secure the translation towards industrial manufacturing. This iterative process based on the same orthogonal methods to characterize the LV raw material down to the final nanoparticle was instrumental in the refinement of the space design around our product.

By combining optimized bioproduction of lentiviral vector payload and high throughput screening of formulations with su-

perior biophysical and functional properties, Alaya.bio has now access to next generation nanoparticles that will be applied to rapid and universal manufacturing of CAR T-cells with preserved naive and memory phenotypes.

12 Towards Off-the-Shelf CAR-T Therapy: Lentiviral Vector-based Polymeric Nanoparticles for In Situ Gene Delivery into T-cells for Haematological Malignancies & Beyond

Cecile Bauche[1], Alice COILLARD[2], Laurence SELLIER[2], Marion LHUAIRE[2], Eva MAUNICHY[2], Céline Jaudoin[3], Julie BERGALET[2], Léa DANDAN[2], Sarra LESCHIUTTA, Rachel PACHERIE[4], Frederic Mourlane, Renaud VAILLANT[1]
[1]Alaya.bio, Paris, Ville de Paris, France, [2]Alaya.bio, PARIS, Paris, France, [3]Alaya.bio, Antony, Paris, France, [4]PARIS, France

With Abecma®, Carvykti®, Yescarta®, Kymriah®, Tecartus® and Breyanzi® marketed therapies for B cell hematological malignancies and more than 600 ongoing clinical trials, genetic modification of T cells with chimeric antigen receptors (CARs) recognizing surface antigens on tumor cells has emerged as a revolutionary therapeutic strategy in immuno-oncology. Despite impressive clinical benefits, the complex logistics required to manufacture *ex vivo* CAR-T cells from individual patients, the impact of such a process on the fitness and efficacy of engineered cells and the associated costs represent major hurdles to the widespread use of these therapies.

Alaya.bio is preclinical-stage biotechnology company developing *in situ* CARs based on nanoparticles consisting of lentiviral vectors lacking the VSV-G immunogenic protein and encoding anti-CD19 CARs coated with oligopeptide-modified poly(beta-amino ester)s biodegradable polymers.

Here we report on a microfluidics-based method that consistently manufactures polymeric nanoparticles able to reprogram *ex vivo* peripheral blood mononuclear cells to express a functional CAR. Importantly, stable delivery of the transgene is mediated without the need of CD3/CD28 activation and cytokines addition normally required with VSV-G pseudotyped LV for immune cells transduction. Fast and efficient nanoparticle-mediated gene editing of quiescent cells is achieved without labor-intensive processing and amplification which preserves reprogrammed cells from exhaustion, differentiation and does not jeopardize their functionality.

In vivo, the biodistribution of NPs has been investigated in immunocompetent and humanized mice after multiple intravenous administrations or infusions and showed a different profile compared to pseudotyped LVs. Long-term expression of GFP or CAR transgenes was detected in blood leukocytes up to 80 days post-treatment. The pronounced tropism for blood cells observed *in vivo* with our polymeric nanoparticles provides an obvious advantage for CAR T-cell therapy of blood malignancies.

Repeat administration was safe and well tolerated as no obvious sign of distress, body weight loss, hepatotoxicity, change in blood cell count or cytokine levels were reported. Preliminary efficacy data have shown in healthy Balb/c mice that repeat administration of anti-CD19 CAR encoding nanoparticles induced B cell depletion. In mice with A20-induced lymphoma, repeat treatment cycles showed promising anti-cancer efficacy and reinfusion at relapse was possible.

This technology shows great potential for further preclinical and clinical development of *in situ* universal CAR T-cell therapy. In addition, Alaya.bio's versatile platform offers the potential to target and reprogram a broad range of therapeutic cells not limited to the immuno-oncology field but also various genetic disorders

13 An Optimal Pairing of scFv and Costimulatory Domain Regulates Dual CAR T Cell Signaling for Better Response Against B-ALL

Divanshu Shukla[1], Sasikanth Manne[1], Shuguang Jiang[2], Marco Ruella[1], Saar Gill[1], Edward Wherry[1], James Riley[3]*
[1]University of Pennsylvania, Philadelphia, PA, [2]University of Pennsylvania Perelman School of Medicine, [3]The University of Pennsylvania, Philadelphia, PA
**Corresponding Author*

We explore whether introducing two chimeric antigen receptor (CAR) constructs with distinct signaling motifs into a single T cell improves efficacy against B cell derived tumors. RNA expression profiling confirmed that CARs targeting CD19 or CD22 linked to CD28 and CD3 zeta (28z) promoted canonical NF-κB pathways whereas CARs linked to 4-1BB and CD3 zeta (BBz) enhanced expression of genes linked to the non-canonical NF-κB signaling cascade. Interestingly, T cells co-expressing CARs in which one CAR was linked with 28z and the other BBz augmented both the canonical and non-canonical NF-κB pathways, as well as Th1/Th17 differentiation upon target engagement, leading to augmented expression of genes associated with effector function and survival of T cells. In vitro, we found that CD19 targeting dual CART cells able to maintain greater sensitivity against low Ag in vitro and killing efficiency after continuous Ag exposure. We also observed that T cells co-expressing CD19 targeting CAR linked to the CD28 and CD3 zeta chain (CD19.28z) were more functional than those expressing CD22 targeting CAR (CD22.28z) whereas no major differences were detected if the T cell co-expressed CD19.BBz or CD22.BBz. In vivo and in T cell exhaustion assays, however, we found that T cells that targeted both CD19 and CD22 rather than just one of these antigens with distinct signaling domain led to durable control of B-ALL with T cells co-expressing CD19.BBz and CD22.28z being the most potent. Together, this data indicates that optimal CAR signaling through both CD28 and 4-1BB bolsters anti-tumor efficacy by improving both T cell durability and effector function.

15 Bone-Marrow-Homing Lipid Nanoparticles for Genome Editing in Leukemia and Leukemic Stem Cells *(Virtual)*

Xizhen Lian, Johns Hopkins University, Baltimore, MD

Introduction: Therapeutic gene delivery and genome editing in hematopoietic stem cells (HSCs) would provide long-lasting treatments for multiple diseases. However, in vivo delivery of genetic medicines to HSCs remains challenging, especially in diseased and malignant settings. Here, we report on a series of bone marrow (BM) homing lipid nanoparticles (LNPs) that deliver mRNA to a broad group of at least 14 unique cell types in the

BM, including HSCs and progenitor cells, B cells, T cells, macrophages, and endothelial cells. On a bone-engrafted, MLL-AF9 driven acute myeloid leukemia (AML) model, bone-homing-LNPs were able to achieve Cre recombinase-mediated genetic deletion in bone- and spleen-residing leukemic stem cells and leukemia cells. Overall, we show evidence that diverse cell types in the BM niche can be edited using BM-homing-LNPs and demonstrate the therapeutic potential of this delivery technology for aggressive cancers in the hematological system.

Materials and Methods: We incorporated biologically active molecules as a supplemental lipid to the LNP base formulation. We started an initial screening from a molecular pool containing 41 molecules, including 16 carbohydrates, 6 vitamins, 7 amino acids, 5 hormones, 5 neurotransmitters, 1 nucleotide, and 1 covalent lipid that has the potential to form covalent bonds with amino acid residue, to identify those that confer a strong HSC affinity to LNPs. We examined transfection efficacy in different cell types in bone marrow on a tdTom reporter mouse strain, in which endogenous tdTom expression can be activated by Cre recombinase delivery. We established a reporter leukemia cell line by lentiviral transduction of MLL-AF9-IRES-YFP-encoding plasmid in Lin- cells isolated from tdTom reporter mice. Transduced cells were then transplanted into lethally irradiated recipient mice to establish bone-engrafted leukemia animals.

Results and Conclusion: Covalent lipid species demonstrated bone marrow homing capability when incorporated in LNP base formulation, and control experiments confirmed that the chemical reactivity, not the hydrolyzed product or head group geometry, or the covalent lipid was driving BM tropism. BM homing capability of covalent lipid species could be well maintained when incorporated in LNPs for COVID-19 vaccines, demonstrating a clinical pathway for tackling hematopoietic diseases. One of the best BM-homing-LNP formulation led to tdTomato expression activation in 44.8% Lin-Sca-1+CD117+ (LSK) population, 40.2% long-term HSCs, 17-66% in different progenitor cells, 7.19% B cells, 17.0% T cells, 32.1% macrophages, 7.55% monocytes, 22.9% neutrophils, and 13.0% endothelial cells after a single, low-dose intravenous injection. After confirming that BM-homing LNPs efficiently delivered mRNA to MLL-AF9 leukemia cells in vitro, we created an AML mouse model that mimics the clinical aggressiveness and sought to determine if BM-homing-LNPs could transfect leukemia cells in vivo. A single, low dose injection of BM-homing-LNPs encapsulating Cre recombinase mRNA led to tdTom expression activation in 13-18% leukemic stem cells in BM, the cell type that is believed to account for drug resistance and leukemia relapse, and in 2-4% BM or spleen residing leukemic cells. Overall, these results demonstrate the potential application of therapeutic genetic medicine delivery to treat aggressive cancerous diseases in hematological system through non-invasive, less toxic LNP strategy.

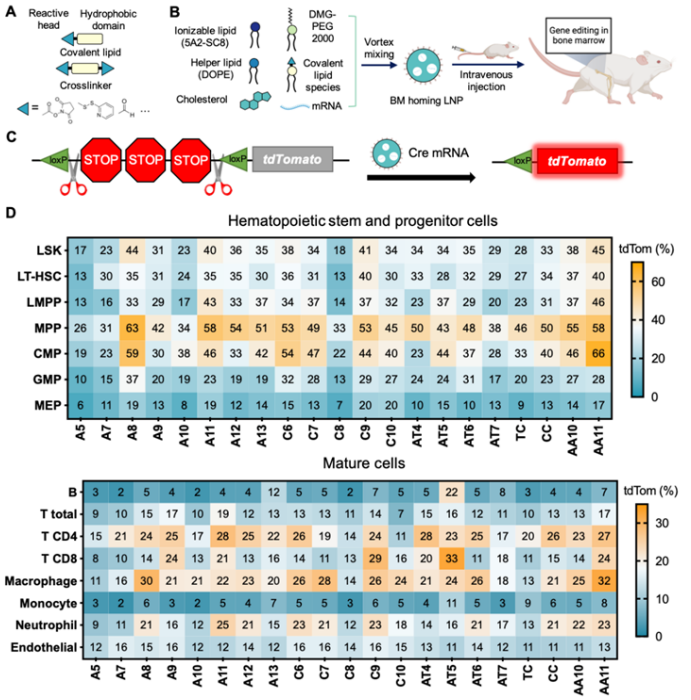

Figure 1. (A) Schematic of LNP preparation including covalent lipid species (covalent lipids and crosslinkers). **(B)** Addition of a covalent lipid or crosslinker to the base-4-lipid LNP formulation leads to BM mRNA delivery and genome editing in a great breadth of unique BM cell types. **(C)** Schematic showing how the delivery of Cre mRNA activates tdTom expression in tdTom transgenic mice via Cre-mediated genetic deletion of the stop cassette. **(D)** Flow cytometry was used to quantify Cre mRNA delivery efficacy in various BM cell types.

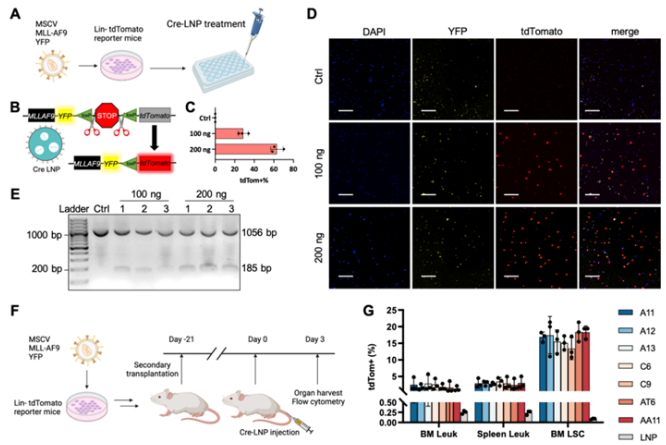

Figure 2. (A) The MLL-AF9-IRES-YFP gene was installed into the genome of Lin⁻ cells extracted from the fetal liver of tdTom reporter mice and the cells were incubated with LNPs containing Cre mRNA in a 24-well plate for 24 h. **(B)** Delivery of Cre mRNA activates tdTom expression in tdTom transgenic mice via the Cre-mediated genetic deletion of the stop cassette. **(C)** Summary of the percentage of tdTom+ cells measured from the confocal microscopy images of the edited MLL-AF9-IRES-YFP Lin⁻ cells. **(D)** Representative confocal microscopy images of the control and LNP-treated cells. Scale bar, 100 µm. **(E)** DNA agarose gel of the PCR amplicon performed from the genomic DNA extracted from the control and LNP-treated cells with primers flanking the Ai14 locus. **(F)** C57BL/6 recipient mice were lethally irradiated and received IV transplantation of MLL-AF9-transfected Lin⁻ cells. Three weeks after the transplantation, the BM and spleen were extracted from the primary transplant recipient animal and the isolated cells were transplanted into a secondary transplant recipient to establish the model for LNP study. Three weeks after the secondary transplantation, Cre mRNA BM-homing LNPs were injected via IV administration. Leukemic cells were harvested and analyzed 72 h after LNP injection. **(G)** Percentage of tdTom+ cells after LNP and BM-homing LNP injection on bone-engrafted leukemia animals. BM-isolated leukemic cells, leukemic stem cells residing in BM (BM LSCs) and spleen-isolated leukemic cells are characterized by flow cytometry.

16 A Novel Virus-Free Platform for Engineering CAR-T Cells with Enhanced Clinical Safety and Therapeutic Efficacy in Acute Lymphoblastic Leukemia

(Virtual)

Philip Chiu-Tsun TANG[1], Kam Tong Leung[2], Margaret Heung Ling Ng[3], Ka-Fai To[4], Patrick Ming Kuen Tang[1]*
[1]Department of Anatomical and Cellular Pathology, State Key Laboratory of Translational Oncology, Prince of Wales Hospital, The Chinese University of Hong Kong, Hong Kong, Hong Kong, Sha Tin, Hong Kong, [2]Department of Paediatrics, The Chinese University of Hong Kong, Hong Kong, Prince of Wales Hospital, Sha Tin, Hong Kong, [3]Blood Cancer Cytogenetics and Genomics Laboratory, Department of Anatomical and Cellular Pathology, Prince of Wales Hospital, The Chinese University of Hong Kong, Hong Kong, The Chinese University of Hong Kong, Sha Tin, Hong Kong, [4]Department of Anatomical and Cellular Pathology, State Key Laboratory of Translational Oncology, Prince of Wales Hospital, The Chinese University of Hong Kong, Hong Kong, The Chinese University of Hong Kong, Sha Tin, Hong Kong
**Corresponding Author*

Chimeric antigen receptor T (CAR-T) cell therapy showed promising therapeutic efficiency for blood cancers. However, the CAR-T cells are conventionally produced with virus-based methods, which causes additional carcinogenic risk and quality assurance costs. However, non-viral alternatives with comparable transfection efficiency is still yet available. Here, we successfully developed a novel virus-free platform for CAR-T engineering by combining our low-energy non-viral gene transfer system with CRISPR/Cas9 gene editing. This approach offers a highly efficient and safe method for CAR-T engineering. Encouragingly, our system showed superior transfection rate (>60%) and cell viability (>80%) on primary human T cells derived from the peripheral blood, representing a non-viral option which is comparable to the conventional methods based on the virus. More importantly, we not only evidenced the safety of the produced T cells by confirming the precision CAR insertion at genomic level, but also demonstrated their enhanced anti-cancer activity for CD19+ leukemia in vitro and in vivo. Thus, our novel virus-free method may represent a safe and effective platform for advancing CAR-T cell therapy in the clinics.

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17 Efficient Combinatorial Adaptor-Mediated Targeting of Acute Myeloid Leukemia with CAR T-Cells

Laura Volta[1], Renier Myburgh[2], Christian Pellegrino[2], Christian Koch[1], Monique Maurer[1], Francesco Manfredi[3], Mara Hofstetter[1], Anne Kaiser[1], Florin Schneiter[4], Jan Müller[1], Marco M. Buehler[1], Roberto De Luca[5], Nicholas Favalli[5], Chiara Magnani[6], Timm Schroeder[4], Dario Neri[7], Markus G. Manz[1]*
[1]University of Zurich Faculty of Medicine, Zurich, Zurich, Switzerland, [2]University of Zurich Faculty of Medicine, SWITZERLAND, [3]University Hospital Zurich, SWITZERLAND, [4]ETH Zurich, Basel, Basel-Stadt, Switzerland, [5]Philochem AG, Otelfingen, Zurich, Switzerland, [6]Universitätsspital Zurich, University of Zurich, Zurich, Switzerland, [7]Philochem AG, SWITZERLAND
**Corresponding Author*

Introduction: Chimeric Antigen Receptor (CAR) T-cell therapies effectively treat B- and plasma-cell malignancies by targeting cell-of-origin antigens, eliminating both tumor cells and healthy counterparts. Their loss can be compensated by immunoglobulin substitution until regeneration from hematopoietic stem and progenitor cells (HSPCs) occurs. However, in HSPC-derived malignancies like acute myeloid leukemia (AML), similar on-target, off-tumor cell-of-origin elimination would be detrimental, as CAR T-cell activity might lead to terminal ablation of hematopoiesis. In principle, CAR T-cells could be used to abrogate both AML and HSPCs, followed by HSPC transplantation, ensuring hematopoietic recovery after termination of CAR T-cell activity and potentially avoiding genotoxic preconditioning. To address this challenge, we developed adaptor-mediated (AdFITC) CAR T-cells expressing an anti-fluorescein single-chain variable fragment, which binds to fluorescein-conjugated antigen-binding adaptors in a diabody format. These adaptors act as safety switches, modulating CAR T-cell activity.

Methods: We developed second-generation AdFITC-CAR T-cells and diabodies targeting AML antigens CD117 and CD33 conjugated with fluorescein to create bridging adaptors. The efficacy of these cells and adaptors was tested against AML cell lines and primary AML blasts *in vitro* and *in vivo* using xenograft models in NSG mice.

Results: We generated AdFITC-CAR T-cells and diabody-based adaptors against selected AML antigens. Diabodies specific for CD117 and CD33 exhibited high purity and retained their antigen-binding properties. Combinatorial staining with both adaptors increased fluorescein decoration of target cells, enhancing AdFITC-CAR T-cell target density. *In vitro* cytotoxicity assays showed that dual adaptors significantly enhanced tumor cell lysis compared to single adaptors. Single-cell time-lapse imaging revealed accelerated tumor cell lysis and enhanced CAR T-cell engagement with dual adaptors. Pharmacokinetic studies showed that the diabodies had a short serum half-life but maintained prolonged on-tumor residence, crucial for sustained CAR T-cell activation. *In vivo* studies using xenogeneic mouse models engrafted with AML cell lines or primary AML blasts demonstrated that AdFITC-CAR T-cells, in combination with CD117 or CD33 adaptors, were as effective as direct CAR T-cells against the same antigens. The combination of both adaptors inhibited tumor growth more effectively than monotherapies, leading in some cases to complete AML elimination in bone marrow and blood, confirmed by bioluminescence and terminal flow cytometry analysis.

Conclusions: We here tested an adaptor CAR T-cell approach using multiple adaptors to on-off modulate and enhance CAR T-cell activity, focusing on AML as a relevant disease model with high clinical need for therapeutic improvement. The heterogene-

ity in antigen expression on AML cells supports a combinatorial targeting strategy individualized based on AML immunophenotype. Due to their relatively small molecular weight (~55kDa), diabody-based adaptors are rapidly cleared via the kidneys, allowing rapid control over AdFITC-CAR T-cell on-off activity prior to HSPC transplantation. We envision that this approach has the clinical potential to improve CAR T-cell safety profiles by enhancing the specificity towards target cells while ameliorating side effects against healthy tissues expressing a single antigen of the combination.

18 Human Cord Blood-Derived Induced Pluripotent Stem Cells into Natural Killer Cells for Advanced Cancer Immunotherapy

RANRAN ZHANG[1], Daniel Terheyden-Keighley[2], Tejashree Redij[3], Boris Greber[2], Xuan Guo[3]*, Junxia Wang[4]*
[1]Catalent Cell & Gene Therapy, Princeton, NJ, [2]Catalent Cell and Gene Therapy, Düsseldorf, Germany, Rheinland, Rheinland-Pfalz, Germany, [3]Catalent Cell and Gene Therapy, Princeton, USA, Princeton, NJ, [4]Catalent, Princeton, NJ
**Corresponding Author*

Induced pluripotent stem cells (iPSCs) have the ability to unique differentiate into nearly any cell type, including natural killer (NK) cells. iPSCs can be genetically engineered to enhance their therapeutic potential, such as by adding chimeric antigen receptors (CARs) to target specific cancer cells. The iPSC technology holds great potential for the development of "off-the-shelf" NK cell therapies for the treatment of cancer. Ongoing research is focused on creating next-generation NK or CAR-NK cells with improved persistence, reduced adverse effects, and enhanced targeting capabilities. However, the process of differentiating iPSCs into NK cells and transducing them with CARs is complex, requires extensive optimization, and often representing a bottleneck for the clinical transition of the potentical therapeutics.

In our study, we successfully established a culture process and differentiated human cord blood-derived iPSCs into iNK cells with phenotypes comparable to healthy primary NK cells, with great efficiency, as evidenced by > 85% of CD56+CD3- expression of the cells. We developed a media formulation to prime the cells for expansion and another formulation to enhance the cell-killing functionality of the iPSC-derived NK cells. These iPSC-derived NK cells demonstrated the ability to quickly recognize and kill surrogate cancer cells (K562) in vitro, indicating a rapid therapeutic response. Additionally, CARs can be delivered to iPSC, iNK cells via viral or non-viral methods to enhance their targeting capabilities.

The differentiation of iPSCs into NK cells and their genetic engineering to create CAR-NK cells holds great promise for cancer therapy. Developing a GMP-compliant differentiation and transduction process is crucial to ensure the efficacy and scalability of next-generation iNK cell therapies. This study is one of the first to report the differentiation and expansion of human cord blood-derived iPSCs into functional iNK cells following the innovative culture method. Continued refinement are essential to fully realize the impact of culture process on the critical quality attributes for the development of next generation iPSC-derived NK and CAR-NK cell therapies.

19 A Dual-Population CAR T Product for Safe and Effective Targeting of T-cell Malignancies

Ruchi Patel[1], Trang Vu[2], Dean Qian[1], Guido Ghirardi[3], Aditya Nimmagadda[1], Rosemary Mazanet[4], Stefan Barta[5], Steven Yang[1], Nicholas Siciliano[1], Adam Snook[1], Marco Ruella[5]
[1]Vittoria Biotherapeutics, Philadelphia, PA, [2]Philadelphia, PA, [3]Upenn: University of Pennsylvania, [4]Chestnut Hill, MA, [5]University of Pennsylvania, Philadelphia, PA

Chimeric antigen receptor T (CAR T) cell therapy has achieved remarkable clinical outcomes in several B cell-associated malignancies. However, despite these promising results, CAR T therapy has not shown similar efficacy in other hematological malignancies such as T cell lymphomas (TCLs). Therefore, it is imperative to develop CAR T products for TCLs, which are characterized by a very poor prognosis and are considered orphan diseases. The key challenge in developing CAR T for TCL is that the tumor antigens used as CAR T targets, such as CD5, CD7, and CD2, are also expressed in normal healthy T cells. Consequently, CAR T therapies against these targets are limited by CAR T fratricide and produce normal T-cell toxicity, leading to poor manufacturing outcomes and potentially severe infections and other complications post-infusion. To address this, we focused on the membrane protein CD5, known to be overexpressed in malignancies like TCL but also chronic lymphocytic leukemia, mantle cell lymphoma, T acute lymphoblastic leukemia, and acute myeloid leukemia. In normal T cells, CD5 acts as a negative regulator of T cell activation. We have previously shown that genetically deleting CD5 in engineered CAR T cells might amplify their effector functions while preventing fratricide in CAR T cells targeting CD5 (*Patel et al., Sci Immunol, 2024*). In this study, we developed clinical-grade CD5 knockout (KO) CD5-targeting CAR T cells (CD5KO CART5). To establish a good manufacturing practice (GMP) product, we refined our manufacturing protocol, reducing the duration from two weeks (conventional) to five days (rapid). We performed a large-scale pilot run of this rapid manufacturing protocol and confirmed that target KO and transduction efficiencies are reasonable and scalable. We compared the efficacy of CD5KO CART5 cells generated using small-scale conventional manufacturing (small-conv) and large-scale rapid manufacturing (large-rapid) in vivo. The results showed more robust anti-tumor effects in mice treated with the large-scale and rapid product. Furthermore, we conducted IND-enabling safety evaluations to establish the product's safety profile. We confirmed the genetic stability of the final product with no chromosomal aberrations and verified the specificity of the CAR5 construct using the Retrogenix Cell Microarray tool and normal tissue screening. Additionally, a full veterinary pathology toxicology study evaluated whether CD5KO CART5 caused unintended tissue damage in NSG mice. This study showed no treatment-related tissue lesions. To mitigate potential toxicity toward a patient's normal T cells, our optimized manufacturing protocol generates two main cell populations in the same infusion product: a CD5-, CAR5+ population for targeted anti-tumor activity and a CD5-, CAR5- population with the potential to repopulate a patient's endogenous T cells. We confirmed that CD5KO T cells maintain reactivity against opportunistic pathogens such as cytomegalovirus and showed long-term in vivo persistence in NSG mice. This study details the development of an autologous GMP-grade CD5KO CART5 product, now ready

for a phase I clinical trial for relapsed and refractory CD5+ nodal T-cell lymphomas, now open at the University of Pennsylvania (NCT06420089).

20 The Simultaneous Measurement of Edit Associated Structural Variation and Transgene Localization in Single Cells

Christopher Tompkins[1], Erin Cross[1], Stephen Gross
[1]KromaTiD, Longmont, CO

Production of cell and gene therapy drug products such as CAR-T or CAR-NK often require complex genome engineering combining nuclease-based edits with the simultaneous genomic insertion of large CAR transgenes. Even in systems with highly specific targeted transgene insertions, there is the potential for off-target insertions at random or off-target editing loci within the genome. The average copy number (and sometimes location) of the CARs in these complex edited products can be measured by NGS or dPCR methods however, averaging divorces structural variant and transgene data from the cellular context, making it impossible to observe co-localization of events or distribution of transgenes in a batch of edited cells. With only dPCR or NGS data, it is not possible to differentiate one cell with ten transgenes from two cells with five transgenes. Simultaneous quantitation and localization is best performed by a direct, definitive, single cell method such as directional Genomomic Hybridization™.

dGH is a unique cytogenetic technique for mapping the structural variation of individual genomes in single cells. Based on images of fluorescently labeled DNA probes designed to target specific loci and hybridized to metaphase chromosomes, dGH in-Site assays provide direct detection of structural variation, including DNA inversions, a common editing by-product. Combining this data with the number and location of transgene insertions, however, is challenging due to the relatively small size of therapeutic CARS, often less than 2Kb. By combining advanced DNA probe design strategies that avoid homology, repetitive sequences, and other sources of fluorescent signal noise with the unique sample preparation inherent in dGH, we have designed and qualified a single cell assay system for the simultaneous measurement of edit associated structural variation and transgene localization in the same single cell. Using a model transgene system, we demonstrate the potential of this system for measuring both the distribution of transgenes as well as identification of co-localized structural variants.

21 Zip-Code Delivery: Creation of a Novel Nucleic Acid based Targeted Delivery System

Anthony Johnson[1], Leon bernal-mizrachi[2], Pavan Kumar puvvula[1], Lourdes Martinez-Medina[1], Andrey Pisarev[1]
[1]Kodikaz, New York, NY, [2]Emory University, Atlanta, GA

Multiple myeloma is the second most common type of blood cancer. In the United States, 35,730 new MM cases will be diagnosed, and 12,590 deaths from the disease will occur in 2023. Most MM patients will progress to the refractory stage. While numerous new drugs and immunological treatments have succeeded over the past few decades, many have failed due to toxicity from a lack of specific targeting. Targeted therapy delivery (TTD) is a method of delivering therapies to specific targets to reduce side effects and improve effectiveness. TTD is increasingly used in oncology, with many FDA-approved products available. While there are many effective therapies in research and clinical trials, many fail due to the inability to deliver effective amounts specifically to cancer cells. The only FDA-approved ADC against BCMA for MM treatment, Belantamab Mafodotin, was recently withdrawn, raising concerns about ADC use in MM. To overcome these obstacles, Kodikaz has developed a DNA-based targeted delivery system known as Zip-Code (ZC) technology.

We have thoroughly characterized the mechanism of ZC internalization and found that Clathrin-mediated endocytosis and endosomal maturation mechanisms play a significant role in ZC internalization in myeloma cells. Several proteins, including Clathrin, Rab5a, and Syntaxin-6, are associated with MM-ZC, and their loss significantly reduces MM-ZC internalization, underscoring their crucial role in the uptake process. Rab5a, Clathrin, and Syntaxin-6 function in a coordinated and stepwise signaling pathway for ZC trafficking, highlighting their interdependencies. These findings paved the way for the development of several ZC-delivered payload classes, demonstrating the effectiveness of ZC technology as a novel targeted delivery system for treating a range of cancers.

One such development is ZC-DM4, named KTS002, which combines the targeted delivery approach of zcDNA with the anti-tumor efficacy of DM4 (a maytansinoid derived from the bark of Maytenus ovatus). DM4's use is often limited by its narrow therapeutic window and associated neurological and gastrointestinal toxicities. Preliminary analysis indicated that ZDC-DM4 (KTS002) effectively targets multiple myeloma cell lines and primary human bone marrow specimens, eliminating malignant cells.

We have also created a ZC synthesized with herpes simplex virus thymidine kinase (HSV-TK) and a CMV promoter which, when combined with ganciclovir, forms a cell-mediated "suicide" gene therapy. HSV-TK is known to convert ganciclovir (GCV) into the toxic product, GCV triphosphate, selectively killing TK-positive cells. This killing also has a bystander effect, increasing its potential for use in cancer therapies. Lastly, we successfully conjugated our Zip-Code (ZC) to Cu-64 and demonstrated its targeted delivery to myeloma tumors in vivo. This radiolabeled ZC proved to be stable in vivo and selectively delivered Cu-64 to the tumor, without affecting other tissues or organs, providing evidence for the potential use of ZC in targeted radiotherapies.

This innovative platform holds potential for advancing targeted tumor delivery (TTD) in myeloma and other cancer types. By harnessing this technology, Kodikaz aims to enhance the specificity and efficacy of cancer treatments. The ability to deliver therapeutic agents directly to tumor sites represents a significant leap forward in cancer therapy, offering new avenues

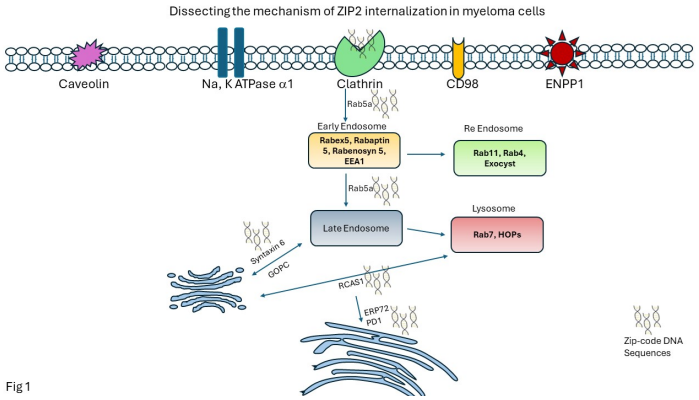


Fig 1

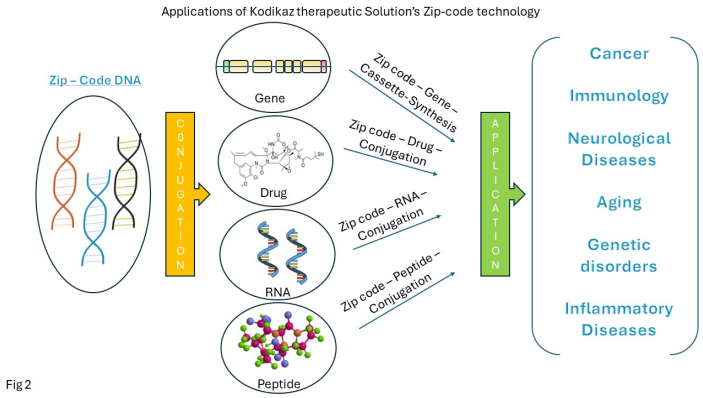


Fig 2

22 INTASYL PH-894: Targeting BRD4 to Enhance NK Cell-Based Immunotherapy

Melissa Maxwell[1], Linda Mahoney, Elfriede Noessner[2]
[1]Phio Pharmaceuticals, Marlborough, MA, [2]Helmholtz Zentrum, Neuherberg, Bayern, Germany

Background: Natural killer (NK) cells are the body's first line of defense against cancer, capable of recognizing and killing tumor cells without prior exposure and without inducing graft-versus-host disease, unlike T cells. Furthermore, using allogeneic NK cells in adoptive cell therapy (NK-ACT) holds promise as a true "off-the-shelf" cellular immunotherapy for cancer, potentially overcoming many challenges associated with autologous cell therapies. However, enhancing NK cell activity in ACT is crucial for improving clinical efficacy, particularly against hematological cancers.

Bromodomain-containing protein 4 (BRD4) is an epigenetic regulator involved in the transcriptional control of key genes associated with cell proliferation and survival. BRD4 plays a critical role in various cellular processes, including inflammation and cancer. Targeting BRD4 has emerged as a potential therapeutic strategy in oncology, as its inhibition can modulate the expression of genes involved in tumor growth and immune responses.

Incorporating RNA interference (RNAi) treatment into ex vivo NK cell expansion protocols prior to ACT is one strategy to enhance NK cell activity. We have developed a new class of stable, self-delivering RNAi compounds (INTASYL™) that incorporate features of RNAi and antisense technology. INTASYL compounds demonstrate potent activity, stability, and are rapidly and efficiently taken up by cells, and can be administered during the expansion process. INTASYL compound PH-894 targets BRD4 and results in an enhanced phenotype for expanded human NK cells.

Methods: Primary human CD56$^+$ NK cells were expanded using the ImmunoCult™ NK Cell Expansion Kit. Following the 14-day expansion protocol, cells were seeded directly into 24-well plates containing PH-894. At the end of the culture period, cell viability and expression levels of BRD4 mRNA and protein were determined. Flow cytometry was performed to explore the impact on various proliferation and activation makers.

Results: PH-894 treatment results in efficient knockdown of BRD4 in NK cells without loss of viability. Following BRD4 silencing by PH-894, there is a significant decrease in the expression of CD94, a receptor involved in NK cell inhibition, and a concomitant increase in CD25, the alpha chain of the interleukin-2 receptor, indicative of enhanced activation and proliferation.

Conclusions: Incorporating PH-894 treatment into ex vivo NK cell expansion protocols prior to ACT represents a promising strategy for improving NK cell efficacy. These findings suggest that BRD4 plays a critical role in regulating NK cell activity and that its silencing may modulate NK cell function, potentially enhancing their antitumor activity. This study provides a foundation for further exploration of BRD4 as a target for enhancing NK cell-based immunotherapies.

23 Therapeutic Targeting of the CBS-CD73 Signaling Axis Attenuates Head and Neck Cancer Progression

Yong Teng[1]*, Fanghui Chen[1], Jianqiang Yang[1], Fan Yang[1]
[1]Emory University School of Medicine, Atlanta, GA
**Corresponding Author*

Cystathionine beta synthase (CBS), the first and rate-limiting enzyme in the transsulfuration pathway, is an important mammalian enzyme in health and disease. Our pilot study has shown that CBS is highly expressed in head and neck cancer (HNC). Knockdown of CBS inhibits HNC growth in preclinical models, highlighting its newly identified oncogenic role. To better understand the regulatory network of CBS, we generated CBS-overexpressing HNC cells by transfection of the full-length human CBS construct tagged with flag and then performed immunoprecipitation (IP) with either anti-CBS antibody (Ab) or anti-Flag Ab. The resulting immunoprecipitants were then subjected to mass spectrometry. This analysis identified more than 100 proteins present in the CBS immunocomplex. Among them, CD73, a glycosylphosphatidylinositol (GPI)-anchored cell surface protein encoded by the NT5E gene, was identified as a novel interacting partner of CBS. The interaction between CBS and CD73 was confirmed by co-IP. Interestingly, knockdown of CBS dramatically reduced CD73 at the protein level but not at the expression level in HNC cells. Our mechanistic study further revealed disruption of CBS signaling in HNC cells by genetic depletion resulted in a reduction in CD73 protein stability via a USP14-dependent ubiquitin-proteasome pathway. More importantly, USP14 inhibition by the treatment with IU1 attenuates HNC growth and aggressiveness, which at least partially due to blocking CBS-CD73 signaling axis. These novel findings offer new insights into the role of the CBS-CD73 axis in promoting HNC progression, suggesting that targeting the CBS-CD73 interaction could be a promising therapeutic approach for HNC treatment.

24 mRNA-mediated Rescue of Lost Tumor Suppressor Function in Mutated p53 or BRCA1 Tumors is a Potent New Strategy for Cancer Therapy Delivered with Tumor-Targeted Peptide (non-LNP) Nanoparticles

Neil Desai[1], Audrey Grunenberger[2], Léa Cabrera[2], Elodie Czuba[2], Veronique Josserand[3], Melanie Guidetti[3], Gilles DIVITA[4]*, Jordi Rodon[5]
[1]Aanastra Inc, Pacific Palisades, CA, [2]DIVINCELL, Nimes, Hérault, France, [3]IAB GRENOBLE University, Grenoble, Isère, France, [4]DIVINCELL, Nimes, Gard, France, [5]University of Texas MD Anderson Cancer Center, Houston, TX
**Corresponding Author*

Tumor suppressors p53 and BRCA-1 play essential roles in tumorigenesis. p53 mutations resulting loss of function are common across variety of tumors and influence tumor cell proliferation and metastasis in more than 50% of cancers, while BRCA-1/2 mutations occur in up to 10% of breast cancers and epigenetic silencing of BRCA-1 (also known as BRCAness) occurs in approximately 30% of sporadic breast cancers. These alterations are associated with a lack of expression and/or lack of tumor suppressor function of the corresponding protein, which induces genomic instability. To date there are no effective treatments for directly rescuing p53 or BRCA-1 loss of function in cancers harboring p53 or BRCA-1 mutations or rescuing loss of function in other tumor suppressors.

Therapeutic mRNAs are potentially a new generation of treatments to restore functional versions of mutated/missing proteins in patients. However, effective *in vivo* delivery of functional mRNAs to tissues other than the liver remains significant limitation in the mRNA therapeutic field and requires new improved drug delivery systems. We have developed a new potent strategy combining functional tumor suppressor mRNAs with tumor-targeted peptide nanoparticles, to selectively rescue tumor suppressor function as a potential cancer treatment.

ADGN-531 is a proprietary p53 derived mRNA delivered in tumor-targeted peptide (non-LNP) nanoparticles to rescue loss of p53 tumor suppressor function in p53 mutated cancers. ADGN-531 showed strong antiproliferative effects across a panel of cancer cells harboring various p53-missense or nonsense mutations resulting from rescue of p53 transcriptional activity and subsequent expression of its target proteins, induction of cell cycle arrest in G1 due to p21 upregulation and apoptosis following BAX and PUMA activation. Weekly IV administration of ADGN-531 resulted in strong dose-responsive (0.5-3.0 mg/kg) tumor growth inhibition and regression of osteosarcoma (SaOs2/p53 null), colorectal (SW403/p53 nonsense mutation) and lung (H358/ p53 null) cancer xenografts.

ADGN-903 combines proprietary BRCA-1 derived mRNA delivered in tumor-targeted peptide (non-LNP) nanoparticles to rescue loss of BRCA-1 tumor suppressor function in BRCA-1 mutated cancers. ADGN-903 markedly inhibited the proliferation of BRCA-1 deficient cell lines and restored BRCA-1 function in homologous recombination. ADGN-903 prevented RAD51 recruitment to the damage site by reducing RAD51 protein level as well as by formation of RAD51 foci following irradiation. Weekly IV-administration of ADGN-903 (2.0 mg/kg) prevented HCC-1937 triple negative breast cancer tumor growth and resulted in strong tumor regression. ADGN-903 was also highly effective, on large tumors of about 500-600 mm^3, preventing further progression and achieving shrinkage of large tumors.

ADGN-531 and ADGN-903 respectively, were well tolerated, with no body weight loss and no signs of clinical toxicity detected after single or repeated weekly administrations.

Our study provides *in vivo* proof-of-concept that rescue or restoration of lost tumor suppressor function with functional p53 or BRCA-1 mRNA, delivered with peptide nanoparticles in cancers with mutated tumor suppressors p53 or BRCA-1, respectively, could be used as a new therapeutic strategy for cancer treatment across a wide range of tumor suppressor alterations or mutations.

25 In Vivo CRISPR Gene Editing of KRAS Mutant Tumors with Tumor-Targeted (non-LNP) Peptide-based Nanoparticles Causes Strong Tumor Regressions with Only 2 Doses and Overcomes Resistance to Existing Small-Molecule KRAS Inhibitors

Gilles DIVITA[1]*, Audrey Grunenberger[2], Léa Cabrera[2], Veronica Guzman[3], Elodie Czuba[2], Veronique Josserand[4], Melanie Guidetti[4], Neil Desai[5], Jordi Rodon[6]
[1]DIVINCELL, Nimes, Gard, France, [2]DIVINCELL, Nimes, Hérault, France, [3]AANASTRA, Pacific Palissades, CA, [4]IAB GRENOBLE University, Grenoble, Isère, France, [5]Aanastra Inc, Pacific Palisades, CA, [6]University of Texas MD Anderson Cancer Center, Houston, TX
**Corresponding Author*

KRAS mutations at codon-12 are among the most commonly observed mutations in pancreatic, colorectal and lung adenocarcinomas. Recently, $KRAS^{G12C}$ inhibitors sotorasib and adagrasib have been approved. However, **fewer than half of patients respond and r**esistance to $KRAS^{G12C}$ inhibitors rapidly arises. Resistance is mainly associated with the emergence of secondary KRAS mutations, activation of feedback pathways, and high amplification of the $KRAS^{G12mutant}$ allele. $KRAS^{G12D}$ inhibitors (MTRX-1133 and INCB161734) have also recently entered clinical development and facing similar resistance limitations. We have developed a potent strategy combining *in vivo* CRISPR-based gene editing delivered with (non-LNP) tumor-targeted peptide-based nanoparticles, to target specific mutations at codon-12 of the KRAS oncogene and overcome acquired resistance to small-molecule KRAS inhibitors.

ADGN-121, ADGN-122 and ADGN-123 are gene-editing complexes containing proprietary sgRNA targeting specifically $KRAS^{G12D}$, $KRAS^{G12C}$or $KRAS^{G12V}$ respectively, complexed with proprietary peptide-based nanoparticles to promote tumor targeted delivery upon systemic administration.

We demonstrated that ADGN-121, 122 and 123 selectively silenced $KRAS^{G12D}$, $KRAS^{G12C}$ and $KRAS^{G12V}$, respectively, in colorectal, pancreatic and lung cancer cells resulting in strong inhibition of cell proliferation (IC50:10-30 nM) and phosphorylation of downstream effector pathways ERK and AKT.

We showed that only two IV-administrations of ADGN-121 tar-

geting $KRAS^{G12D}$ are required to abolish Panc1 tumor growth in a dose dependent manner with more than 80% tumor regression at 1.0 mg/kg. Similarly, only two IV administrations of ADGN-123 targeting $KRAS^{G12V}$ abolished colorectal SW403 tumor growth with strong tumor regression at 1.0 mg/kg. The combinations of ADGN-121 and ADGN-123 with drugs commonly used in the clinic (nab-paclitaxel and capecitabine respectively) showed strong synergy in these models. In contrast, no effect on tumor growth was observed with a nonspecific gRNA control or vehicle controls. No long-term resistance was detected *in vivo* at 90 days post-dosing.

The gene-editing treatments were well tolerated and "redosable" with no evidence of clinical toxicity, inflammatory response, no detectable off target effects or emergence of other KRAS mutations.

ADGN-122 targeting $KRAS^{G12C}$ can overcome sotorasib/adagrasib acquired resistance *in vitro*. ADGN-122 blocks the proliferation (IC50 : 10-20 nM) of sotorasib or adagrasib-resistant cells with secondary $KRAS^{G12C/R68M}$ and $KRAS^{G12C/Y96D}$ acquired mutations or with KRAS in a permanent active state and effectively inhibits ERK phosphorylation. ADGN-121 targeting $KRAS^{G12D}$ can overcome MRTX1133 acquired resistance in vitro. ADGN-121 can effectively inhibit the proliferation (IC50 : 10-15 nM) of ASPC-1 MRTX-1133R and PANC-1 MRTX-1133R cells with high level of $KRAS^{G12D}$-GTP active state.

Our study provides a strong proof-of-concept that CRISPR gene-editing using (non-LNP) peptide-based nanoparticles can target driver mutations such as KRAS *in vivo* and permanently disrupt the oncogenic alleles, leading to major tumor regression. This *in vivo* targeted gene-editing approach can overcome acquired resistance commonly seen with small molecule inhibitors without *in vivo* evidence of toxicity. This strategy may have significant advantages over current small-molecule approaches.

26 Characterization and Preclinical Development of Tumor-Specific CRISPR/Cas9 Editing as a Combinatorial Therapy for Treatment-Resistant Solid Tumors

Kelly Banas[1], Pawel Bialk[2], Tori Reiner[1], Komal Khan[1], Kristen Pisarcik[3], John Rogowskyj[1], Lauren Skelly[1], Natalia Rivera-Torres[1], Eric Kmiec[4]
[1]ChristianaCare, Newark, DE, [2]Christiana Care, Newark, Delaware, [3]ChristianaCare Gene Editing Institute, Newark, DE, [4]Christiana Care Health System, Newark, DE

Introduction: We are advancing a novel strategy for the treatment of solid tumors by employing CRISPR-directed gene editing to augment the standard of care therapies by halting the progression of tumor growth. Current therapies, including innovative ones, fail over time because the tumor develops resistance and cell-based or antibody therapies are incapable of adapting to genetic changes. Few patients tolerate systemic therapy without severe toxicities that lead to serious morbidity; they often stop treatment, which profoundly impacts their outcomes. We have developed a clinically relevant gene editing strategy to disable the master regulatory gene, Nuclear Factor Erythroid 2-Related Factor (NRF2), specifically and only within tumor cells, to overcome resistance and restore responsiveness to standard of care, while healthy cells remain unedited and genetically unchanged. NRF2, overexpressed in many solid tumors, controls the transcriptional response of over 200 genes, genes whose activation leads to inflammation, cell migration, and cell proliferation.

Results: We successfully disable NRF2 in lung squamous cell carcinoma demonstrating tumor-specific genome disruption in cell models. We demonstrate through targeted deep sequencing that 30% gene editing activity is sufficient to improve survival and response to chemotherapy in animal models. Using an orthogonal approach, off-target analyses reveal a very low level of unwanted genetic changes.

Conclusion: Following guidance from our FDA-INTERACT meeting, we demonstrate how we developed cell models to examine tumor-specific gene editing efficacy, measured phenotypic outcomes, and analyzed off target effects in the disruption of NRF2 in non-small cell lung carcinoma (NSCLC) cells. Our work centers on the design and generation of a clinically relevant cell system and its translation into an animal model, characterizing the efficacy of disabling NRF2 concomitant with the restoration of chemosensitivity. We provide a template for clinical development of CRISPR/Cas gene editing as a platform-based combinatorial therapy for solid tumors. Our foundational goal is to enable patients to complete their treatment regimens more successfully, at lower dosages and with far fewer adverse effects.

27 Focused Ultrasound Mediated Thermogenetic Control of CAR T Cell Activity in Breast Cancer Brain Metastasis

Chulyong Kim[1], Ali Zamat[1], Zizhen Zha[1], Shwetha Sridhar[1], Gabriel Kwong[1], Costas Arvanitis[2]
[1]Georgia Institute of Technology, Atlanta, GA, [2]Heorgia Institute of Technology, Atlanta, GA

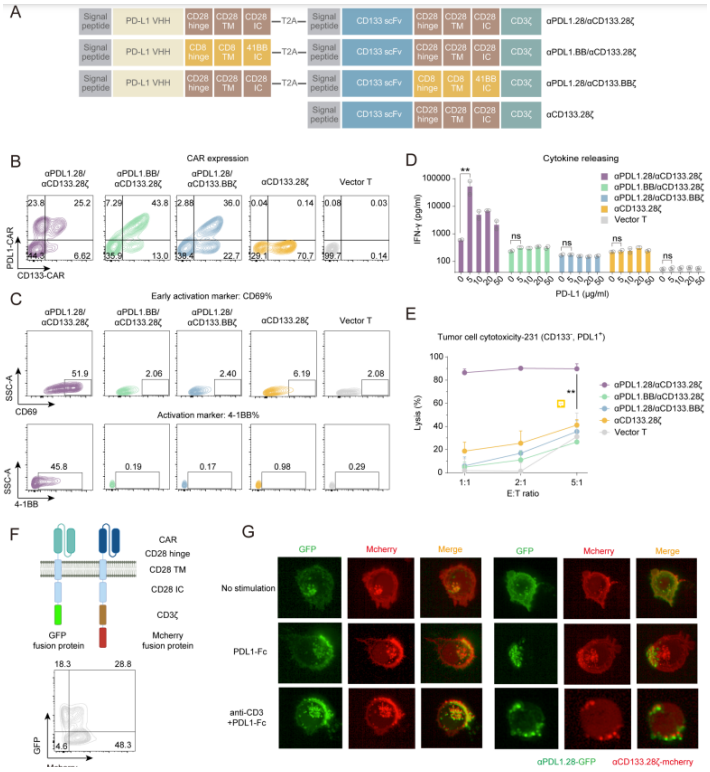
HER2-targeted therapies are promising treatment options for metastatic breast cancer and have improved the median overall survival. However, breast cancer brain metastasis (BCBM), observed in up to 50% of HER2-positive breast cancer patients, remains a clinical challenge. While Chimeric Antigen Receptor (CAR) T cell therapy is promising therapeutic strategy to address the unmet need in treating BCBM, improving its effectiveness and safety is critical for clinical translation. We hypothesized that spatially and temporally controlled activity of CAR-T cells with a thermal gene switch (TS) under MRI-guided focused ultrasound (MRgFUS) would potentiate anti-tumor responses in difficult-to-treat BCBM. To test our hypothesis, we developed a closed-loop controlled MR-thermometry (MRTI) based FUS system (MRgFUS) and αHER2 CAR-T cells engineered with TS expressing the reporter gene firefly luciferase (TS.Fluc) or bi-specific engager on NKG2D ligands (TS.BTE) upon MRgFUS-hyperthermia (41.5°C). *In vivo* quantification of TS activity from intratumorally delivered TS.Fluc αHER2 CAR-T cells in HER2+ BCBM mice model (HER2+ MDA-MB-468 in NSG mice with primary human CAR-T cells) followed by pulsed MRgFUS-hyperthermia resulted in a ~10-fold increase in luminescent activity compared to unheated mice. To assess longitudinal activation, we quantified TS activity with a second sonication 4 days post the initial FUS treatment. Prior to the second sonication, TS.Fluc expression had returned to baseline levels, while following it robust TS.Fluc expression was observed again, demonstrating that MRgFUS can

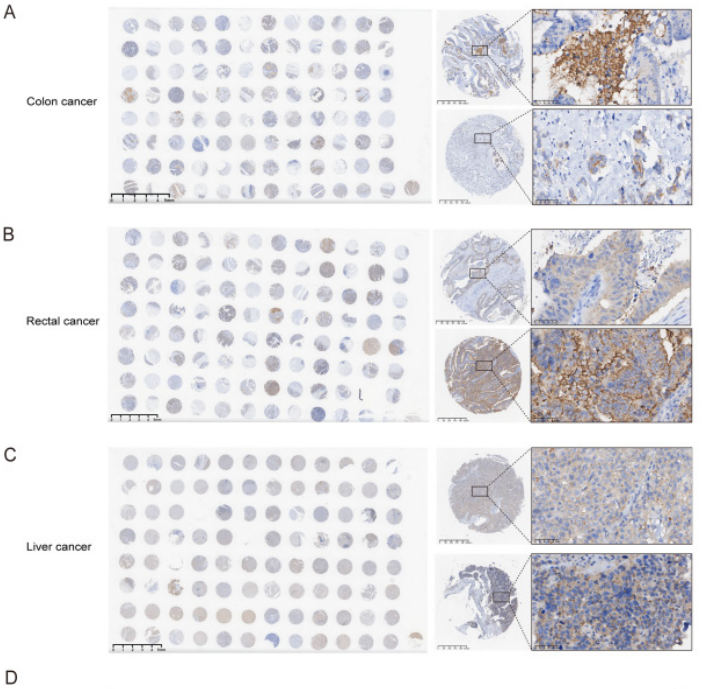
activate engineered T cells with TS in brain tumors with high spatiotemporal control. Subsequently we investigated the therapeutic efficacy of intratumorally delivered TS.BTE αHER2 CAR T cells in mixed tumor model of heterogeneous HER2 MDA-MB-468 (75% HER2+) and observed significant tumor regression and survival of HER2- tumor cells in the treated group compared to controls, demonstrating the potential of the proposed strategy to noninvasively drive the local production of key transgenes and potentiate anti-tumor responses in difficult-to-treat BCBM.

28 Dual-targeting CD133/PD-L1 CAR-T Overcame Suppressive Microenvironment in Solid Tumor and Synergized with Radiotherapy and PD-1 Blockade

Zhuoran Yao[1], **Kai Kang[2]**, Peiheng Li[3], Jianxin Xue[4], You Lu[5]*
[1]Chengdu, China, China, [2]West China Hospital, Chengdu, Sichuaun, China, [3]West China Hospital, Chengdu, Sichuan, China, [4]Sichuan University/West China Hospital of Medicine, Chengdu, Peoples Republic of China, [5]West China Hospital, Sichuan University, Chengdu, Peoples Republic of China
**Corresponding Author*

Adoptive cell transfer therapies (including chimeric antigen receptor-T cell therapy) have transformed the treatment landscape for hematological malignancies. However, the application of chimeric antigen receptor-T therapies in solid tumors remains limited. We developed and assessed a novel dual-targeting chimeric antigen receptor-T therapy that combines an αPDL1.CD28 chimeric receptor with a second-generation anti-CD133 to target CD133⁺ tumors. The αPDL1.CD28 structure activated the CD3ζ signaling in cis by clustering with CD133 CAR via CD28 dimerization. Dual chimeric antigen receptor-T exhibited improved cytotoxicity against CD133⁺ tumor cells in vitro and in vivo, regardless of basal PD-L1 expression. Furthur combination with PD-1 blockade augments tumor control by blocking excessive PD-L1 signaling. Triple-modality therapy with hyperfractionated radiation (10 Gy/1f), chimeric antigen receptor-T cells, and anti-PD-1 maximized antitumor responses induced complete tumor regression in mice. Radiation induced the formation of a unique tissue-resident memory (Trm) chimeric antigen receptor-T cell cluster highly expressing CXCR6 and CD103. The E-cadherin-CD103 interaction emerged as a vital ligand-receptor pair between tumor cells and chimeric antigen receptor-Trm after irradiation. Our study introduces a novel dual-targeting CD133/PD-L1 chimeric antigen receptor-T cell line and further demonstrates the efficacy and rationale of combining novel chimeric antigen receptor-T cells with anti-PD-1 and radiotherapy in solid tumors.





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CD133 expression	Colon cancer (n=47)	Rectal cancer (n=48)	Liver cancer (n=45)
Intensity, n (%)			
0	1 (2.1)	0 (0.0)	2 (4.4)
0~1	20 (42.6)	11 (22.9)	16 (35.6)
1+	24 (51.1)	31 (64.6)	22 (48.8)
2+	2 (4.3)	6 (12.5)	5 (11.1)
3+	0 (0.0)	0 (0.0)	0 (0.0)
Percentage of positive cells, n (%)			
<1%	1 (2.1)	0 (0.0)	3 (6.7)
1-24%	6 (12.8)	0 (0.0)	9 (20.0)
25-49%	5 (10.6)	2 (4.2)	6 (13.3)
≥50%	35 (74.4)	46 (85.8)	27 (60.0)

29 A Platform for the Development of Personalized Oncolytic Adenoviruses

Andrew Munoz[1], Aditya Pandya[1], Carmen Kut[1], Andrew Flies[2], Harry Quon[1], **Fred Bunz[1]**
[1]Johns Hopkins University School of Medicine, Baltimore, MD, [2]Menzies Institute for Medical Research, University of Tasmania, Hobart, Tasmania, Australia

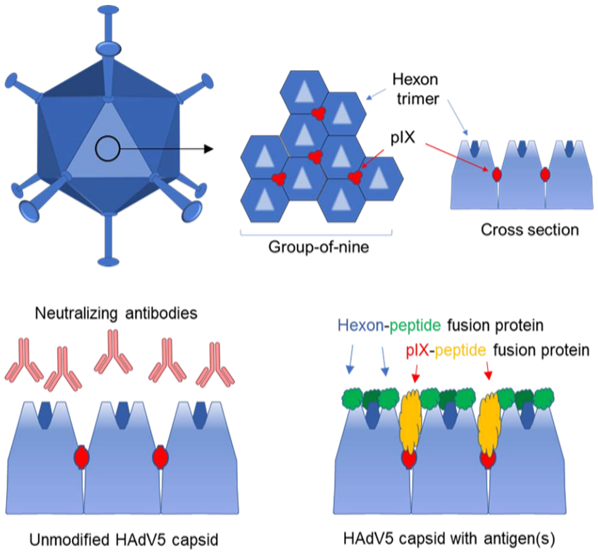
Replication-competent adenoviruses have been extensively employed as anticancer therapeutics. These small viruses have a lytic life cycle and can be modified to selectively replicate in tumor cells. An important attribute of oncolytic adenoviruses (OAds) is their immunogenicity. Upon infecting and lysing tumor cells, OAds elicit robust local and systemic immune responses that can counteract an immunosuppressive tumor microenvironment. Neoantigens released upon viral lysis of tumor cells can stimulate T cell activation by professional antigen presenting cells (APCs), thereby increasing the immune response to the tumor. However, the most effective immune responses elicited by an invading adenovirus are undoubtably directed against the virus itself. Indeed, infection by naturally occurring adenoviruses during childhood generally results in lifelong host immunity. While OAds are highly immunostimulatory, the responses they elicit may have limited cross reactivity with the targeted tumor.

We are employing a platform technology called AdenoBuilder to develop OAds that can stimulate robust immune responses to specified neoantigens. A common characteristic of solid tumors is the loss of neoantigen presentation. A suppressive tumor microenvironment typically has numerous contributing factors, including the activation of immune checkpoints, the loss of HLA expression and limiting levels of neoantigen peptide available for presentation on the tumor cell surface.

Our personalized OAds are designed to functionally restore neoantigen presentation by reconstituting the pHLA/neoantigen peptide complex on the surface of the infected cancer cell. These replication-competent OAds express tumor-specific neoantigens and compatible HLA alleles that can be matched to the genotype of individual patients. Neoantigen peptides are incorporated into several proteins that form the capsid of human adenovirus serotype 5 (HAdV5) and are thus processed shortly after infection and virus disassembly. In addition to increasing intracellular neoantigen abundance, this capsid-incorporation approach to neoantigen delivery allows us to eliminate several immunodominant epitopes on the HAdV5 capsid that are recognized by neutralizing antibodies. Virus neutralization in people previously infected with wild type HAdV5 is a well-recognized limitation to the use of HAdV5-based vectors.

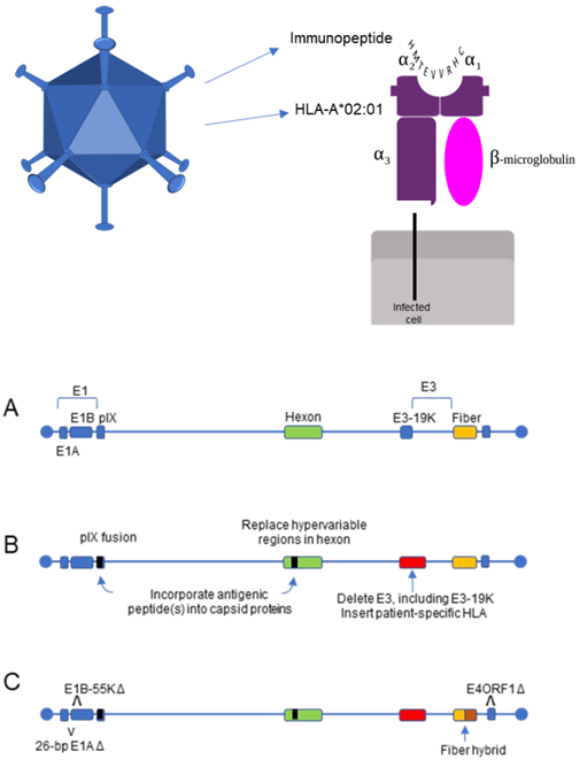
Our results show that patient-specific HLA proteins can be expressed from bicistronic transgenes designed to co-express immune stimulatory molecules such as GMCSF. We are exploring various modifications to optimize tumor selectivity, including the deletion of E1B-55K, E3-19K, and E4orf1, which plays an important role in creating a glycolytic environment that favors viral replication.

The production of customized, multicomponent viruses is facilitated by the AdenoBuilder system. With this accessible toolkit, synthetic adenovirus genomes are enzymatically assembled from plasmid components and directly packaged. This modular approach allows us to rapidly produce recombinant viral vectors with ready-made and bespoke modifications across the viral genome.



Engineering the adenovirus capsid.

We have successfully incorporated neoantigen peptides into the capsid of human adenovirus serotype 5 (HAdV5) by creating fusions in the capsid proteins hexonand pIX (p9). These fusions can delivera high concentrationof neoantigento the infectedcell. In addition, the modificationseliminateimmunodominant epitopes that are targeted by neutralizing antibodies.



Restoring neoantigen presentation

With a single personalized virus, we can reconstitute any neoantigen-HLA complex on the surface of infected cells. These viruses are replication-competent and therefore retain their lytic life cycle. We expect to generate robust antitumor responses by the combination of tumor cell lysis and enhanced neoantigen presentation

30 Controlled, Riboswitch-Regulated Gene and Cell Therapy Targeting HER2+ Tumors

Xuecui Guo[1], Zhaojing Zhong[1], George Wang[2], Jae Gyun Oh[1], Alexandria Forbes[1]
[1]MeiraGTx, New York, New York, [2]New York, NY

Controlled expression of delivered transgene is important for both gene and cell therapies to be efficacious and safe. For this purpose, we have developed a platform for transgene expression control in response to a small molecule inducer. The platform utilizes a mammalian synthetic riboswitch with an aptamer sequence, which binds the small molecule inducer in the context of an alternative exon flanked by 5' and 3' introns. This gene regulation cassette is embedded in the coding sequence (CDS) of the transgene, creating an alternative splicing regulated gene expression platform, in which the alternative splicing is modulated by aptamer/small molecule binding. In the absence of the small molecule inducer (aptamer ligand), the splice site sequence at the 3' end of the alternative exon is accessible, allowing splicing of the alternative exon into the transgene mRNA. The inclusion of the alternative exon generates an in-frame stop codon in the transgene mRNA, leading to mRNA degradation and no protein expression. In the presence of the small molecule inducer, binding of the inducer to the aptamer causes aptamer RNA conformational change that sequesters the splice site, leading to exclusion of the alternative exon from the mRNA, generation of functional mRNA and expression of transgene product. The other component of this gene regulation platform—the small molecule inducers—were designed to specifically bind to the aptamer sequence with high oral bioavailability and a favorable safety profile.

We have applied this novel gene regulation platform to regulation of the expression of various therapeutic genes in response to a small molecule inducer. One of these therapeutic genes is the anti-HER2 antibody. *In vitro*, the expression of anti-HER2 antibody is induced in response to a small molecule inducer in a dose dependent manner. Likewise, *in vivo* (using AAV-mediated transgene delivery), anti-HER2 antibody expression was also dose dependently induced in response to orally administered small molecule inducer. Further, the induced anti-HER2 antibody halted the progression of $HER2^+$ tumors. To further develop treatment targeting the HER2 antigen, we incorporated a riboswitch gene regulation cassette into the coding sequence of a chimeric antigen receptor (CAR) targeting the HER2 antigen. The expression level of CAR on the cell surface is dependent on the presence of the small molecule inducer. During the generation of CAR-T cells, the small molecule inducer is not present in the cultures and the CAR is not expressed—largely preventing tonic signaling and tonic signaling-induced T cell differentiation and exhaustion. We have shown in our preclinical studies that the T cells expressing riboswitch-controlled CAR (RiboCAR-T) are enriched in naïve and stem cell-like memory T cell populations. RiboCAR-T cells' anti-tumor activity can be remotely controlled by orally administered small molecule inducers and is superior to T cells expressing CAR constitutively in eliminating the $HER2^+$ tumor cells.

31 Seamless Integration of a Universal Epitope into Recombinant TCRs for Tagging and Tracking of TCR-T Cells Expressing 3S TCRs

Dolores Schendel[1]*, Kanuj Mishra[2], Justyna Ogonek[2], Anne-Wiebe Mohr[2], Doris Brechtefeld[2], Kathrin Mutze[2], Barbara Lösch[3]
[1]Medigene AG, Planegg / Martinsried, Bayern, Germany, [2]Medigene Immunotherapies GmbH, Planegg/Martinsried, Bayern, Germany, [3]Medigene Immunotherapies GmbH, Planegg, Bayern, Germany
**Corresponding Author*

We developed *UniTope & TraCR* as a universal detection system for 3S recombinant TCRs (rTCRs) used in development of TCR-T cell therapies, based on the 3S characteristics of high specificity, sensitivity, and safety. Seamless integration of UniTope in 3S rTCR sequences eliminates the need for co-expression of separate gene tags (e.g., truncated NGFR or RQR8) to enumerate TCR-transduced T cells and provides an absolute marker of identity distinguishing 3S rTCRs from all other TCRs present in human peripheral blood T cells. Thereby, evaluation of proliferation, persistence, and composition of TCR-T cells expressing 3S rTCRs is easily studied *in vitro* and *ex vivo*. Importantly, unambiguous identification of TCR-T cells with UniTope-tagged 3S TCRs is made using the single TraCR antibody that binds exclusively to the integrated UniTope sequence in 3S rTCRs, bypassing need for staining with TCR V-beta-specific antibodies, peptide-HLA (pHLA) multimers or antibodies detecting an external tag.

Versatility of the *UniTope & TraCR* detection system was shown using different 3S rTCRs demarked with UniTope as compared to non-tagged 3S rTCRs for surface expression and functionality in TCR-T cells. Binding of relevant TCR V-beta- and C-region specific antibodies, as well as peptide-HLA multimers, was used to study structural integrity of tagged rTCRs. Examination of specificity, sensitivity and safety profiles was used to assess critical 3S characteristics upon integration of UniTope in 3S rTCR sequences.

TCR-T cells generated using 3S rTCRs which recognize the cancer-testis antigen NY-ESO-1, with and without the UniTope tag, were characterized in extensive experiments. No differences were noted in any assessment of 3S rTCR surface expression, showing that inclusion of the integrated UniTope tag in the TCR sequence did not alter the 3S rTCR structural integrity. TCR-T cells, with or without UniTope, displayed identical specificity, sensitivity, and safety profiles, exemplifying complete maintenance of functional integrity upon inclusion of UniTope in the 3S rTCR sequence. Similar results with other 3S rTCRs demonstrated that UniTope serves as a universal tool to tag diverse rTCRs, without disturbing their expression or functionality.

The *UniTope & TraCR* detection system elegantly simplifies identification and enumeration of TCR-T cells expressing transduced 3S rTCRs with use of a single antibody reagent to uniquely detect the integrated UniTope tag, thereby reducing complexity in vector design and product development. This detection system allows easy monitoring of in-process steps during GMP production, simplifies quality control for product release and enables exquisite immune monitoring of patient samples *ex vivo*. It enables easy visualization and isolation of TCR-T cells for direct study. As a universal detection system, *UniTope & TraCR* allows

rapid validation of quality control assays for TCR-T therapies utilizing 3S rTCRs of different specificity and provides highly precise information for drug product dosing. GMP-grade TraCR antibody can also be used for drug product enrichment. The *UniTope & TraCR* detection system is a high precision technology that facilitates optimized development of TCR-T therapies.

32 T Cell Immune Characterization of the h-Met ß-Catenin Sleeping Beauty Model of HCC and its Therapeutic Response to Immune Checkpoint Blockade

Thanich Sangsuwannukul[1], Jill Thompson[2], Benjamin Kendall[2], Olivia Liseth[3], Maria Chiriboga[4], Kamonlapat Supimon[5], Jason Tonne[1], Richard Vile[1]
[1]Mayo Clinic, Rochester, MN, [2]Mayo Clinic Minnesota, [3]Mayo Clinic School of Medicine, Rochester, MN, [4]Mayo Clinic, Rochester, Rochester, MN, [5]Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Krung Thep Maha Nakhon Bangkok, Thailand

Current treatments for hepatocellular carcinoma (HCC) are poorly effective and developing novel therapies represents addresses an unmet clinical need. The immunosuppressive tumor microenvironment (TME) is a major challenge to effective immunotherapy in this disease. Atezolizumab with bevacizumab is FDA-approved as a first-line treatment for unresectable HCC. Our previous work found VSV-IFNβ inhibited antitumor T cell responses in a model in which anti-PD-L1 immune checkpoint blockade (ICB) alone cures about 50% of the mice. To better understand how oncolytic VSV modulates tumor-infiltrating T cells (TILs), we characterized the profiles of TILs upon VSV-IFNβ, anti-PD-L1 (ICB), or a combination at different stages of disease, and its therapeutic outcomes.

Hydrodynamic injection of hMet/β-Catenin SB oncogene-expressing plasmids (the Sleeping Beauty model) induced slowly developing progressive HCC in immunocompetent C57BL/6 mice. Six doses of anti-PD-L1 ICB (i.p.) were given with or without three doses of VSV-mIFN-β (i.v.) at different stages of disease. Tumor infiltrating lymphocytes were extracted from liver samples for single cell analysis using flow cytometry and mass cytometry (CyTOF).

Treatment with VSV-mIFN-β and anti-PD-L1 monoclonal antibody led to significantly increased CD8+ T cell infiltration into SB HCC tumors compared to ICB alone. Although we predicted that treatment with VSV would facilitate ICB by inflaming the TME, there was no enhanced antitumor effect when VSV was administered and in fact, addition of VSV-mIFN-β decreased the efficacy of anti-PD-L1 ICB alone.

Immune characterization showed modulation of the PD-L1 inhibitory marker and CD39+CD8+ tumor infiltrating T cells (TILs). CD39intPD-1int CD8+ T cells were increased upon VSV-mIFN-β treatment; however, the effect was not sustained. Alternative sequencing of VSV-mIFN-β with ICB therapy did not improve treatment outcomes compared to ICB alone. However, by encoding a library of HCC-derived tumor associated antigens in the virus it was possible to combine VSV- mIFN-β-TAA therapy with ICB to achieve complete tumor cures (Webb, Sangsuwannukul et al. Nat. Comm. 2024). These data show that chimerization of the anti-viral T cell response with the anti-tumor T cell response is an effective method to combine the immunostimulatory properties of oncolytic viruses with the targeted priming of anti-tumor T cells. In order to understand the effect of different sequencing of oncolytic virus with ICB on the anti-tumor T cell response induced by PD-L1 therapy we investigated how the anti-viral response shifted the phenotypes of the CD8 and CD4 T cell responses in vivo. We observed significant changes in the levels of several markers including CD38, CD39, PD-L1, PD-1 Granzyme B and CD44 in mice responding to ICB alone compared to those in which VSV was added. These data help to inform the subsets of T cells which respond to tumor alone, those which are induced by an immunodominant virus such as VSV and how those subsets interact and will help to develop rational sequencing of virus and ICB for tumor immunotherapy.

Here, we report that T cell profiling can show how anti-viral and anti-tumor T cell responses interact, combine and compete during oncolytic virotherapy in combination with ICB in a murine SB HCC model. Further studies on the antigenic targets of ICB, and VSV-TAA, in this model will inform development of improved immunotherapies for this disease.

33 Assessing Patient Risk, Benefit, and Outcomes in Drug Development: A Decade of Ramucirumab Clinical Trials *(Virtual)*

Adam Khan[1], Hassan Khan[2], Griffin Hughes[2], Chase Ladd[2], Ryan McIntire[2], Brooke Gardner[2], Adriana Pena[2], Abigail Schoutko[2], Jordan Tuia[3], Kirstien Minley[2], Alyson Haslam[3], Vinay Prasad[3], Matt Vassar[2]
[1]Oklahoma State University, Tulsa, OK, [2]Oklahoma State University Center for Health Sciences, Tulsa, OK, [3]University of California San Francisco, San Francisco, CA

Objective: This study aims to evaluate published clinical trials of ramucirumab to assess the risk/benefit profile and burden over time for patients. Background The burden of oncologic drug development on patients paired with increasing clinical trial failure rates emphasizes the need for reform of drug development. Identifying and addressing patterns of excess burden can guide policy, ensure evidence-based protections for trial participants, and improve medical decision-making.

Methods: On May 25, 2023 a literature search was performed on Pubmed/MEDLINE, Embase, Cochrane CENTRAL, and ClinicalTrials.gov for clinical trials using ramucirumab as monotherapy or in combination with other interventions for cancer treatment. Authors screened titles and abstracts for potential inclusion in a masked, duplicate fashion. Following data screening, data was extracted in a masked, duplicate fashion. Trials were classified as positive when meeting their primary endpoint and safety, negative or indeterminate.

Results: Ramucirumab was initially approved for gastric cancer but has since been tested in 20 cancers outside of its FDA approved indications. In our analysis of ramucirumab trials, there were a total of 10,936 participants and 10,303 adverse events reported. Gains in overall survival and progression-free survival for patients were 1.5 and 1.2 months, respectively. FDA-approved indications have reported more positive outcomes in comparison to off-label indications.

Conclusion: We found that FDA-approved indications for ramucirumab had better efficacy outcomes than non-approved indications. However, a concerning number of adverse events were observed across all trials assessed. Participants in ramucirumab randomized controlled trials saw meager gains in overall survival when evaluated against a comparison group. Clinicians should carefully weigh the risks associated with ramucirumab therapy given its toxicity burden and poor survival gains.

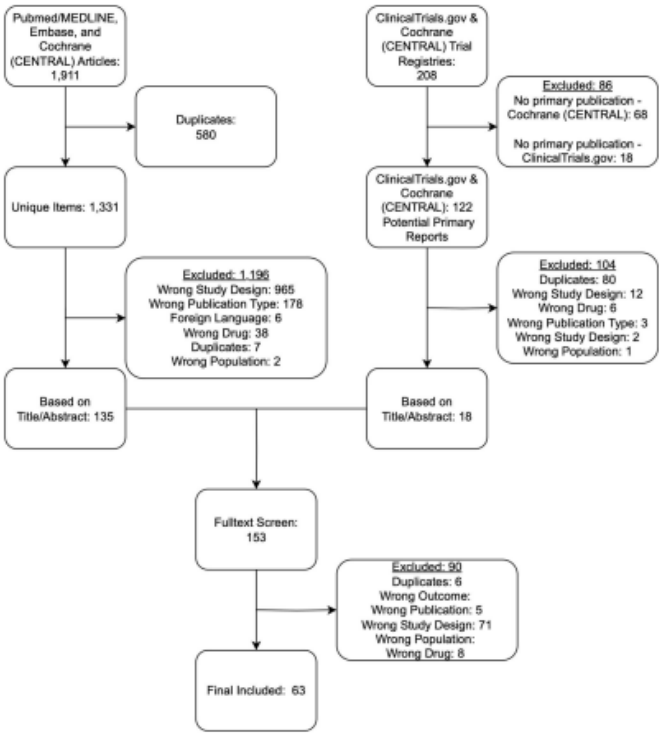


Figure 1. Flow diagram for study inclusion

34 Single Cell Bioinformatics Uncovers a Novel T Cell Origin as Effective Immunotherapy for Solid Tumors

Max Kam-Kwan Chan[1], Philip Chiu-Tsun Tang[1], Calvin Sze-Hang NG[2], David J. Nikolic-Paterson[3], Ka-Fai To[4], Patrick Ming Kuen Tang[5]*

[1]Department of Anatomical and Cellular Pathology, State Key Laboratory of Translational Oncology, The Chinese University of Hong Kong, Hong Kong, Sha Tin, Hong Kong, [2]Department of Surgery, The Chinese University of Hong Kong, Hong Kong, Sha Tin, Hong Kong, [3]Department of Nephrology and Monash University Department of Medicine, Monash Medical Centre, Clayton, Australia, Clayton, Victoria, Australia, [4]Department of Anatomical and Cellular Pathology, State Key Laboratory of Translational Oncology, Prince of Wales Hospital, The Chinese University of Hong Kong, Hong Kong, The Chinese University of Hong Kong, Sha Tin, Hong Kong, [5]Department of Anatomical and Cellular Pathology, State Key Laboratory of Translational Oncology, Prince of Wales Hospital, The Chinese University of Hong Kong, Hong Kong, Hong Kong, Sha Tin, Hong Kong
**Corresponding Author*

T cell-based immunotherapies are effective for blood cancers but show low efficiency in solid tumors. Single-cell RNA-sequencing (scRNA-seq) can retrospectively capture the dynamics of T cells at the transcriptome level, allowing us to better understand their development in solid tumor. By using transformer-based artificial intelligence (AI) tools, we analyzed scRNA-seq data by converting gene expression information into gene set tokens to identify cell types and developmental trajectories. Surprisingly, we discovered a novel T cell population in non-small-cell lung carcinoma (NSCLC), which is absent in leukemia and positively associated with better survival of solid cancer patients. Advanced bioinformatics and fate-mapping studies experimentally further confirmed this T cell type originated from a novel origin. This novel T cell type showed strong anti-tumor activity against syngeneic lung cancer LLC and melanoma B16F10 in vivo and in vitro, highlighting their clinical potential for solid tumor immunotherapy. Mechanistically, we identified a conserved pathway for blocking their development in tumor, which can be targeted by pharmaceutical inhibition in vivo. Most importantly, we successfully engineered and mass-produced this new T cell type from human peripheral blood in vitro which effectively blocked human NSCLC progression in vivo, representing a novel and effective immunotherapy for solid tumors.

Acknowledgement: This study was supported by Research Grants Council of Hong Kong (14111720, 24102723, 14107624); RGC Postdoctoral Fellowship Scheme (PDFS2122-4S06); Health and Medical Research Fund (10210726, 11220576); Innovation and Technology Fund (ITS/177/22FP, ITS/016/23MS), CU Medicine Passion for Perfection Scheme (PFP202210-004) and Faculty Innovation Award 2019 (4620528), CUHK Strategic Seed Funding for Collaborative Research Scheme (178896941), Direct Grant for Research (2024.090), and Postdoctoral Fellowship Scheme (NL/LT/PDFS2022/0360/22lt, WW/PDFS2023/0640/23en)

35 In Vitro and In Vivo Lipid Nanocrystal (LNC) Delivery of Small Oligonucleotides and Small Molecules in Oncology

Hui Liu[1], Miriam Mikhael[2], Amra Tabakovic[2], Partha Samadder[3], Tzong-Jen Sheu[2], Eugene Dank[2], Shan Liu[2], Thomas Hoover[4], **James Ferguson**[5]

[1]Bridgewater, NJ, [2]Matinas BioPharma, Bedminster, NJ, [3]Matinas BioPharma, Bridgewater, NJ, [4]Matinas BioPharma, Concord, MA, [5]Matinas BioPharma, Bedminster, NJ, NJ

Background: Delivery of therapeutic nucleic acids has advanced considerably, but with little progress in orally delivered therapeutics beyond the liver, particularly in oncology. Matinas BioPharmas' lipid nanocrystal (LNC) delivery platform is a highly stable, multi-layered nanoparticle that self-assembles when phosphatidylserine (PS) liposomes and calcium are combined, incorporating cargo between concentric lipid bilayers. LNCs have been successfully used in an oral, non-nephrotoxic formulation of amphotericin-B in patients with severe systemic fungal infections. Recent in vivo data has suggested additional potential for delivery of small oligos targeting inflammatory cytokines. PS appears to be a key targeting moiety of LNCs for infected or inflamed cells and professional phagocytes targeting surface PS-expressing apoptotic cells, but the ability of LNCs to target tumor cells (with varying amounts of surface PS) remains undefined. Live cell imaging in somatic cells (HEK293) is strongly suggestive of cell

entry via endocytosis, but mechanism(s) of LNC cargo delivery to tumor cells have not been described. To assess the potential of LNCs for oncology applications, we performed in vitro studies evaluating intracellular delivery of small oligos to tumor cells and in vivo studies with LNC oral delivery of chemotherapeutics in a number of animal tumor models.

Methods: Live cell imaging studies were conducted in SKBR3 breast cancer cells using FITC-labeled LNCs with an inactive Cy5-labeled ASO cargo. Uptake studies were also performed in MCF7 breast cancer cells using rhodamine-labelled LNCs with docetaxel cargo. As a precursor to in vivo use, stability studies evaluated the ability of LNCs to preserve the activity of small oligonucleotide cargos in GI fluid. Finally, in vivo studies utilizing oral LNC docetaxel were conducted in a variety of tumor models.

Results: In SKBR3 cells LNC uptake and ASO delivery occurred extremely rapidly, with immediate uptake and very early appearance of cargo in cell membrane ruffles, followed shortly thereafter by the appearance of cargo in the cytosol, with delivery complete by 3 hours. The diffuse appearance of cytosolic ASOs and the very rapid time course of delivery is suggestive of cell membrane fusion (Figure 1). MCF7 cells also showed uptake of labeled LNCs within 1 hour. LNC with cytokine-inhibiting small oligos cargos maintained full biological activity in vitro even after 30 minute exposure to pH of 1.2. In vivo studies of oral LNC formulations of docetaxel in multiple tumor models (B16 melanoma, RM-1 prostate and MDA-MB-231 TNBC) demonstrated reductions in tumor size, with little impact on body weight (Figure 2), validating successful tumor targeting and delivery. The observed efficacy despite low tumor concentrations also suggests increased efficiency of delivery - and the very low plasma docetaxel levels (in contrast to IV) – confirm cargo protection in the blood, with an improved therapeutic index.

Conclusions: LNCs are avidly and rapidly taken up by tumor cells, and can successfully deliver small oligo cargos to tumor cells. LNC formulations maintain their biological activity in a highly acidic environment, and in plasma, permitting oral administration. LNCs can be used to orally deliver chemotherapeutic agents to tumors in vivo, with an improved therapeutic index for otherwise toxic agents. LNCs hold considerable promise for future applications of tumor-targeted small oligonucleotides.

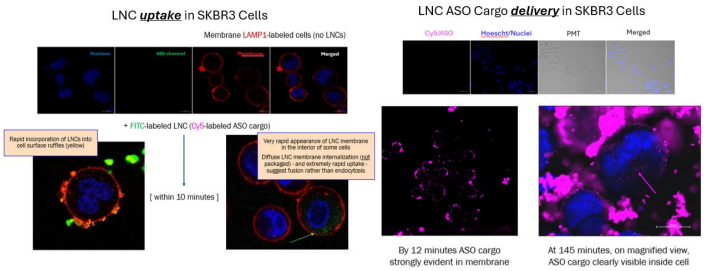


Figure 1

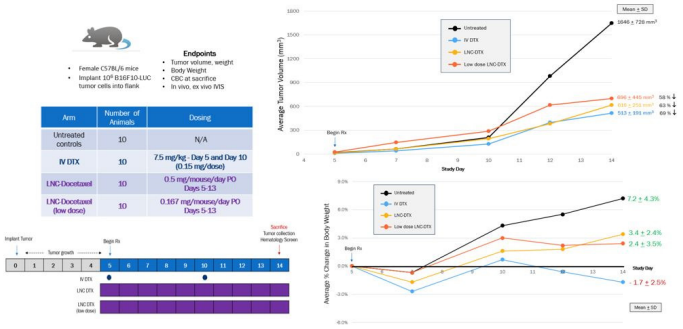


Figure 2

36 C/EBPß Drives Tumor Growth, Chemoresistance, and Immune Evasion Through the Stabilization of Senescent Secretory Cell Populations in the Tumor

Kevin Holm[1], John Rossi[2]
[1]City of Hope Beckman Research Institute, Monrovia, CA, [2]Beckman Research Institute of City of Hope, Monrovia, CA

Triple-negative breast cancer (TNBC) is a form of breast cancer with poor prognosis in large part due to a dearth of available and effective therapeutics. CCAAT-enhancer binding protein β (C/EBPβ) is a leucine zipper transcription factor with a traditional function in mammary gland development and macrophage differentiation. In tumors, C/EBPβ is associated with metastatic and chemoresistant forms of breast cancer. Previous efforts at targeting this transcription factor in the tumor have been hampered by off-target effects and low penetrance into the intratumoral space. Furthermore, studies into C/EBPβ knockdown *in vitro* have been mixed, owing in part to two distinct isoforms that are differentially expressed in healthy and cancerous tissues and the seemingly contradictory role C/EBPβ plays in inducing tumor growth and senescence.

Given that C/EBPβ's function is closely tied to hypoxia factors such as HIF-1alpha, we hypothesized that the hypoxic intratumoral space may be driving specific isoform development, and consequently the pro-metastatic phenotype observed clinically. To this end, we induced a C/EBPβ-deficient cell line which showed increased sensitivity to oxidative stress and hypoxic conditions, as well as reduced growth and migration in vitro. Xenografts derived from these cells displayed reduced myeloid-derived suppressor cell formation, increased senescent secretory cells surrounding the necrotic core, and reduced systemic inflammation. Curiously, the most significant cell populations with a pro-tumorigenic phenotype were found near the hypoxic core of the tumor. Further studies revealed that C/EBPβ can stabilize cells from DNA damage by both oxidative stress and PARP inhibition, possibly through a non-canonical pathway of DNA binding and stabilization.

To therapeutically target this hypoxia-driven phenotype, we then developed an aptamer-siRNA conjugate containing a transferrin receptor 1 (TfR1) aptamer (a receptor activated under hypoxic conditions) linked to a C/EBPβ siRNA. We have measured C/EBPβ's suppression of metastasis in traditional cell culture under hypoxic conditions, as well as in a spheroid model. These results point toward a novel approach to C/EBPβ's contradictory role as a driver and mediator of metastasis, and a potential therapeutic for its treatment.

37 Addressing the HEK293 Host Cell Protein Monitoring Challenges for Viral Vector Gene Therapy Manufacturing

Sonia BRUN[1], Stephane Martinez[2], **Olivia Kelada[3]**
[1]Codolet, France, [2]Revvity Inc, Codolet, France, [3]Revvity Inc, Hopkinton, MA

Introduction: Host cell proteins (HCP) are process-related protein impurities present in biological drug products derived from host organisms, including bacteria, yeast, or mammalian production cell lines. Human Embryonic Kidney (HEK) 293 cells are commonly used in the expression and production of therapeutic proteins and viral vectors for gene therapy. During the manufacturing process, besides expression of the drug product of interest, HEK 293 cells express numerous endogenous proteins, referred to as HCPs. The presence of HCP contaminants is a key concern due to the potential of a decrease in drug product efficacy and stability, as well as the possibility of adverse clinical effects. Hence, the clearance and monitoring of HCPs during downstream process is critical for biopharmaceutical companies to comply with regulatory guidelines. Addressing this concern, the new no-wash AlphaLISA® HEK 293 HCP detection kit has been designed to monitor and quantitatively measure HCPs contamination in products manufactured by transgenic expression in HEK 293 host cells. It covers the entire spectrum, from crude cell harvest material to the final drug product, offering a highly sensitive, quantitative, reproducible, and user-friendly quality assessment workflow. Methods: Limits of detection (LOD) and quantification (LOQ) were respectively calculated by interpolating the average background counts + 3 or 10 x standard deviation value on the standard curve. To characterize the immunoassay performances of the new AlphaLISA® HEK 293 HCP detection kit, experiments of dilutional linearity, antigen spike recovery, inter- and intra-assay precision were performed on crude lysates samples derived from biomanufacturing process using two various subtypes of the HEK 293 cell line. Moreover, the assay compatibility of several commonly used Adeno-Associated Virus (AAV) manufacturing buffers and the lack of cross-reactivity between HEK 293 HCP detection and AAV presence (AAV2 and AAV5) were assessed. Finally, to estimate the percentage of total HCPs that the assay can detect, a coverage analysis by 2D-DIBE (2D Differential in Blot Electrophoresis) was performed with the Revvity anti-HEK 293 HCP polyclonal antibody. Results: The AlphaLISA® HEK 293 HCP detection kit is characterized by very low LOD (0.302 ng/mL) and LOQ (1.013 ng/mL) and a very broad quantitative range (1.013-1,000 ng/mL). It also displays exceptional dilutional linearity (global mean % dilution recovery close to 100% and linearity R2>0.99), reliable antigen spike recovery (acceptance criteria: 85-115%), and robust reproducibility and repeatability (coefficient variation % of measured concentrations < 15%). The kit is compatible with the most commonly used biotherapeutics manufacturing buffers and in presence of the drug substance (80% < antigen recovery < 120%). Moreover, the Revvity anti-HEK 293 HCP polyclonal antibody has an excellent coverage of HEK 293 HCPs across the two HEK 293 subtypes tested (>95%). Conclusion: The exceptional immunoassay performance of the AlphaLISA® HEK293 HCP detection kit and the excellent coverage of the associated anti-HEK 293 HCP antibody demonstrate the great interest of using this new ready-to-use no-wash kit to quantify HEK 293 HCP impurities quickly and easily during biopharmaceutical and gene therapy viral vector process manufacturing. The off-the-shelf kit delivers a streamlined workflow, a broader quantitative range, and greater sensitivity compared to trad

38 A Novel Therapeutic Target of MDSC-dependent Immunosuppression in NSCLC *(Virtual)*

Siu Fan Jane Li[1], Kam Kwan Chan[2], Philip Chiu-Tsun TANG[3], Jiaoyi Chen[4], Sydney Chi-Wai Tang[4], Chun-Kwok Wong[5], Ka-Fai To[6], Patrick Ming Kuen Tang[3]
[1]The Chinese University of Hong Kong, Hong Kong, Wan Chai, Hong Kong, [2]Department of Anatomical and Cellular Pathology, State Key Laboratory of Translational Oncology, The Chinese University of Hong Kong, Hong Kong, Sha Tin, Hong Kong, [3]Department of Anatomical and Cellular Pathology, State Key Laboratory of Translational Oncology, Prince of Wales Hospital, The Chinese University of Hong Kong, Hong Kong, Hong Kong, Sha Tin, Hong Kong, [4]The University of Hong Kong, Hong Kong, Central and Western, Hong Kong, [5]The Chinese University of Hong Kong, Shatin, Sha Tin, Hong Kong, [6]Department of Anatomical and Cellular Pathology, State Key Laboratory of Translational Oncology, Prince of Wales Hospital, The Chinese University of Hong Kong, Hong Kong, The Chinese University of Hong Kong, Sha Tin, Hong Kong

Myeloid-derived suppressor cells (MDSCs) play a crucial role in promoting immunosuppression and cancer progression within the solid tumor microenvironment, contributing significantly to major resistance against T-cell immunotherapy. A deeper understanding of the regulatory mechanisms governing MDSC development and function may help identify new therapeutic strategies for solid cancers. By single cell RNA-sequencing (scRNA-seq), we observed hyperactivation of TGF-beta/Smad signaling in MDSCs of non-small-cell lung carcinoma (NSCLC), the Smad+ve MDSCs are associated with a poorer overall survival in patients. Interestingly, by using syngeneic Lewis Lung Carcinoma (LLC) mouse model, we observed a significant deceleration of tumor growth and reduction of MDSC abundance in Smad-KO mice. In addition, scRNA-seq further revealed a regulatory role of the Smad protein in MDSC developmental fate under cancer conditions. Genetic deletion and pharmacological inhibition of the Smad protein in MDSCs effectively reprogrammed their developmental fate and enhanced anticancer immunity, thereby suppressed cancer progression in both LLC and A549-bearing mice in vivo. Thus, we discovered Smad signaling as a novel therapeutic target for enhancing clinical immunotherapy by effectively inhibiting MDSC-dependent immunosuppression in NSCLC.

Acknowledgement: This study was supported by Research Grants Council of Hong Kong (14111720, 24102723, 14107624); RGC Postdoctoral Fellowship Scheme (PDFS2122-4S06); Health and Medical Research Fund (10210726, 11220576); Innovation and Technology Fund (ITS/177/22FP, ITS/016/23MS), CU Medicine Passion for Perfection Scheme (PFP202210-004) and Faculty Innovation Award 2019 (4620528), CUHK Strategic Seed Funding for Collaborative Research Scheme (178896941), Direct Grant for Research (2024.090), and Postdoctoral Fellowship Scheme (NL/LT/PDFS2022/0360/22lt, WW/PDFS2023/0640/23en)