Distribution Agreement

In presenting this thesis or dissertation as a partial fulfillment of the requirements for an advanced degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis or dissertation in whole or in part in all forms of media, now or hereafter known, including display on the world wide web. I understand that I may select some access restrictions as part of the online submission of this thesis or dissertation. I retain all ownership rights to the copyright of the thesis or dissertation. I also retain the right to use in future works (such as articles or books) all or part of this thesis or dissertation.

Scott Andrew Becker

Date

Strategies for engineering γδ T cells to treat pediatric cancers

By

Scott Andrew Becker
Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences
Molecular and System Pharmacology

_____
H. Trent Spencer, PhD
Advisor

_____
Christopher Doering, PhD

_____
Renhao Li, PhD

_____
Lily Yang, PhD

_____
David Archer, PhD

Accepted:

_____
Kimberly Jacob Arriola, PhD
Dean of the James T. Laney School of Graduate Studies

_____

Date

Strategies for engineering γδ T cells to treat pediatric cancers

By

Scott Andrew Becker
B.S. University of South Carolina, 2016

Advisor: H. Trent Spencer, PhD

An abstract of a dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
in the Graduate Division of Biological and Biomedical Science,
Molecular and Systems Pharmacology
2023

Abstract (350 words)

Strategies for engineering γδ T cells to treat pediatric cancers

By Scott Andrew Becker

Developing novel treatments to fight cancer is essential for improving patient outcomes and safety. Cancer immunotherapy has become an attractive alternative to chemotherapy that harnesses the immune system to target cancer cells. Adoptive cell therapy (ACT) is a therapeutic technique where immunocompetent cells are isolated from a patient or healthy donor, expanded and engineered *ex vivo*, and then administered to the patient. Traditionally, ACT utilizes autologous αβ T cells that are stably engineered using viral vectors, such as lentivirus. One of the most common engineering strategies is the expression of chimeric antigen receptors (CARs) that are specific to an antigen on cancer cells. While CAR T cell therapy has seen promising clinical results, there are several limitations that have been identified during clinical testing. Transient engineering of γδ T cells offers a promising alternative to the traditional stable engineering of αβ T cells and can overcome some of the obstacles with CAR T cell therapy. γδ T cells are a small subset of lymphocytes that bridge the gap between the adaptive and innate immune systems, which makes them the ideal candidate for ACT. They recognize antigens in an MHC-independent manner and have several inherent cytotoxic mechanisms that allow them to recognize infected and cancerous cells. Therefore, the goal of this dissertation was to develop a transient engineering platform for γδ T cells through the use of recombinant AAV and mRNA electroporation. Despite limited gene transfer and poor viability with AAV6 techniques, mRNA electroporation resulted in high transfection efficiency and CAR expression up to 60-70%. We also show that mRNA electroporation can be used to express a secreted bispecific T cell engager (sBite) in γδ T cells. The sBite-modified γδ T cells were specific and improved the cytotoxicity of modified and unmodified cells. The CAR- and sBite-modified γδ T cells not only exhibited increased cytotoxicity *in vitro*, but also reduced tumor burden and improved survival in two *in vivo* cancer models. Overall, we show that transient engineering of γδ T cells can be an effective platform to develop the next generation of cancer therapeutics.

Strategies for engineering γδ T cells to treat pediatric cancers

By

Scott Andrew Becker
B.S. University of South Carolina, 2016

Advisor: H. Trent Spencer, PhD

A dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
in the Graduate Division of Biological and Biomedical Science,
Molecular and Systems Pharmacology
2023

Acknowledgments

I would like to first thank my mentor, Dr. Trent Spencer. Your guidance and mentorship during my graduate school career has been instrumental in my training and education. You introduced me to the exciting world of Gene and Cell Therapy and, in turn, revealed a promising path to accomplish one of my biggest dreams, developing the next generation of cancer therapeutics. I've learned so much from you over the years and appreciate your continued support and dedication. You have helped make me the scientist I am today, and I am truly grateful for that.

I would also like to thank Dr. Chris Doering. You have played an integral role in my training, and I am so thankful for your input on my project. You were always someone I could go to with questions and always willing to help when needed. I am so grateful for your expertise and assistance during my time in graduate school. I would like to thank my other committee members: Dr. Renhao Li, Dr. Lily Yang, and Dr. David Archer. Your questions and comments throughout graduate school not only improved my project, but also pushed me to think critically and be a better scientist. Also, thank you to the past and present members of the Spencer and Doering Lab. You created an amazing working environment that was full of comradery and collaboration.

Finally, I would like to thank my family. Thank you to my parents for helping me get to where I am today. Your support and encouragement helped me get through the good times, as well as the tough times during graduate school. Thank you to my brothers for supporting me and listening when I complained when things didn't go as planned, even if you didn't understand. To my lovely wife Gaby, you were one of the best things that came out of graduate school. You are an amazing partner and I can't thank you enough for always being by my side.

Table of Contents

Abstract

Acknowledgments

Table of Contents

List of Figures and Tables

List of abbreviations

Chapter 1: Introduction to Cancer Immunotherapy …….....1

1.1 Cancer immunotherapy overview ……2

A. Adoptive cell therapy……..……..2

B. CAR T cell therapy…….……..4

C. CD19 CARs and clinical landscape……...5

1.2 Challenges in traditional CAR T cell therapy……9

A. Graft-versus-host disease……..……9

B. On-target-off-tumor toxicity……...……13

C. Cytokine release syndrome……..……...15

D. Antigen escape……..……..17

1.3 Transient engineering for cancer therapeutics…….……19

A. Gene delivery techniques……....……19

B. Genetic engineering of TCRs……...……...25

C. Expression of CARs……..……..27

D. Expression of cytokines and other recombinant proteins…….……..32

1.4 γδ T cells…….……37

A. Classification, characterization, and functions…….……..37

B. Mechanisms of antigen recognition ……39

C. Vγ9Vδ2 T cells for ACT……...……..42

Chapter 2: Engineering γδ T cells using AAV6 and microfluidics devices … ……..……43

2.1 Abstract……..44

2.2 Introduction……44

2.3 Results……46

2.4 Discussion……..64

2.5 Materials and Methods……67

2.6 Supplemental Figures, Tables, and Legends…….……..70

Chapter 3: Enhancing the effectiveness of γδ T cells by mRNA transfection of chimeric antigen receptors or bispecific antibodies ……...……..74

3.1 Abstract……..75

3.2 Introduction……75

3.3 Results……77

3.4 Discussion……..……99

3.5 Materials and Methods……..102

3.6 Supplemental Figures, Tables, and Legends…….……107

Chapter 4: General Discussion…….……...115

4.1 Summary of Results…….…….116

4.2 Implications of Findings……...122

4.3 Limitations and Future Directions…...……126

4.4 Conclusions……..129

Literature Cited……130

List of Figures and Tables

Table 1-1: Current FDA-approved CAR T cell therapies

Figure 1-1: Limitations of the current CAR T cell therapy platform

Figure 1-2: γδ T cells exhibit several natural mechanisms of cancer cell recognition

Figure 2-1: Second ultracentrifugation improves purity of AAV6 vector

Figure 2-2: Second ultracentrifugation increases the proportion of full capsid particles

Figure 2-3: AAV6 transduction of γδ T cells results in variable GFP expression and reduced cell viability

Figure 2-4: Microfluidics device improves transduction of Jurkat cell line

Figure 2-5: Microfluidics device improves transduction of γδ T cells but reduces cell viability

Figure 2-6: Transduction of AAV6-MND-CD5 CAR results in limited GFP and CAR expression in Jurkats and γδ T cells

Figure 2-7: Jurkat cells transduced with AAV6 mutant vectors express GFP and CD5 CAR

Figure 2-8: CD5 expression is reduced as CD5 CAR expression increases

Figure 2-9: AAV6 mutant transduction of γδ T cells results in limited transduction efficiency and low cell viability

Supplemental Figure 2-1: Schematic of AAV6 GFP and CD5 CAR vectors

Supplemental Figure 2-2: GFP expression increases with increasing vector percentage of culture volume

Supplemental Figure 2-3: HEK293 cells express GFP after transfection of newly cloned AAV6 vector

Supplemental Figure 2-4: Downregulation of CD5 CAR after interaction with CD5

Figure 3-1: Optimization of γδ T cell electroporation with bicistronic CD19 CAR-GFP mRNA

Figure 3-2: Electroporation of γδ T cells before freezing results in lower CAR expression and reduced cytotoxicity

Figure 3-3: CD19 CAR- and CD22 CAR-expressing γδ T cells enhances cytotoxicity against two B-ALL cell lines

Figure 3-4: γδ T cells express and secrete CD19 sBite after mRNA electroporation

Figure 3-5: γδ T cells are not able to kill cancer cells once they extravasate from circulation

Figure 3-6: Engineered γδ T cells expressing a CD19 CAR reduce tumor burden and improve survival in 697 model

Figure 3-7: Engineering γδ T cells with CD19 CAR or sBite mRNA reduce tumor burden and improves survival in Nalm6 model

Figure 3-8: Longer treatment regimen does not lengthen survival benefit for Nalm6 model

Supplemental Figure 3-1: Schematic of CD19 CAR and CD19 sBite mRNA constructs

Supplemental Figure 3-2: Freezing engineered cells reduces cell viability

Supplemental Figure 3-3: Comparison of newly cloned codon optimized CD19 CAR construct

Supplemental Figure 3-4: Western blot analysis of CD19 sBite in γδ T cell conditioned media

Supplemental Figure 3-5: Migration patterns of cancer cells and γδ T cells *in vivo*

Supplemental Figure 3-6: Treatment starting on day 7 is unsuccessful in improving tumor burden

Supplemental Figure 3-7: Co-expressing CD19/CD22 CARs did not further improve efficacy

Supplemental Figure 3-8: Representative data from characterization of γδ T cell expansion

List of Abbreviations

7AAD	7-aminoactinomycin
AAV	Adeno-associated virus
ACT	Adoptive cell therapy
Ad	Adenovirus
ADCC	Antibody-dependent cellular cytotoxicity
AML	Acute myeloid leukemia
B-ALL	B-cell acute lymphoblastic leukemia
BCMA	B-cell maturation antigen
BTN	butyrophilin
CAR	Chimeric antigen receptor
CEA	Carcinoembryonic antigen
CIK	Cytokine induced killer
CMV	cytomegalovirus
CRP	C-reactive protein
CRS	Cytokine release syndrome
CTLA4	Cytotoxic T-lymphocyte-associated antigen 4
DAMP	Damage-associated molecular pattern
DRIL18	Decoy resistant IL18
DsiRNA	Diver-substrate small interfering RNA
E:T	Effector to target
EGFR	Epidermal growth factor receptor
FasL	Fas ligand
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GvHD	Graft-versus-host disease
HBV	Hepatitis B virus
H+E	Hematoxylin and eosin
HER2	Human epidermal growth factor 2
HLA	Human leukocyte antigen
HSA	Human serum albumin
HSPC	Hematopoietic stem and progenitor cell
IFNγ	Interferon γ
IL	interleukin
IL15RA	IL15 receptor alpha chain
ITR	Inverted terminal repeat
IPP	Isopentenyl pyrophosphate
i.v.	intravenously
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MICA/B	MHC class 1-related proteins A and B
MOI	Multiplicity of infection
NHL	Non-Hodgkin lymphoma
NK	Natural killer
NKT	Natural killer T

NSCAR	Non-signaling CAR
OTOT	On-target-off-tumor
PAg	phosphoantigen
PBMC	Peripheral blood mononuclear cell
PD1	Programmed cell death protein 1
sBite	Secreted bispecific T cell engager
scAAV	Self-complimentary AAV
scFv	Single chain variable fragment
rAAV	Recombinant AAV
TALEN	Transcription activator-like effector nuclease
T-ALL	T cell acute lymphoblastic leukemia
TCR	T cell receptor
TIL	Tumor infiltrating lymphocytes
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAC	T cell receptor constant alpha chain
TRAIL	TNF-related apoptosis-inducing ligand
TRBC	T cell receptor constant beta chain
ULBP	UL16-binding protein
WT1	Wilms' tumor protein 1

Chapter 1

Introduction to Cancer Immunotherapy

1.1 Cancer Immunotherapy Overview

Cancer immunotherapy has become a focal point in improving treatment options for cancer patients. The first observation that aspects of immunotherapy can be applied to cancer treatment occurred in the late 19th century with the discovery that inoculating cancer patients with heat-inactivated bacteria resulted in many experiencing either short or long term remission [1]. Over the course of the 20th century, scientific discoveries in the field of immunology advanced our understanding of the immune system and further revealed the potential of harnessing the body's natural mechanisms of immunity to fight cancer. For example, interferons were discovered to play a role in the immune system's response to foreign or injured cells, and the discoveries of several immune cells including T cells, dendritic cells, and natural killer (NK) cells were instrumental in learning how the body's immune system functions [2]. With a better grasp of how the immune system works, scientists began investigating how it can be harnessed to target and eliminate cancer. This led to breakthroughs in the field of cancer immunotherapy like cancer vaccines, immune-modulating agents like cytokines and immune checkpoint inhibitors, and adoptive cell therapy.

A. Adoptive cell therapy

Adoptive cell therapy (ACT) is a type of immunotherapy where immunocompetent cells are isolated from a patient, expanded and engineered *ex vivo*, and re-infused back into the patient. ACT dates back to the 1980s when Rosenberg et. al. reported the first clinical benefit of co-infusing autologous lymphokine-activated killer cells with interleukin (IL) 2 to patients with metastatic cancers [3]. Despite only achieving complete or partial responses in 11 out of 25 patients, this groundbreaking study laid the foundation for adoptive transfer of immunocompetent cells to treat cancer. The group later improved upon their initial work by applying their ACT technique to tumor infiltrating lymphocytes (TILs), which resulted in significant improvements in response rates, both *in vivo* and clinically [4, 5].

Since these preliminary studies, the field of ACT progressed quickly, with one of the main focal points initially being the use of TILs. TILs are isolated from a primary tumor by either digestion into a single

cell suspension or by manual cutting into small fragments. The subsequent addition of IL2 to the culture results in the preferential expansion of lymphocytes that can be infused back into the patient [6]. Many of the preliminary clinical studies investigating the use of TILs for ACT focused on targeting melanoma, with varying complete response rates ranging between about 30-60% [5, 7-10]. As more studies were conducted, researchers optimized the culturing and administration of TILs to patients. An important study in 2002 by Dudley et. al. demonstrated that pretreating patients with a lymphodepleting regimen before TIL infusion resulted in increased cell persistence and improved response rates [8]. Researchers also found more advanced methods of culturing and expanding highly effective TILs from tumors like using gas permeable G-REX flasks, identifying screening techniques to determine the likelihood of successful tumor regression, as well as other methods to improve the efficacy of TIL-based ACT [11, 12].

Another source of immunocompetent cells for ACT is the use of lymphocytes isolated from peripheral blood. Initial attempts focused on infusing T cells that were isolated from a patient and simply activated *ex vivo*. The idea that T cells can be directed toward cancer cells through specific activation stems from the work mentioned above by Dudley et. al., when they showed that clonal populations of T cells specific to a certain tumor antigen can improve proliferation and persistence *in vivo* and can help eradicate tumors [8]. This concept improved response rates compared to simply infusing *ex vivo* expanded lymphocytes, however, as technology advanced, researchers began investigating the advantages of engineering T cells. Engineering T cells significantly advanced the field of ACT because it allowed for a more targeted approach by redirecting the cytotoxic functions specifically to cancer cells, based on expression of an antigen. Since then, researchers have investigated methods to enhance the specificity of adoptively transferred cells, with two main techniques being developed: T cell receptor (TCR) engineering and expression of chimeric antigen receptors (CARs).

The concept of TCR engineering originated from the work conducted by Dembic et. al. in 1986, when they first showed that the successful transfer of TCR genes into lymphocytes can redirect their specificity [13]. Once TCR genes for specific antigens were identified and cloned, this concept was later applied to

introduce TCR genes specific to a single tumor antigen into lymphocytes. In 1999, Clay et. al. published their work on transducing peripheral blood lymphocytes with cDNA encoding a MART1-specific TCR and showed the engineered cells were able to lyse a melanoma cell line via the introduced TCR [14]. Since this first groundbreaking publication, researchers have investigated the introduction of TCRs specific to numerous tumor antigens; including MDM2, LMP2, and many others [15]. Another technique researcher developed to enhance the specificity of T cells is through the expression of chimeric antigen receptors. The design of CARs is based on the components of the endogenous TCR, with an extracellular antigen binding domain and an intracellular signaling domain. The development, advancement, and success of CAR T cell therapy is discussed below.

B. CAR T cell therapy

The development of CAR T cell therapy has revolutionized cancer immunotherapy, especially ACT, by allowing for a more targeted therapeutic approach, compared to chemotherapeutics. This is achieved by engineering T cells to express a receptor that recognizes a specific tumor antigen. The first reports of the development of chimeric receptors that were able to redirect the cytotoxic capabilities of T cells occurred in the late 1980s and early 1990s [16-18]. These studies showed that expression of these chimeric receptors can focus the cytotoxic mechanisms of T cells based on the specificity of antibodies, which can help target certain cells and results in IL2 secretion and T cell activation. Importantly, it also revealed that T cells can be activated through mechanisms that are not dependent on antigen presentation via the major histocompatibility complex (MHC).

CARs combine the signaling components of a T cell's TCR with the specificity of an extracellular antigen binding domain. The most common type of CAR uses the single chain variable fragment (scFv) of an antibody as the antigen recognition domain, which allows for a high affinity interaction between the T cell and cancer cell [19]. In addition to scFv-based CARs, researchers have also developed CARs based on natural receptors and ligands [20]. A spacer region is typically placed after the antigen binding domain

and before the transmembrane region. This region is critical for some CAR constructs because it gives flexibility to the antigen binding domain and can allow for more effective binding to target cells [21]. The next component of a CAR is the transmembrane domain and is typically comprised of type I membrane proteins, such as CD3, CD4, CD8, and CD28. Researchers discovered that the transmembrane domain plays an important role in the activation of CAR T cells. In 2010, Bridgeman et al. showed that CARs containing CD3ζ become homodimerized via the transmembrane domain and form complexes with the endogenous TCR, which improves CAR T cell signaling and activation [22]. The intracellular portion of the CAR comprises the signaling domains. The most common CARs use CD3ζ or the signaling components of an Fc receptor for the activation domain and use CD28, 4-1BB, or OX40 as the costimulatory domains [23].

Initial work with first generation CARs included only the extracellular antigen recognition domain and an intracellular signaling domain, without a costimulatory domain. Despite showing signs of minor activation, these first generation CARs did not elicit production of IL2 and full activation upon binding to the target [24]. In addition, first generation CAR T cells had very poor proliferative potential *in vivo* and therefore showed limited clinical efficacy [25, 26]. As scientists further studied the activation of T cells and the importance of co-stimulation, researchers applied this concept to the development of CARs. The current, second and third generation CARs have incorporated one or two costimulatory domains, respectively. The addition of costimulatory domains, such as CD28 and 41BB, has been shown to enhance proliferation, persistence, and efficacy of CAR T cells [27-29]. These second and third generation CARs have shown great promise in preclinical and clinical studies and have greatly advanced ACT for the treatment of cancer.

C. CD19 CARs and clinical landscape

Although the most successful application of CAR T cells in clinical trials is the treatment of B-cell malignancies using CD19 CARs, the initial attempts to translate CAR T cells into the clinic focused on

solid tumors, however they were unsuccessful due to limited *in vivo* persistence and the development of severe toxicities [30, 31]. Since these initial attempts, the CAR T cell field has focused on B-cell malignancies and the improvement of CAR design led to more promising results. The first evidence that CAR T cell therapy was safe and had clinical applications occurred in 2008 at the Fred Hutchinson Cancer Research Center and the City of Hope National Medical Center [26]. Seven patients with indolent or mantle cell lymphoma were treated with CAR T cells targeting CD20, with two patients maintaining previous complete responses, one achieving partial response, and four achieving stable disease. Despite not showing outstanding responses, this first clinical trial revealed that CAR T cell therapy can be tolerated by cancer patients and had the potential to be an effective treatment option. A follow-up study was performed by the group from the City of Hope on patients with relapsed diffuse B cell lymphoma using CD20 or CD19 CARs, however CAR T cell persistence was limited due to an anti-transgene immune response [32]. The first successful report of CD19 CAR T cells occurred in 2010 at the National Cancer Institute [33]. Despite only treating one patient with advanced follicular lymphoma, the impressive response and cancer regression showed that CD19 CARs had the potential to be an effective option for treating B-cell malignancies.

After these results from the preliminary studies to advance CAR T cell therapy into the clinic, hundreds of cancer patients have been treated in clinical trials. CD19 CARs have been the most common because CD19 is an attractive target for CAR T cell therapy due to its expression on most B cell precursor cells and mature B cells. Based on these first clinical trials, several conclusions have been made on the advancement of CAR T cell therapy, including the importance of preconditioning with lymphodepleting chemotherapy, second and third generation CARs are more beneficial than first generation CARs, there is no definitive dose-response relationship between the number of CAR T cells administered and outcomes, and the most promising response rates were seen in patients with B-cell acute lymphoblastic leukemia (B-ALL) [34]. This led to the next wave of clinical trials that were more extensive and had bigger cohorts with adult and pediatric patients. The results from these trials showed impressive responses with most observing complete or objective response rates of over 70% [35, 36].

These clinical trials, as well as others, laid the foundation for treating cancer patients with CAR T cell therapy and ultimately resulted in the FDA approval of several CAR T cell products (Table 1-1). In 2017, the FDA approved the first CAR T cell therapy, Kymriah. Kymriah is a CD19 CAR therapy that was initially approved for relapsed or refractory B-cell acute lymphoblastic leukemia. The ELIANA trial, consisting of 75 pediatric patients, showed an overall remission rate of 81% and event free survival of 73% after 6 months [37]. Based on results from the JULIET trial, Kymriah was later approved for the treatment of relapsed or refractory lymphoma as well [38]. The approval of Yescarta, another CD19 CAR product, followed soon after for the treatment of several types of lymphomas. The approval was based on the ZUMA-1 phase II clinical trial that included 111 patients and showed an objective response rate of 82% and overall survival rate of 52% at 18 months [39]. These first two approved CAR T cell therapies were instrumental in advancing ACT and revealed the potential benefit of this treatment technique for cancer patients. Since then, there have been four additional FDA approved CAR T cell products, including Tecartus, Breyanzi, Abecma, and Carvykti. Tecartus and Breyanzi are both CD19-based CARs, while Abecma and Carvykti target the B-cell maturation antigen (BCMA).

Table 1-1: Current FDA-approved CAR T cell therapies

Name	Brand Name	Company	Target	Cancer type
Tisagenlecleucel	Kymriah	Novartis	CD19	B-ALL B-cell NHL
Axicabtagene ciloleucel	Yescarta	Kite Pharma	CD19	B-cell NHL Follicular lymphoma
Brexucabtagene autoleucel	Tecartus	Kite Pharma	CD19	B-ALL Mantle cell lymphoma
Lisocabtagene maraleucel	Breyanzi	Bristol Myers Squibb	CD19	B-cell NHL
Idecabtagene vicleucel	Abecma	Bristol Myers Squibb	BCMA	Multiple myeloma
Ciltacabtagene autoleucel	Carvykti	Novartis	BCMA	Multiple myeloma

B-ALL: B- cell lymphoblastic leukemia; B-cell NHL: B-cell non-Hodgkin lymphoma

Although most of the success of CAR T cell therapy has been with B-cell malignancies, researchers have also focused on applying this treatment to other types of cancers. Many different types of solid tumors have been targeted and reached clinical trials including pancreatic [40, 41], gastric [42], ovarian [31, 43], colorectal [44], several types of tumors in the brain [45, 46], as well as others. Most CAR targets are specific to certain cancer types, however some have been shown to be effective against several cancers such as epidermal growth factor receptors (EGFR), NKG2D ligands, human epidermal growth factor receptor 2 (HER2), B7-H3, Mucin-1, and carcinoembryonic antigen (CEA) [47]. Despite not performing as well as CARs targeting B-cell malignancies, CAR T cell therapy for solid tumors has the potential to be an effective treatment option and improve patient outcomes.

1.2 Challenges in traditional CAR T cell therapy

As more clinical trials are conducted, and long-term data becomes available, our understanding of how CAR T cell therapy performs clinically becomes clearer and more extensive. Despite its early success and great promise, there are obstacles and limitations that must be addressed for CAR T cell therapy to advance (Figure 1-1). The difficulties of using autologous cellular products complicates preparations for treatments and the risk of graft-versus-host disease must be addressed for allogeneic products. In addition, several toxicities have been identified after CAR T cell therapy including on-target-off-tumor side effects and cytokine release syndrome. Lastly, a major setback to CAR T cell therapy is the concept of antigen escape that renders the treatment unsuccessful, and many times results in relapse.

A. Graft-versus-host disease

Currently, the most common immunocompetent cell used for CAR-based therapeutics is the αβ T cell. They are an attractive source for cellular therapeutics, however one of the major limitations of using αβ T cells is the need for autologous products where the cells are isolated from the patient, expanded and engineered *ex vivo*, and re-infused back into the patient. This process can be costly and time consuming, which poses a problem, especially when dealing with highly aggressive cancers [48-51]. In addition, there is no

Figure 1-1: Limitations of the current CAR T cell therapy platform

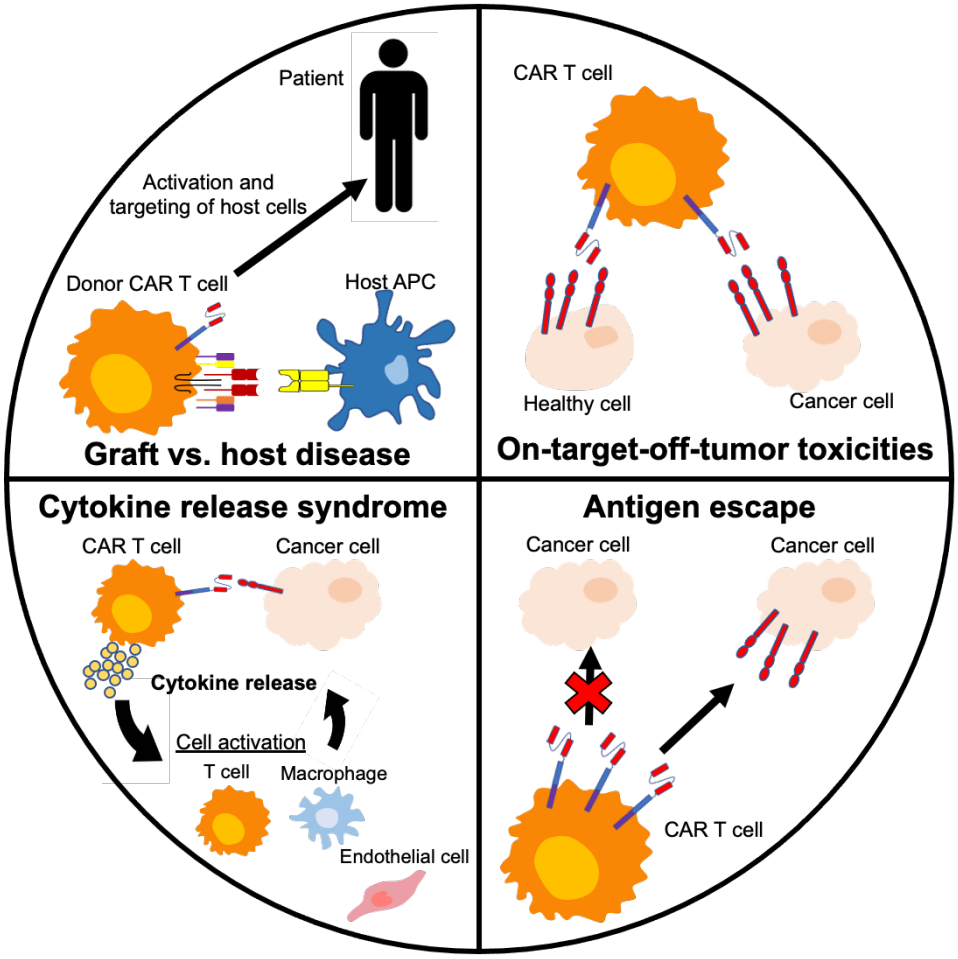


Figure Legend 1-1: Despite the promising early success, several limitations of CAR T cell therapy were identified during clinical testing. Graft-versus-host disease and on-target-off-tumor toxicities were caused by donor cell activation and targeting of the patient's healthy cells. The development of cytokine release syndrome due to excessive cytokine production resulted in harmful side effects, while antigen escape led to treatment resistance and relapse.

guarantee of successful production of an effective therapeutic product, especially for patients who undergo lymphodepleting chemotherapy or radiation therapy, and many times patients are excluded from clinical trials altogether due to insufficient manufacturing [46, 52]. Allogeneic CAR T cell products are quickly becoming an attractive alternative to autologous products because they are readily available, highly effective, and easily manufactured [53]. Despite the great promise, a significant limitation of these off-the-shelf allogeneic products is the risk of causing graft-versus-host disease (GvHD). GvHD occurs when immunocompetent cells within the donated tissue or graft recognize the recipient's cells as foreign and results in the activation of their cytolytic functions [54]. The resulting immune response causes the symptoms seen in GvHD and can lead to life-threatening side effects and engraftment failure.

Since the discovery of GvHD after allogeneic transplantation, there have been several avenues to overcome this obstacle. One approach to avoid GvHD after allogeneic transplant is through human leukocyte antigen (HLA) matching of donor and recipient. The HLA system is the human version of the MHC and is used by the body to detect foreign cells from "self" [55]. The concept of HLA matching dates back to the 1960s when a collaborative effort to develop a standardized nomenclature and techniques for HLA matching resulted in the first International Workshop [56]. Since that first conference, HLAs have been categorized into two subgroups, class I and class II, with HLA class I comprised of HLA- A, -B, and -Cw loci and HLA class II comprised of HLA-DR, -DQ, -DP, -DM, and -DO loci. After extensive investigations, researchers have identified the HLA-A, -B, -C, and -DRB1 loci as the most important for HLA matching, and mismatches within these loci results in poorer outcomes for patients [57-60]. While HLA matching can be effective and improve the chances of successful transplantation, finding a perfect 8/8 HLA match can be difficult, with the likelihood varying greatly based on racial and ethnic background, and ranges from 16-75% [61]. Therefore, alternative techniques are needed to ensure allogeneic grafts are safe and effective.

To minimize the risk of GvHD after adoptive cell transfer, researchers turned their focus to genetically engineering the immunocompetent cells. Since the main driving force of GvHD is the recognition of host

tissue by T cells through their TCR, a large portion of research has focused on genetic manipulation of the T cell receptor constant alpha chain (TRAC) and the T cell receptor constant beta chain (TRBC) [53]. In 2012, Torikai et. al. first reported a technique of using zinc finger nucleases to disrupt and eliminate expression of endogenous TCRs and showed the T cells were still able to target CD19 positive cancer cells through expression of a CD19-specific CAR [62]. Since then, the discovery and advancement of other gene editing techniques have allowed further research into TCR engineering, including transcription activator-like effector nucleases (TALENs) [63], meganucleases [64], and CRISPR/Cas9 [65]. To compare these techniques, Osborn et. al. conducted studies examining their gene disruption efficiency, off target cleavage, and toxicity; and identified CRISPR/Cas9 and a type of meganuclease known as megaTAL nucleases as being superior [66]. Although gene editing platforms targeting the TCR have shown promising results, it does not fully address the immunogenicity of the allogeneic cells. Since HLA expression on these transplanted cells also present as foreign to the patient, a strong immune response against the graft can still occur. Therefore, recently, researchers have not only focused on disrupting the TCR genes, but also HLA genes. In fact, several studies have investigated the use of gene editing techniques for the simultaneous knockdown of the TCR and HLA molecules [67, 68]. These preclinical studies have been instrumental in advancing the potential of allogeneic CAR T cell therapy and have ultimately resulted in promising clinical trials [69-72].

Another method to avoid the risk of GvHD altogether is the use of alternative immunocompetent cells for CAR-based therapeutics. In order for this method to be effective, the cells must have cytotoxic mechanisms to target cancer cells, initiate cross-talk between other immune cells, and limit the risk of causing alloreactivity and autoimmunity [73]. NK cells, natural killer T (NKT) cells, and γδ T cells are among the most commonly used alternatives for CAR-based therapeutics [74, 75]. These cell types have mechanisms of antigen recognition that are not dependent on antigen presentation through MHC and have been shown to be safe and effective as an allogeneic CAR-based product [45, 76, 77].

B. On-target-off-tumor toxicity

One of the main obstacles of developing CAR T cell therapies is the limited number of tumor-specific antigens that can be targeted. Despite being a more targeted treatment compared to chemotherapeutics, finding targets on cancer cells that are not also expressed on healthy cells can be challenging. If there is expression on healthy cells, this can lead to on-target-off-tumor (OTOT) toxicities and can result in harmful side effects [78, 79]. For some cancers, like B-cell malignancies, OTOT toxicities are acceptable because, for example, the development of B cell aplasia is tolerable with treatment of immunoglobulin [80]. However, for most cancers these toxicities can be severe and lead to termination of the treatment [81].

After the discovery of OTOT toxicities, studies investigating strategies to limit these side effects have been instrumental in advancing CAR T cell therapy. One of the major problems is the difficulty of predicting clinical OTOT side effects in preclinical models. For example, although preclinical studies on a CLDN18.2-specific CAR for gastrointestinal cancers showed no OTOT, there were cases of such toxicities, albeit minor, during a phase I clinical trial, which was attributed to limited CLDN18.2 on healthy mouse tissues and the rapid tissue regeneration of mouse gastric stem cells [42]. The first step in solving this issue is to develop *in vivo* models that can effectively simulate OTOT in mice. In 2020, Castellarin et. al. developed a mouse model using AAV8 to express target antigens on healthy tissue in order to examine OTOT toxicities *in vivo* after CAR T cell therapy [82]. Similarly, Qibin Liao et. al. recently published their work on developed a model using adenovirus to express human antigens on healthy mouse tissue to evaluate toxicity after injecting CAR T cells [83]. These preclinical models will be essential to test the potential risk of causing harmful OTOT side effects.

Despite being important, developing preclinical models to study and test OTOT is not sufficient to advance CAR T cell therapies. Strategies to limit the risk of OTOT altogether is critical. Researchers have started searching for alternative targets that are more specific to cancer cells. One such avenue researchers have focused on is targeting tumor-specific post-translation modifications, with the most common being changes

in glycosylation of proteins [84]. This idea comes from the observation that many cancer cells have abnormal glycosylation, as well as increased glycosylation. The majority of work targeting these aberrant glycosylation sites focused on monoclonal antibodies, however CARs targeting these epitopes have seen some success [85]. Current research on glycan-targeting CARs are promising and the clinical safety and efficacy have been reported for CARs targeting TAG72 [86], Lewis y [87], GD2 [88], as well as others.

Another technique to limit OTOT is the use of low affinity CARs that only target cancer cells that overexpress a certain antigen. This concept was again first applied to treatments using monoclonal antibodies; however, it can also be applied to CARs [89]. Caruso et. al. tested and compared two different CARs that target EGFR, one based on the lower affinity monoclonal antibody Nimotuzumab and one based on the high affinity monoclonal antibody Cetuximab [90]. They showed that the CAR T cells based on Nimotuzumab selectively targeted cancer cells with high expression of EGFR, while the CAR T cells based on Cetuximab targeted any cells expressing EGFR, regardless of cell surface density. This suggests that CARs can be developed with the intention of selectively targeting antigens that are overexpressed on cancer cells, while not targeting healthy cells with normal expression.

Researchers have also focused on the expression of inhibitory receptors or CARs to limit OTOT. Inhibitory receptors are an immune cell's natural method to regulate and terminate T cell activation [91]. The most commonly studied inhibitory receptors are programmed cell death protein 1 (PD1) and cytotoxic T-lymphocyte-associated antigen 4 (CTLA4), which are immune checkpoint receptors that are essential in dampening immune responses after infection and preventing autoimmune reactions. Researchers began using that concept to limit the activation of CAR T cells when they recognize the targeted antigen on healthy cells. For example, Fedorov et. al. published their work on the development of PD1- and CTLA4-based inhibitory CARs [92]. They utilized the concept of CARs, however instead of an intracellular activating signal domain, they used the inhibitory signaling domains. They showed that T cell activation was reversible when the inhibitory CAR was bound to its target, thus selectively targeting cancer cells. Despite

these techniques showing signs of success in limiting OTOT, more research is needed to address the issue of OTOT and the limited preclinical models for OTOT.

C. Cytokine release syndrome

Another side effect of CAR T cell therapy that was discovered during clinical testing is cytokine release syndrome (CRS). Despite being a serious issue for CAR T cell therapies, CRS was first observed in a 1990 clinical trial investigating the use of the OKT3 monoclonal antibody as an immunosuppressant for kidney transplantation recipients [93]. The authors reported high levels of tumor necrosis factor (TNF), interferon γ (IFNγ), and IL2 in the serum of all patients as early as the first or second infusion of OKT3. Importantly, they also noted that pre-treatment with corticosteroids resulted in a much smaller increase in these cytokines, which laid the foundation for co-administration of corticosteroids to dampen and reduce the overactivation of the immune response. With CAR T cell therapies advancing quickly since the 2000s, more and more studies have focused on understanding its role in causing CRS, the pathogenesis after CAR T cell infusion, and the symptoms of CRS.

Although not fully understood, the pathogenesis is believed to be attributed to several factors including release of cytokines by CAR T cells, release of cytokines and damage-associated molecular patterns (DAMPs) by lysed cancer cells, activation of other immune cells, and initiation of a systemic inflammatory response [94]. The first step in the progression of CRS is the secretion of cytokines after CAR T cell activation upon binding to its target. The activation of the T cells results in the production and secretion of IL2, IL6, IL10, TNFα, granulocyte-macrophage colony-stimulating factor (GM-CSF), IFNγ, as well as other cytokines [95, 96]. This increase in cytokine serum levels trigger the next step in the progression of CRS, the activation of other immune cells. Researchers identified macrophages as one of the major mediators of CRS and plays an important role in its progression [97, 98]. The activation of macrophages occurs in response to the cytokines released by CAR T cells, the recognition of DAMPs released by lysed cancer cells, and direct interactions between CD40/CD40 ligand [99]. This activation further exacerbates

the influx of cytokines and leads to increases in IL6, IL1, IL10, TNF, and nitric oxide. These signals released by macrophages, as well as the signals released by CAR T cells, can then result in the next step of CRS, a systemic inflammatory response. The resulting inflammatory toxicities include endothelial injury, vascular leakage, cytokine and chemokine production, and hypotension [100]. Altogether, these responses to CAR T cell treatment can cause harmful and sometimes life-threatening symptoms for patients.

The increase in cytokine levels and the induction of an inflammatory response can eventually result in the symptoms seen in CRS cases. The onset of symptoms can occur within hours or days after CAR T cell infusion and typically starts with a fever and fatigue. Additionally, patients may experience sinus tachycardia, hypotension, depressed cardiac function, hypoxia, as well as other symptoms [101, 102]. As CRS progresses, these side effects can ultimately lead to cardiac and respiratory dysfunction, renal and hepatic failure, and intravascular coagulation [103]. The identification of risk factors and biomarkers, in order to limit the risk of patients developing sever CRS is an important topic of research. Although not fully defined, it is believed that treatment of patients with higher tumor burdens have increased risk and more severe symptoms because it leads to increased T cell activation and cytokine accumulation. In addition, based on a patient with metastatic colon cancer, it was discovered that very high doses of CAR T cells can cause faster onset of symptoms and more severe side effects [104].

Despite helping clinicians determine which patients are suited for CAR T cell therapy, it is still necessary for scientists to identify specific biomarkers that correlate with increased CRS symptoms. One such biomarker that was identified is serum levels of C-reactive protein (CRP), which is produced in the liver and is directly related to IL6 signaling [105]. CRP levels have been shown to significantly increase during CRS and was found to be an indicator of potentially severe symptoms, leading to constant monitoring of patient serum CRP levels during treatment [106]. In addition, a recent study by Hay et. al. found that increased serum levels of monocyte chemoattractant protein-1 was another important predictor of patients developing more severe CRS [107]. The development of laboratory assays to test and monitor the levels of

these, and other, important biomarkers have not only limited the severity of CRS symptoms during CAR T cell therapy, but also have helped improve patient outcomes.

D. Antigen escape

Therapeutic resistance has always been an issue for cancer therapeutics, and it is no different for CAR T cell therapy. One of the major mechanisms of resistance to CAR T cell therapy is the concept of antigen escape and is a leading cause of relapse [108]. Antigen escape was first observed in the initial clinical trials testing CD19 CAR treatment for B-ALL. Despite the promising response rates, CD19-negative relapse was prevalent and was most common in patients who experienced long term CD19 CAR persistence [109]. The extensive clinical data show that antigen escape can play a major role in relapse. During the phase II trial for Kymriah, 15 out of the 16 patients who relapsed had CD19-negative cancer [37]. As more clinical trials were published, the extent of this issue was made clear. Although comparing different clinical trials and CAR constructs is difficult, the rate of CD19-negative relapse ranges from 30-70% [84]. Researchers also discovered that the phenomenon of antigen escape is not specific to CD19 CARs and has been seen during treatment with BCMA-targeting CARs, as well as CARs targeting solid tumors [110-112].

This acquired resistance occurs when cancer cells develop mechanisms to modulate expression of the targeted antigen through gene manipulation or physical loss of antigen detection. There are several mechanisms for this phenomenon. The first occurs when a small population of CD19-negative or CD19-variant cancer cells proliferate and expand due to immune pressure by the CAR T cell therapy. Despite playing an important role in normal human protein diversity and growth, alternative splicing can be used by tumors to evade targeted treatments [113, 114]. Researchers found that certain isoforms of CD19 from B-ALL patient samples were missing exon 2, the extracellular epitope that is recognized by CD19 CAR T cells. In another study, investigators not only found hemizygous deletions in the CD19 locus of cancer cells, but also found frameshift and missense mutations within the CD19 gene in samples from B-ALL patients after relapse [115]. Another major cause of antigen escape is lineage switch. Lineage switch occurs when

relapses contain genetically similar but phenotypically different cancer cells [116]. The most common lineage switch that has been observed clinically occurs in B-ALL patients with a shift from a lymphoid phenotype to a myeloid phenotype [117, 118]. Investigators not only found the relapsed cancer cells to be CD19-negative, but also observed a reduction in lymphoid cell markers and an increase in myeloid cell markers. Although not fully understood, the potential cause of the lineage switch is believed to be due to reprogramming and dedifferentiation of B-lymphoid blasts or differentiation of noncommitted leukemic stem cells [119].

Once antigen escape was identified as a significant limitation of CAR T cell therapy, researchers began investigating techniques to limit the risk and occurrence. The major method to overcome antigen escape is targeting multiple antigens on the cancer cell. Researchers have developed several methods to accomplish this goal, including expressing two different CARs on the same cell and combining two cell populations that each express one specific CAR. In addition, tandem CARs were another approach developed to target multiple antigens on cancer cells. Tandem CARs are created by linking two separate antigen binding regions on the same molecule. One example of this was reported by Hegde et. al. in 2016, when they developed a tandem CAR targeting HER2 and IL13Rα2 to treat glioblastoma and showed enhanced potency and efficacy *in vivo* [120]. This preclinical study, as well as others, have shown that targeting multiple antigens improves efficacy of CAR T cells compared to single targeted CAR T cells, as well as counter antigen escape after loss of one target [121-123]. Based on the promising preclinical data, dual targeting CAR T cell therapies have progressed into clinical trials and have shown encouraging results. The majority of clinical data on dual targeting CAR T cell therapies has focused on B-cell malignancies using CD19 CARs with either CD20 or CD22 CARs. In 2020, Wang et. al. published their clinical data on treating patients with refractory or relapsed B-cell malignancies [124]. They showed that treating patients with sequential infusions of CD19 CAR- and CD22 CAR-expressing cells was safe, efficacious, and a viable option to reduce antigen escape. Although targeting multiple antigens on cancers is becoming a potential

option to counter antigen escape, it remains a limitation for CAR T cell therapy and must be addressed to advance its use as a cancer therapeutic.

1.3 Transient engineering for cancer therapeutics

A. Gene delivery techniques

Adenovirus

Adenovirus (Ad) is a non-enveloped virus containing a linear, double-stranded DNA genome. It was first discovered in 1953 and isolated from adenoid tissue cultures from patients after surgery [125]. There are 6 species of Ad that can infect humans, which are further classified into more then 50 serotypes. The most commonly studied serotypes for its use as a viral vector are serotypes 2 and 5 of the species C. These serotypes result in only mild respiratory diseases and have been found to be non-oncogenic, which gives them a favorable safety profile compared to other serotypes [126]. Research into the basic biology, structure, and infection of Ad was instrumental in developing Ad into a viral vector. The first step during an infection is the binding of Ad particles to cells through interactions of the capsid's knob domain and cell surface receptors. The specific receptors vary for each serotype of Ad, with the coxsackie/adenovirus receptor being the primary receptor for serotypes 2 and 5 [127]. The viral particles are then internalized via receptor-mediated endocytosis and disassembled in order for the viral genome to be transported to the nucleus [128-130]. The genome is flanked by two short inverted terminal repeats (ITR) that act as the origin of replication and contains four major groups that are classified based on the timing of expression: early region, delayed units, late region, and VA regions [131, 132]. The early genes encode proteins related to transcription of other viral genes for replication, while the late genes encode structural proteins.

Once the general biology of Ad was determined, researchers started studying its use as a viral vector. The initial hurdle that needed to be addressed was the safety of Ad vectors and the need to eliminate Ad replication. There were three generations of recombinant Ad developed to address this. First generation Ad vectors were developed by eliminating the E1A/E1B region of the genome, which was found to be essential

for transcription and replication [133]. Removing these regions eliminated replication and allowed for the insertion of a transgene to be expressed by the transduced cells. In order to produce the Ad viral particles, researchers engineered complimentary cell lines, like HEK293 cells, to express these genes [134]. Despite the successful assembly and production of recombinant Ad vectors, it was soon discovered that expression of Ad proteins resulted in the activation of an immune response and clearance of transduced cells [135]. Therefore, second generation recombinant Ad vectors were developed that were missing additional early gene regions and were shown to limit the immune response and improved transgene expression *in vivo* [136-138]. Despite seeing improvements compared to first generation Ad vectors, the production of second generation Ad vectors was hindered and the expression of late genes still resulted in an immune response [139]. Third generation recombinant Ad vectors, also called "gutless" and "helper-depended", completely lack all native Ad genes and only contain the ITRs. This allowed for a substantially larger transgene cassette reaching about 36Kb and eliminated the risk of natural Ad protein expression [140]. This technique required an alternative source for the proteins involved in the assembly and package of the viral particle. This was achieved by using a helper adenovirus that contained a defective packaging signal [141]. These helper Ads only contained the portions of the genome that were essential for packaging and resulted in the assembly of the recombinant Ad and helper Ad, however this posed a new problem as the recombinant Ad must be isolated from the helper Ad. This led to the development of a Cre recombinase-mediated separation step, which was developed by Parks et. al. [142]. This technique utilized a helper Ad that contained the packaging sequence flanked by two loxP sites. When Ad production was conducted in cell lines expressing Cre recombinase, the helper Ad packaging signal was excised and prevented its genome from being packaged into the Ad vector particles. This technique revolutionized the production of recombinant Ad and ultimately helped its advancement as one of the most popular viral vectors.

There are many advantages of using Ad as a viral vector for *ex vivo* engineering. For example, compared to retroviral vectors, Ad vectors have high transduction efficiencies and are able to transduce a wide range of cell types because their transduction is not dependent on the cell cycle. This allows for transduction of

differentiated and postmitotic cells. In addition, they are able to deliver larger transgenes compared to many other viral vectors and Ad production is able to be easily scaled up for clinical use. There are, however, limitations of using Ad vectors. For example, although efforts have been made to limit the risk, the activation and initiation of an immune response remains a possibility. In addition, despite being widely known as a nonintegrating viral vector, there have been reports of random integration after Ad transduction, which poses a potential risk of causing insertional mutagenesis [143-145]. Adenovirus is considered one of the first engineering techniques used for the delivery of transgenes to cells, however nowadays it is not as commonly used because of its limitations and the advantages of other viral and nonviral techniques. There have been studies conducted on improving the transduction of T cells, however they are simple proof-of-concept reports that show gene transfer of reporter genes, rather than functional anti-cancer genes [146-148]. While these studies do not show specific delivery of functional genes to improve the cytotoxicity of immunocompetent cells, they do present promising data suggesting Ad vectors have the potential to be used in cancer immunotherapy development.

Adeno-associated virus

Adeno-associated virus (AAV), a part of the Parvoviridae family, is a nonenveloped virus with a single-stranded DNA genome. In 1965, scientists first discovered AAV as a contaminant in preparations of adenovirus [149]. Initial views on these particles focused on them being subunits of adenovirus, however further investigations revealed they were small viruses that were simply dependent on adenovirus replication [150]. During the late 1960s and 1970s, several groups sought to characterize this newly discovered virus. During these studies, several aspects of the AAV biology were determined and characterized including genome structure and sequence [151-153], replication [154, 155], and infectious latency [156]. AAV was found to have a ~4.7kb genome flanked by two ITR sequences, which, like Ad, act as the packaging signal and origin of replication. Another important aspect of AAV biology is the capsid design. The genome is encapsulated by an icosahedral capsid comprised of three subunits: VP1, VP2, and VP3. Researchers found variations in the capsid structure that led to the discovery of many different

serotypes of AAV. There are currently at least 12 natural serotypes, each with tropisms for specific tissue based on the primary and secondary receptors that are required for binding [157, 158]. The next important step of AAV research on its path to becoming a viral vector was its molecular cloning. In 1982, a group led by Drs. Nic Muzyczka and Ken Berns developed a method to clone AAV2 duplexes into the pBR322 plasmid [159]. Despite being successful, their methods resulted in partial genomic fragments. It wasn't until 1983 that an intact AAV DNA clone was established by a group led by Dr. Barrie Carter [160]. Once the successful cloning of AAV was accomplished, researchers began investigating its use as a vector to deliver genetic material.

Soon after the successful cloning of AAV, scientists quickly realized the potential of recombinant AAV (rAAV) as a viral vector. rAAV originally was produced with the help of a secondary virus, such as adenovirus, to provide essential genes for the successful assembly of AAV particles, however the need to remove contaminating viral particles and proteins and the risk of causing immune responses complicated rAAV production [161, 162]. The development of plasmids containing the essential genes for AAV packaging was instrumental in advancing the use of rAAV vectors and eliminated the need for helper viruses [163, 164]. This led to the three-plasmid approach as a means to produce the rAAV particles, which included a pHelper plasmid, a Rep/Cap plasmid, and a plasmid encoding the gene of interest [165, 166].

The use of rAAV for *ex vivo* genetic engineering offers several advantages. For example, AAV is able to transduce many different types of cells. Based on their tropisms, specific serotypes can be utilized for targeted transduction of tissues and cells. Researchers are able to preferentially choose the necessary serotype for targeting a certain organ. This is especially beneficial when developing *in vivo* engineering techniques. In addition, like Ad, AAV is able to transduce both dividing and non-dividing cells. However, there are limitations of using AAV. One limitation is the difficulties and cost of scaling up manufacturing. Despite being more efficient and safer, the most common three-plasmid technique using HEK293T cells is difficult to scale up because they are adherent cells and would require large surface areas. Researchers are

beginning to develop suspension HEK293 cell cultures, which have seen some success, however more research is needed to advance and perfect large scale production [167]. Another constraint of using AAV for gene therapy is the small packaging compacity, which is only about 4.7Kb. This limits the possible proteins that can be delivered to immunocompetent cells. Additionally, the genome of AAV is single-stranded DNA, which requires second strand synthesis. This is generally perceived as the rate limiting step during transduction and can lead to slower expression of the transgene. However, the development of self-complimentary AAV (scAAV) transgenes have allowed researchers to bypass the need for second strand synthesis and offers a simple solution to this problem [168]. The technique has revolutionized the use of rAAV vectors and is a promising option to improve transduction efficiency, however the method does require a smaller transgene as the scAAV can only hold half of the packaging capacity of single-stranded rAAV.

Electroporation

Electroporation is a common non-viral technique for delivering genetic material to a wide range of cell types. The first use of applying electrical fields to deliver DNA to cells occurred in 1982 by Neumann et al. [169]. They proposed that applying an electrical field to cells results in permeation sites within the cell membrane and allows for cross-membrane transport. This initial study laid the foundation for electroporation, however at the time very little was known about the specific mechanism. Many theories were developed early on including deformation of lipids within the cell membrane [170], a phase transition of phospholipid bilayers [171], and breakdown of heterogenous domains within the membrane [172]. Since these initial theories, however, a more likely mechanism was proposed, the formation of aqueous pores within the membrane, which is now widely accepted [173-176]. In the aqueous pore theory, water molecules first penetrate the lipid bilayer, causing the reorientation of the lipid heads toward the water thus stabilizing the aqueous pore [177]. This allows for the passive transport of nucleic acids across the membrane for expression into functional proteins.

DNA plasmids have traditionally been the focal point of early electroporation research; however, the advancement of mRNA synthesis and technologies in the last two decades have led the field to focus on the delivery of mRNA [178]. Compared to DNA, mRNA does not need to be transported into the nucleus, which allows for faster and more efficient translation into protein. Traditionally, DNA has been considered the more stable molecule, however improvements to mRNA structure and modifications have allowed for a longer half-life within cells. For example, the importance of a 5' cap and poly(A) tail has been extensively studied and shown to limit the degradation of mRNA within the cytoplasm [179-182]. With the advancement of mRNA vaccines, a great deal of effort has been placed on investigating the optimal modifications for mRNA. Commercially available synthetic mRNAs are offered with varying types of caps and tails that can be chosen based on specific requirements for downstream applications [183].

Electroporation offers several advantages over viral vectors that makes it an attractive technique for engineering immunocompetent cells for cancer immunotherapy. One of the major advantages of electroporation is the ability to engineer a wide range of cell types with high transfection efficiency, as well as the ability to modify dividing and non-dividing cells [184]. Researchers have published the successful electroporation of dendritic cells [185-187], αβ T cells [188, 189], γδ T cells [190, 191], NK cells [192, 193], and stem cells [184, 194]. Another benefit of electroporation is the ability to deliver larger payloads. Many viral vectors have limitations to their payload capacity which restricts the potential expression of certain larger transgenes. In addition, electroporation can be scaled up for clinical use with the development of microfluidic devices [192, 195]. These large-scale electroporators have even been developed to ensure full GMP compliance, which has been a pivotal step in the advancement of mRNA technologies into the clinic [196]. Furthermore, this has been highlighted by the increased number of companies developing large-scale electroporators, including Thermo Fisher Scientific, Miltenyi Biotec, and MaxCyte. Electroporation is also safer compared to viral vector, which poses a risk of inducing an immune response and insertional mutagenesis. These advantages have led to the use of electroporation in several clinical trials for a wide range of applications including the development of vaccines and delivery of CRISPR/Cas9 for

gene engineering as a means to develop cancer therapeutics [197-201]. Despite its many advantages, the use of electroporation for *ex vivo* engineering does have limitations. The major limitation is the need for high voltages that can result in increased cell death. This not only effects the viability of the cellular product, but also limits the cell yield during production. In addition, specific parameters must be identified and optimized for individual cell types, as well as for each electroporation device.

B. Genetic engineering of TCRs

One of the first strategies developed to enhance the cytotoxicity of immunocompetent cells was the engineering and expression of TCRs specific to a certain tumor antigen. Although the vast majority of research on TCR engineering has utilized integrating viral vectors, there has been some effort applying transient engineering technique. Early research into the optimization of DNA plasmid electroporation for T cell engineering was shown to be safe, however low transfection efficiencies and viability remained an issue [202, 203]. Therefore, the field shifted to the use of mRNA-based electroporation, which saw several groups publish their work in the early 2000s. In 2005, one of the first recorded findings on electroporation for the introduction of specific TCRs was conducted by Zhao et. al. [204]. Despite being used as a preliminary test for their cDNA constructs for retroviral transduction, they showed about 60% transgene expression in stimulated primary human lymphocytes after electroporation. In addition, the NY-ESO-1-specific and Mart-1- specific TCRs were able to induce production of IFN-γ upon recognition of the subsequent antigens. The same group later published their work on further optimizing the mRNA electroporation of human and murine T cells [189]. They tested several electroporation parameters using GFP mRNA to evaluate transfection efficiency and cell viability for unstimulated and stimulated human peripheral blood lymphocytes. They then tested the introduction of NY-ESO-1- and MART-1-specific TCRs and found increased IFNγ production. At almost the same time, Schaft et. al. also published their work on optimizing and investigating the use of TCR engineering through electroporation [205]. They tested their electroporation technique using GFP mRNA, with over 90% transfection efficiency. Although the expression was low, they did show successful expression of a TCR specific to gp100 and found that the

engineered cells exhibited increased IFNγ production and improved cytotoxicity against gp100 peptide-positive target cells.

These *in vitro* studies were helpful as proof of concept for transient TCR engineering in T cells and led to further investigations into the efficacy of this therapeutic technique in preclinical studies. In 2013, Koh et. al. published their work on a unique method for targeting cancer cells [206]. They produced T cells expressing an anti-hepatitis B virus (HBV) TCR based on the fact that HBV infections are a major etiological factor for the development of hepatocellular carcinoma and that viral DNA integration is common in cancer cells and leads to expression of viral peptides that can be targeted. By using mRNA electroporation, they were able to achieve about 80% anti-HBV TCR positive CD8 T cells and showed these cells had increased IFNγ production and enhanced cytotoxicity. In subsequent *in vivo* experiments, the group showed the electroporated T cells were not only able to limit tumor growth but were also able to prevent tumor development. Another group in 2019 published their promising preclinical data on electroporated TCR engineering. Mensali et. al. tested their methods of electroporating T cells with a Radium-1- specific TCR, which recognizes a frequent mutation common in colon cancer [207]. They first evaluated the mRNA electroporated T cells *in vitro* and found that about 80% of T cells expressed the Radium-1 TCR, which resulted in production of TNFα and IFNγ and enhanced cytotoxicity upon incubation with target cells. They then showed that treating mice with the T cells expressing the Radium-1 TCR significantly lowered tumor burden and improved survival, however this was only observed when administering the T cells intraperitoneally, not intravenously. This study led to the initiation of the first clinical trial (NCT03431311) testing transient TCR therapy, however the study was terminated due to limited patient enrollment.

During the early stages of TCR engineering research, scientist quickly discovered issues with expressing antigen-specific TCRs in T cells. They found that the introduced TCRs can interact with the endogenous TCR causing TCR mispairing, which could lead to graft-versus-host disease [208]. Scientists found that the

best way to eliminate the risk of TCR mispairing was to downregulate the expression of the endogenous TCR. In the field of transient TCR engineering, one group developed a method to achieve this. Campillo-Davo et. al. used sequential electroporations to deliver Diver-substrate small interfering RNAs (DsiRNA) to suppress the endogenous TCR, followed by DsiRNA- resistant tumor-specific TCR mRNA [209]. The group showed that introducing the DsiRNA 24 hours before a Wilms' tumor protein 1 (WT1)-specific TCR resulted in improved expression of the WT1-specific TCR, increased production of cytokines, and enhanced cytotoxicity. This study provides evidence that introducing cancer-specific TCRs, while also silencing endogenous TCRs, is possible using a completely transient engineering platform. This provides evidence that could lead to the testing of other gene silencing techniques using transient engineering platforms, such as other RNAs like small interfering RNA and short hairpin RNA.

C. Expression of CARs

Besides TCR engineering, the expression of CARs is the most common engineering technique for adoptive cell therapy and has been the most widely studied application for transient engineering techniques. Several groups during the span of 2008-2011 developed methods to express various CARs in T cells using mRNA electroporation, including CARs specific to CD19 [210-212], Her-2/neu [213], and mesothelin [214]. Based on the studies that reported transfection efficiency, they found that between 80-95% of T cells expressed the CARs and highest expression occurred on Day 1 or Day 2 post electroporation. Collectively they not only showed increased cytokine production and *in vitro* cytotoxicity in the engineered cells, but also improved anticancer activity in mouse tumor models. Several groups have also published their work on directly comparing mRNA electroporation to other viral vector delivery methods. In 2009, Birkholz et. al. compared the use of mRNA electroporation to retroviral gene transfer [215]. They tested their electroporation techniques using a Her-2/neu CAR and a CEA CAR and reported about 90% CAR expression. The engineered cells exhibited increased tumor cell lysis and cytokine secretion, including IFNγ, IL2, and TNF. The comparison between electroporation and retroviral gene delivery revealed that both techniques resulted in IFNγ production and improved cytotoxicity, however the increase was more

substantial for the retroviral transduced cells. Another study conducted by Barret et. al. investigated the use of mRNA electroporation for the expression of a CD19 CAR and compared it to a clinically relevant lentiviral vector approach [216]. As expected, the lentiviral-engineered T cells resulted in a sustained reduction in tumor burden *in vivo*, however, after optimization of doses and treatment schedules, multiple infusions of the electroporated T cells also resulted in substantial tumor burden reduction and a median overall survival that was similar to treatment with the lentiviral-engineered T cells.

Despite being the most common technique, electroporation is not the only transient gene delivery method used to express CARs in T cells. While most of the research on the use of AAV to engineer T cells has focused on the delivery of gene editing nucleases, Wang et. al. published their work on investigating and optimizing the use of AAV6 to express a CD19 CAR in T cells [217]. Initial tests were conducted using GFP to optimize the AAV transduction of T cells and found that pre-treating T cells with OKT3 and genistein, a tyrosine kinase inhibitor which improves the replication and transcription of AAV genes, significantly enhanced T cell transduction. They then applied these techniques to express a CD19 CAR in primary T cells, which resulted in about 40-50% CAR positive cells. Furthermore, the engineered cells exhibited improved anticancer cytotoxicity both *in vitro* and *in vivo*.

These preclinical studies paved the way for using transient engineering techniques as a treatment platform for cancer therapeutics and have led to several clinical trials. Two groups investigated the use of electroporation to generate CAR T cells for the treatment of B-cell malignancies. In 2008, Till et. al. published their results on a phase I clinical trial testing the use of autologous CD20 CAR T cells generated using DNA electroporation for the treatment of relapsed or refractory indolent B-cell non-Hodgkin lymphoma and mantle cell lymphoma [26]. CAR expression ranged from about 25-90% and the cells exhibited an activated effector T cell phenotype with improved anticancer killing *in vitro*. They found that the cellular product was safe and well tolerated with no grade 3 or 4 toxicities observed. In addition, they reported the engineered cells persisted *in vivo* for between 5-21 days alone and up to 9 weeks with

subcutaneous IL2. The efficacy of the engineered T cells was also examined and found that two patients maintained their previous complete responses, one achieved a partial response, and four displayed stable disease. In 2018, Svoboda et. al. investigated the use of mRNA electroporation to generate autologous CD19 CAR T cells for patients with Hodgkin lymphoma [218]. They examined the persistence of CAR expression and found that CAR RNA was detected in the blood between 48 hours and 7 days after infusion. The treatment was found to be safe and well tolerated with no development of cytokine release syndrome. Treatment response was assessed after one month and resulted in one complete response, two partial responses, and one stable disease.

Several clinical trials testing CAR T cells generated by electroporation for solid tumors have also been conducted by Dr. Carl June's group. In 2014, they published a first-in-human study investigating the use of a mesothelin-specific CAR, generated by mRNA electroporation [219]. They treated one patient with metastatic pancreatic cancer and one patient with malignant pleural mesothelioma. They examined persistence of the engineered cells and found that the persistence was transient, with maximal transgene expression at 2 hours post infusion. They also found the engineered cells were able to traffic and infiltrate into the solid tumors and that the initial recognition and lysis of cancer cells resulted in a systemic antitumor immune response via humoral epitope-spreading. They did, however, report the first incidence of anaphylactic shock following CAR T cell infusion, which they believed was mediated by an IgE response due to the specific dosing schedule [220]. The same group conducted another clinical trial with an altered dosing schedule to test their mesothelin-specific CAR T cells developed using mRNA electroporation to treat patients with metastatic pancreatic ductal adenocarcinoma [40]. They treated six patients and administered the cellular product three times per week for three weeks to examine safety and therapeutic potential. They observed no incidences of cytokine release syndrome or neurotoxicity for any patient, demonstrating the cellular product was safe and tolerable. Despite the transient nature of the treatment, they also reported stable disease for two patients with progression-free survival lasting about 4 and 5 months. Based on their promising clinical results from the mesothelin-specific CAR, the group also tested their

mRNA electroporation technique to develop a c-Met-specific CAR for the treatment of metastatic breast cancer [221]. In contrast to their previous clinical trials, this study sought to investigate the safety and efficacy of an intratumoral injection of the transiently engineered CAR T cells. They first tested the c-Met CAR *in vitro* and in an *in vivo* xenograft mouse model. They found the engineered cells exhibited increased anticancer killing and intratumoral injections were effective at controlling tumor growth. In the phase 0 clinical trial, they tested two doses of mRNA c-Met CAR T cells and ultimately found that the treatment was well tolerated and resulted in an inflammatory response within the tumor. Taken together, these studies show the progress, as well as the potential of transient engineering techniques for developing CAR T cell therapies.

Although αβ T cells are the most commonly used cells to express CARs, researchers are also focusing on the use of innate and innate-like immune cells. NK cells are widely considered as one of the most cytotoxic immunocompetent cells and the ability to direct the effector function of these cells toward cancer cells through the expression of CARs offers an attractive platform for adoptive cell therapy. However, engineering this cell type has traditionally been difficult and success has been variable. For example, a group led by Dr. Hans Klingemann studied the expansion and engineering of primary NK cells, as well as the NK cell line NK92. During their initial testing with GFP, they found very limited expression and significant cell death in primary NK cells after testing several conditions [222]. In a subsequent study, they did show expression of a CD19- and CD20-specific CAR in the NK92 cell line, reaching about 55% transfection efficiency [223]. The engineered NK92 cells exhibited improved cytotoxicity against NK-resistant lymphoid cell lines, as well as against primary leukemia cells. Although these results are promising, the need to engineer primary NK cells remains an important hurdle for their use as a cancer immunotherapy. There have, however, been reports of successful transfer of CARs into primary NK cells. Rabinovich et. al. showed that mRNA electroporation of primary NK cells isolated from a donor to express a CD19 CAR resulted in significantly improved cytotoxicity against autologous B cells, however transfection efficiency was not reported [212]. In addition, Li et. al. developed a technique for a scalable

and translatable mRNA electroporation protocol for expanded and purified unstimulated NK cells [224]. They tested the expression of a CD19 CAR and found, on average, 58% transfection efficiency, which resulted in an increase in cytotoxicity toward leukemic cell lines *in vitro*. They also investigated the use of a novel computer-controlled flow electroporation device and showed in a proof-of-concept experiment that the NK cells expressed similar levels of GFP compared to the static transfection. Lastly, a more recent study conducted by Ingegnere et. al., tested their methods of using DNA electroporation to transfect NK cells [193]. They first used GFP expression to optimize the conditions for NK cell electroporation and, once optimized, they tested their technique for the expression of a CD19 CAR. The transfection efficiency for the CAR was found to be about 40% and the engineered cells exhibited increased cytotoxicity *in vitro* against several CD19-positive cell lines. They also briefly tested their electroporation protocols for the expression of CCR7 and, although found lower transfection efficiency, did see an increase in their migratory capacity.

Although NK cells are the most common innate cell type used for cancer immunotherapy, there have also been studies investigated the use of less common innate immunocompetent cells. For example, NK T cells have also been investigated as an immunocompetent cell for the expression of CARs. This cell type has characteristics similar to NK cells and T cells, which make them a valuable tool for adoptive cell therapy. In 2018, Simon et. al. investigated the use of mRNA electroporation to generate CSPG-4-specific CAR-NK T cells and compared them to conventional CD8 CAR T cells [225]. After electroporation with CSPG4-specific CAR mRNA, both cell populations displayed about 80-90% CAR expression and produced IL2, TNF, and IFNγ in the presence of target cells, however the CD8 T cells produced larger quantities of the cytokines. They also showed the engineered cells exhibited improved cytotoxicity *in vitro* against a CDPG4-positive cell line and, importantly, their intrinsic cytotoxic capacity was not affected by the electroporation. Another group studied the use of mRNA electroporation of cytokine induced killer (CIK) cells [226]. CIK cells are characterized by their expression of CD3 and CD56 and exhibit non-MHC restricted cytotoxicity against several cancer cell types. The group utilized mRNA electroporation to

express an anti-Her-2/neu CAR in the CIK cells and found over 95% of the cells expressed the CAR and the engineered cells secreted elevated levels of cytokines in the presence of target cells. They also showed that the electroporated CIK cells displayed enhanced anticancer activity *in vivo* by inhibiting tumor growth in an ovarian cancer model.

The final cell type that has been studied for the transient expression of CARs is the γδ T cell. γδ T cells bridge the gap between the adaptive and innate immune system and have several inherent mechanisms of cytotoxicity toward cancer cells. Despite being a traditionally difficult cell type to engineer, electroporation has seen encouraging results. Harrer et. al. reported their findings on the use of mRNA electroporation to express a CAR specific to the melanoma-associated-chondroitin-sulfate-proteoglycan antigen, as well as an αβ TCR specific to gp100/HLA-A2 [191]. Transfection efficiency for the CAR reached over 80% and the engineered cells exhibited antigen-specific cytokine secretion, as well as improved cytotoxicity of melanoma cells.

Overall, these studies show that the transient expression of CARs in immunocompetent cells offers an attractive alternative to stable integrating vectors. The transient engineered cells are able to limit many of the risks of traditional engineering technique, like cytokine release syndrome, while still maintaining a high level of cytotoxic potential.

D. Expression of cytokines and other recombinant proteins

Although the most common engineering strategies to improve the anti-cancer functions of immunocompetent is the expression of specific TCRs and CARs, there are other techniques that have been studied. Traditionally, cytokines have been co-administered with immunocompetent cells to improve efficacy, however the systemic administration can lead to harmful inflammatory side effects [3, 227-229]. Therefore, researchers began investigating the expression of cytokines in immunocompetent cells, which can improve efficacy and persistence *in vivo*. Although the majority of work on the expression and secretion

of cytokines in immunocompetent cells has focused on stable engineering techniques, there are groups that have investigated the use of transient engineering methods. In 2010, Lee et. al. utilized mRNA electroporation to simultaneously express IL2 with a Her-2/neu-specific CAR in peripheral blood lymphocytes [230]. They showed that the engineered cells expressed the CAR and secreted IL2 for up to 6 days after electroporation and the cells secreting IL2 exhibited increased proliferation *in vitro*. Furthermore, the engineered cells were able to inhibit tumor growth in an ovarian cancer mouse model.

Another cytokine that has been investigated for its transient expression in T cells is IL12, due to its beneficial effects on T cells and NK cells. Etxeberria et. al. published their work on using mRNA electroporation to express single chain IL12 in mouse CD8 T cells and tested whether these engineered cells exert anticancer activity *in vivo* [231]. Their electroporation technique resulted in high levels of IL12, and the engineered cells not only eradicated subcutaneous tumors upon intratumoral injection but also reduced tumor burden in distant contralateral tumors. A more in depth analysis revealed that the expression of IL12 not only enhanced persistence, but also tumor infiltration. They then tested whether they could apply their electroporation technique to tumor infiltrating lymphocytes (TILs). They isolated PD1+ and PD1- TILs from MC38 and B16OVA mouse tumors and showed that IL12 mRNA electroporation resulted in about 40% transfection efficiency and significant production of IFNγ. Both the engineered PD1+ and PD1- TILs were efficacious in treated tumors *in vivo*, however the PD1+ TILs performed better. Lastly, they applied their electroporation technique to primary human TILs isolated from melanoma, renal cell carcinoma, ovarian, and endometrial cancer patients. mRNA electroporation of these primary TILs resulted in substantial IL12 expression and IFNγ production. In 2023, the same group sought to improve upon their technique of transiently expressing IL12 in T cells by testing the addition of a decoy resistant IL18 (DRIL18), either through co-electroporation or mixing separate populations of T cells expressing either IL12 or DRIL18 [232]. They found that co-electroporating the T cells with both mRNAs resulted in reduced IL12 expression and IFNγ production, compared to mixing the single cell populations. In addition, treating tumor bearing mice with the co-electroporated cells was not as efficacious compared to treating with the

separate cell populations. To further examine the effects of IL12 and DRIL18 expression, the group conducted experiments to investigate the cause of the improved anticancer activity. They found that the IL12 and DRIL18 expression improved T cell metabolism, enhanced control of immunosuppressive genes, increased expression of other cytokines, and altered glycosylation of surface proteins that improved E-selectin adhesion. Overall, these studies conducted by Dr. Ignacio Melero's group further demonstrates the great potential of using transient engineering to express cytokines, specifically IL12 and IL18, to improve the anticancer efficacy of T cells.

Another group led by Dr. Chang-Xuan You investigated the use of AAV to enhance the stimulation and function of T cells [233]. Although their ultimate goal was to optimize the activation and priming of T cells through dendritic cell engineering, they also tested AAV transduction of the T cells directly. After transduction of an AAV2-IL7 vector, they showed an increase in IL7 expression in about 88% of T cells. They also characterized the engineered T cells after AAV-IL2 and AAV-IL7 transductions and found increased CD8:CD4 ratios, increased production of IFNγ, and increased activation. In contrast to the expression of cytokines, one group led by Dr. Jesse Rowley investigated the expression of a cytokine receptor in T cells. IL15 is a cytokine that plays an important role in the adaptive immune response and its interaction with IL15 receptor alpha chain (IL15RA) has been shown to improve T cell survival and proliferation. The group utilized RNA nucleoporation to express IL15RA in unstimulated CD8 T cells and showed that these cells exhibited enhanced survival and proliferation in the presence of IL15, as well as through cis-presentation of IL15 on the T cells [234]. Furthermore, they developed an RNA chimeric construct containing a linked IL15 and IL15RA. This construct not only resulted in improved proliferation and increased IFNγ production *in vitro*, but also enhanced proliferation of the engineered cells *in vivo*.

An alternative technique to having the immune competent cells secrete cytokines, Weinstein-Marom et. al. sought to anchor cytokines to the cell membrane, in order to enhance cell survival and anticancer activity while avoiding systemic distribution [235]. They developed mRNA constructs that contained human or

mice IL2, IL12, and IL15. The cytokines were anchored to the cell membrane using the transmembrane and cytoplasmic domains of HLA-A2 or H-2Kb for the human and mouse cytokines, respectively. Using mRNA electroporation, they successful expressed these membrane-attached cytokines and showed that they support viability and promote proliferation. In addition, a synergistic effect on enhancing IFNγ production and upregulating activation markers was observed when coexpressing the membrane cytokines with a constitutively active toll-like receptor 4. This work showed the possibility of expressing membrane-anchored cytokines through transient engineering techniques.

Besides cytokines, the expression of other recombinant proteins has also been investigated. Toll-like receptors (TLRs) have important roles in linking the adaptive and innate immune systems and has been shown to enhance T cell functions [236]. In 2015, Pato et. al. published their work on the expression of TLR-4 though mRNA electroporation [237]. They showed successful expression of constitutively active TLR-4 on human peripheral blood lymphocytes and primary TILs obtained from a melanoma patient biopsy. These engineered cells exhibited an activated phenotype based on CD25, CD69, and CD137 surface expression and increased IFNγ and cytokine secretion. In addition, the transient expression of TLR-4 improved the cytotoxicity of the T cells and TILs against melanoma cells *in vitro*. The same group later studied the expression of CD40 in T cells, which has been shown to improve T cell functions and protect against apoptotic signals and exhaustion upon interaction with CD40 ligand [238]. They developed an mRNA construct that contained a constitutively active form of CD40 and showed that the electroporation of peripheral blood lymphocytes and TILs resulted in the induction of an activated phenotype [239]. They also showed that the engineered cells exhibited increased production of IFNγ and that a synergistic effect was observed when the T cells were electroporated with CD40 and TLR-4.

The expression of chemokine and chemokine receptors has also been a popular topic of research. The ability to modulate the migratory patterns of immunocompetent cells *in vivo* offers a valuable tool to develop a more targeted treatment approach. Several groups have studied the use of mRNA electroporation to

transiently delivery chemokine or chemokine receptors to T cells and NK cells. In 2008, Mitchell et. al. published their work on expressing CXCR2 in activated T cells [240]. They showed that about 60-85% of the T cells expressed CXCR2 and the engineered cells exhibited improved chemotactic responses to three CXCR2 ligands in a trans-well migration assay. They then tested the engineered cells *in vivo* and found CXCR2 expression resulted in increased migration toward CXCR2 ligands, with the largest chemotactic response to UL146. In 2011, Almasbak et. al. published their work on combining the expression of a CD19 CAR with the expression of either CXCR4 or CCR7 [210]. Although their major goal was to investigate the expression of the CAR, they also conducted a simple experiment to test whether the simultaneous electroporation of both CAR mRNA and chemokine receptor mRNA was possible in T cells and found that the cells expressed both proteins and the expression of each was not affected by the other.

The transient expression of chemotactic molecules has not only been studied in T cells, but also in NK cells. In 2016, Carlsten et. al. reported their findings on expressing CCR7 in human NK cells using mRNA electroporation [241]. They showed that CCR7 expression resulted in improved migration toward the CCL19 chemokine *in vitro*. They also reported on the successful expression of CD16 in NK cells using mRNA electroporation and found these engineered cells exhibited improved antibody-dependent cellular cytotoxicity. Another paper by Ng et. al. showed expression of CXCR1 in CAR-NK cells enhanced the anticancer efficacy in a preclinical study [242]. Using mRNA electroporation, they were able to express CXCR1 in over 95% of NK cells and the engineered cells exhibited improved tumor infiltration *in vivo*, which resulted in enhanced tumor control. To test whether the efficacy of CXCR1-expressing NK cells could be further improved, they co-expressed CXCR1 with an NKG2D CAR. The co-expression resulted in a synergistic effect *in vivo* that led to a reduction in tumor burden, compared to the NK cells only expressing the NKG2D CAR.

Taken together, the use of transient engineering technique, specifically mRNA electroporation, for the expression of cytokines and other recombinant proteins can be an effective platform to improve the cytotoxicity and anticancer efficacy of immunocompetent cells.

1.4 γδ T cells

A. Classification, characterization, and functions

The αβ T cell is the most abundant and commonly studied type of lymphocyte, however γδ T cells play important roles in a wide range of applications. γδ T cells have characteristics that make them the ideal cell type for immunotherapy. They are a small subset of lymphocytes that bridge the gap between the adaptive and innate immune systems and are classified into three major populations based on their δ chain: Vδ1, Vδ2, and Vδ3 [243]. γδ T cells expressing the Vδ1 chain are found in epithelial tissues and mucosal regions, such as the gut, skin, and liver. They play major roles in mucosal immune surveillance, responding to stress antigens on epithelial cells after infection or damage, and maintaining epithelial tissue integrity [244, 245]. Research conducted by Davey et. al. found that Vδ1 T cells play a pivotal role in immune surveillance and were shown to exhibit clonal expansion over time [246]. The group also showed that these cells display a more effector phenotype after their TCR becomes more focused. Furthermore, Vδ1 T cells have been shown to play important roles in the response to viral infections, especially cytomegalovirus (CMV) and Epstein Barr virus [247-249]. In addition, researchers discovered that Vδ1 express several natural cytotoxicity receptors that confer immune surveillance toward cancer cells such as NKp30, NKp44, and NKp46 [250, 251]. These innate receptors help the γδ T cells initiate an immediate response to abnormal, infected, or cancerous cells.

In contrast to Vδ1 T cells, Vδ2 T cells are predominantly circulating in the blood and have been characterized to have two subsets, one with more adaptive features and one with more innate-like features [252]. These two subsets are defined by the γ chain, with $V\gamma9^+$ cells exhibiting more innate-like features and $V\gamma9^-$ exhibiting more adaptive-like features. The work by Davey et. al. revealed that $V\gamma9^+V\delta2$ T cells

maintain clonotypic plasticity, while Vγ9^{-}Vδ2 T cells exhibit more of a focused clonal expansion and differentiation [253]. Vγ9Vδ2 T cells play important roles in initiating immune responses after microbial infection, which is achieved by responding to phosphoantigens (PAg) produced by the microbial non-mevalonate pathway [254, 255]. Similar to Vδ1 T cells, they also play key roles in detecting and targeting cancer cells through several receptors including the TCR, NKG2D, CD16, as well as other [256]. In addition, Vγ9Vδ2 T cells have been shown to play important roles in initiating and activating other immune cells, such as αβ T cells, neutrophils, and dendritic cells [257-259]. Similar to Vδ1 T cells, Vγ9^{-}Vδ2 T cells exhibit a more naïve phenotype within the blood and undergo a shift toward a more effector phenotype and clonal expansion upon viral infection [253]. Far less is known about Vγ9^{-}Vδ2 T cells, however it has been shown that they play important roles in adaptive immunity against infection.

Vδ3 T cells are the third type of γδ T cell and are found in the liver, gut, and in small numbers within the blood [260, 261]. Although the levels are typically low in the blood, researchers have found increased levels of circulating Vδ3 T cells after CMV infections in recipients of renal and stem cell transplants [247, 262], in patients with HIV-negative CD4 T cell deficiency [263], and in patients with B cell chronic lymphocytic leukemia [264]. Not much is known about specific antigens that are recognized by Vδ3 T cells, however researchers have identified CD1d as a target [265]. Mangan et. al. published their work on expanding Vδ3 T cells and showed they become activated and kill CD1d^{+} target cells and release Th1, Th2, and Th17 cytokines. In addition, they showed that the Vδ3 T cell induce the maturation of dendritic cells and cause the upregulation of markers associated with stimulated dendritic cells, such as CD40, CD83, CD86, and HLA-DR. Research conducted by Petrasca et. al. found that Vδ3 T cells also induce maturation of B cells [266]. The group found that co-culturing Vδ3 T cells with B cells caused an upregulation in costimulatory molecules on both cells and induced the production of IgM by B cells. Despite not being widely studied, these publications reveal an important function of Vδ3 T cells in activating and inducing an immune response.

B. Mechanisms of antigen recognition

The major mechanism of antigen recognition for γδ T cells is through the TCR. As mentioned above, the γδ T cells recognize PAg, but more specifically they recognize prenyl pyrophosphate metabolites that are produced by the host mevalonate pathway or the microbial non-mevalonate pathway [267]. One such PAg is isopentenyl pyrophosphate (IPP), which is a substrate for farnesyl pyrophosphate synthase in the mevalonate pathways. In cancer cells, the overproduction of bisphosphonates blocks the action of this enzyme and causes levels of IPP to increase, which can be recognized by γδ T cells. For a long time, the mechanism of recognizing PAg through the TCR was unclear, however research conducted by Harly et. al. proved instrumental in understanding how the γδ TCR functions [268]. They showed that the family of butyrophilin (BTN) receptors, specifically BTN3A, directly interacts with PAg through its intracellular domains, which, in turn, causes a conformational change that is detected by the γδ TCR. Work done by other researchers have confirmed these findings and have even implicated other members of the BTN receptor family, such as BTN2A1 [269, 270]. Through PAg recognition via the TCR, γδ T cells are able to identify cancer cells and infections, which allows the cells to initiate an immune response.

NKG2D is a C-type lectin-like receptor that plays an important role in ligand recognition and the activation of γδ T cells. The NKG2D receptor is most notably considered a costimulatory receptor and has been shown to enhance the signaling of γδ T cells and other immunocompetent cells upon TCR activation [271, 272]. Although widely known as a costimulatory receptor, NKG2D has also been shown to activate γδ T cells in a TCR-independent and antigen-independent manner [273]. The interaction between NKG2D and its ligands play essential roles in immune surveillance. A study conducted by Guerra et. al., showed that NKG2D-deficient mice were more susceptible to epithelial and lymphoid malignancies and that tumors in these mice expressed higher levels of NKG2D ligands compared to wild type mice, suggesting NKG2D plays a critical role in tumor surveillance [274]. Its main function is to recognize stress markers that are related to heat shock and genotoxic stress. Several ligands for NKG2D have been identified including the MHC class 1-related proteins A and B (MICA/B) and the members of the UL16-binding protein family

(ULBP), ULBP1-4 [275]. These ligands are not expressed by most healthy cells and are typically upregulated in cancer cells and infected cells [276, 277]. Activation of the NKG2D receptor has been shown to increase production of cytokines like TNFα, as well as increase the release of cytolytic granules [273]. As more research was conducted on NKG2D and its expression on γδ T cells, it became clear that this receptor plays a critical role in initiating and enhancing γδ T cell effector functions.

There are many other receptors and proteins that are expressed on γδ T cells that are important for its effector functions. One such receptor is CD16, which binds to the Fc region of IgG antibodies and plays a critical role in its antibody-dependent cellular cytotoxicity (ADCC) activity. ADCC is an immune mechanism where certain immunocompetent cells can recognize and kill target cells that are coated with antibodies on their surface. The most common cell type involved in ADCC is NK cells, however γδ T cells have also been shown to play a role in ADCC through expression of CD16 [278]. In fact, researchers have found increased expression of CD16 on γδ T cells after CMV infection and upon activation induced by nonpeptidic antigens [279, 280]. In the context of cancer, this observation led to the testing of combining antibody-based therapeutics with γδ T cells and has shown promising results in preclinical studies [281-283]. Another mechanism of cytotoxicity of γδ T cells is the induction of programmed cell death and apoptosis in target cells via expression of the TNF receptors, Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL). The expression of FasL and TRAIL on γδ T cells has been shown to play a role in its anticancer activity by interacting with Fas receptors and TRAIL receptors on target cells [282, 284, 285]. These interactions induce apoptosis pathways and ultimately results in target cell death. The ability for γδ T cells to recognize antigens in several different ways and through several different receptors and proteins makes them the ideal cell type for ACT and offers a promising option for developing novel cancer therapeutics (Figure 1-1)

Figure 1-2: γδ T cells exhibit several natural mechanisms of cancer cell recognition

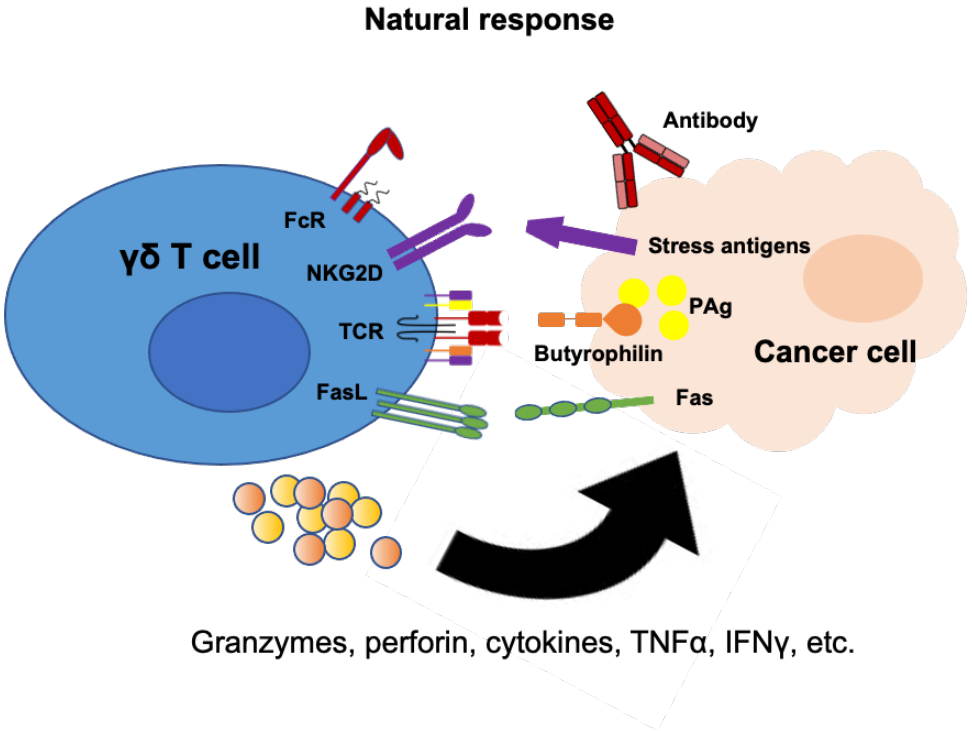


Figure Legend 1-2: γδ T cells have several inherent cytotoxic mechanisms that make them the ideal candidate for adoptive cell therapy. One of the major advantages of this cell type is their MHC-independent antigen recognition that allows for a rapid response to infection and cancerous cells. This is accomplished by the expression of several different receptors and proteins including Fc receptors, NKG2D, FasL, and the TCR that recognizes phosphoantigens.

C. Vγ9Vδ2 T cells for ACT

One of the most widely studied γδ T cell is the Vγ9Vδ2 T cell. γδ T cells account for 1-10% of circulating lymphocytes with Vγ9Vδ2 T cells being the major population of within the blood. Vγ9Vδ2 T cells have certain characteristics that make them the ideal cell type for cancer immunotherapy and ACT. One of the main advantages is their ability to be preferentially expanded from healthy donor blood. Our lab, as well as others, have developed protocols to culture these cells and expand them from peripheral blood mononuclear cells (PBMCs) to numbers sufficient for clinical use. There have been two major techniques for expansion; a feeder cell-based technique and the use of bisphosphonates like Zoledronate and the synthetic bromohydrin pyrophosphate [286-290]. Another important feature of Vγ9Vδ2 T cells is their ability to be used as an allogeneic product. As mentioned above, γδ T cells recognize antigens in an MHC-independent manner, which significantly limits the risk of causing GvHD [291]. In addition, Vγ9Vδ2 T cells have been shown to be well tolerated in clinical trials and have shown some success for several cancer types including hematological malignancies, lung, liver, pancreatic, as well as others [292-294]. The use of unmodified or engineered γδ T cells has the potential to be an effective cancer treatment and holds great promise as the next generation of ACT products.

Chapter 2

Engineering γδ T cells using AAV6 and microfluidics devices

2.1 Abstract

Gene and cell therapy is becoming a valuable therapeutic tool to treat a variety of diseases. The development of CAR T cell therapy has revolutionized cancer immunotherapies and has seen great success in clinical trials. The use of γδ T cells is becoming an attractive alternative to the traditional αβ T cells because of their innate and adaptive immune functions, however engineering this cell type has been difficult. In contrast to AAV2, AAV6 has emerged as a promising viral vector for the transduction of T cells. Therefore, we investigated the use of AAV6 to engineer γδ T cells with a CD5 CAR, capable of targeting T-cell malignancies. First, we improved upon the AAV manufacturing process by optimizing the purification step, which led to a purer and cleaner AAV vector. After initial attempts using traditional wildtype AAV6 well transductions were unsuccessful, we show that the use of a microfluidics device with mutant AAV6 vectors improves transduction efficiencies and CD5 CAR expression in the Jurkat cell line, however γδ T cell transduction resulted in limited gene transfer and increased cell death. Although AAV6 has the potential to be an effective gene engineering technique for γδ T cells, we illustrate the need for further optimization.

2.2 Introduction

In the past two decades, the field of gene and cell therapy has revolutionized therapeutic approaches for treating a variety of diseases. The development of viral vectors to deliver specific genes has been instrumental in advancing patient care and outcomes for diseases like cancer, heart failure, neurodegenerative disorders, as well as many others [295]. For cancer, this is evident by advances in cancer immunotherapy, especially adoptive cell therapy, where engineering immunocompetent cells has been necessary for improving the success of these treatments. T cells have been the most widely used cell type for the development of various cancer immunotherapies like CAR T cell therapy and TCR-based therapies, thus a great deal of effort has been put into engineering these immunocompetent cells. As mentioned in Chapter I, γδ T cells are a small subset of lymphocytes that are the ideal candidate for use as an immunotherapy to treat cancer, however engineering these cells has traditionally been difficult with low and variable transduction efficiencies. Lentiviral vectors have been the most common technique used for

engineering T cells, however the stable integration of transgene poses risks that can ultimately lead to side effects and toxicities (see section 1.2) and lentiviral transduction of γδ T cells is limited and only results in about 30% transduction efficiency [288]. Therefore, more effective engineering strategies for γδ T cells are necessary, in order to enhance their potential for clinical success.

AAV has become a promising tool for gene therapies and has seen success in preclinical and clinical studies. AAV2 was one of the first serotypes discovered and is one of the most common viral vectors used for gene therapy. It has natural tropisms for a variety of tissues and cell types including skeletal muscle [296], cells of the central nervous system [297, 298], retinal cells [299, 300], and hepatocytes [301]. Scientists have taken advantage of these specific tropisms by developing targeted treatments for many diseases. For example, the liver tropism has increased appeal for the development of *in vivo* treatments for hemophilia by engineering hepatocytes to produce Factor VIII or Factor IV [302, 303]. Furthermore, AAV2 has seen great success in clinical trials for a variety of hereditary eye conditions where monogenic diseases are able to be treated with the delivery of specific genes [304, 305]. In fact, studies like these have led to the development of Luxturna, an AAV2-based treatment developed by Spark Therapeutics for patients with RPE65-mutation-associated retinal dystrophy [306].

Although AAV2 is the most widely studied serotype, it has seen very little success in transducing T cells. AAV6 has recently become an attractive candidate as a viral vector due to its enhanced transduction efficiency for a wide range of cell types [307]. It was found to be a natural recombinant of AAV1 and AAV2 and is structurally similar to the AAV2 capsid [308-310]. Importantly, it has been shown to engineer hematopoietic cells with higher efficiencies than other viral vectors [311-313]. Therefore, we tested whether AAV6 is able to successfully engineer γδ T cells through the expression of CARs. In addition, we examined techniques to improve gene transfer using AAV6 by investigating the purification step during manufacturing, as well as utilizing techniques to enhance γδ T cell transduction. The first technique used to improve γδ T cell transduction with AAV6 was the use of a perfusable polystyrene microfluidics device,

which we have shown improves transduction of primary cells [314-316]. The device works by spatially constraining the viral particles and cells to allow for more virus-cell interactions and limits wasted virus due to prolonged diffusion times. The second technique that was tested to improve AAV6 transduction efficiency was the use of mutant AAV6 vectors. These vectors contain modified capsids that were shown to not only improve the intracellular trafficking and nuclear translocation of transgenes that resulted in enhanced transduction of primary cells, but also prevent kinase-mediated phosphorylation that causes ubiquitination and proteosome-mediated degradation of transgene DNA [317-319]. By optimizing the *in vitro* transduction of γδ T cells using AAV6, we hypothesize the limitation of low gene transfer can be overcome, resulting in sufficient numbers of engineered γδ T cells for downstream applications.

2.3 Results

Two ultracentrifugation steps in AAV6 production process results in purer product

The production process of AAV was simplified with the development of the three-plasmid technique and consists of HEK293T cell transfection, harvesting, concentration and purification, and a buffer exchange step. There are, however, ways that the process can be improved and optimized. Here, we evaluated the concentration and purification step and tested whether the addition of a second ultracentrifugation improves the final product, compared to only one ultracentrifugation. Using SDS page, we evaluated the purity of using one ultracentrifugation (denoted sample one) and two ultracentrifugations (denote sample two) and found the addition of the second ultracentrifugation resulted in a purer final product with less debris and contaminants (Figure 2-1). Both preparations did not affect the detection of all three capsid proteins, as seen by bands at 87kDa, 72kDa, and 62kDa. While making a purer AAV batch is essential for producing a safe product for down-stream applications, the viral particle yield is important when considering clinical applications. Therefore, we next examined whether the addition of the second ultracentrifugation affected the number of particles that were collected. We conducted a physical titer on both samples to determine the viral yield. The physical titers were found to be similar with sample one containing 2.69×10^{12} gc/mL and sample two containing 2.21×10^{12} gc/mL. To further evaluate the two samples, electron microscopy was

performed to examine the two preparations and evaluate the percentage of empty and full particles (Figure 2-2). Interestingly, the addition of the second ultracentrifugation resulted in a higher percentage of full capsids, with sample one containing 86.6% full particles and sample two containing 98.1% full particles. In addition, the microscopy images supported the SDS page results that sample two exhibited improved purity compared to sample one. Taken together, these results show that the addition of a second ultracentrifugation step during the concentration and purification step of AAV production improves purity and increases the full to empty particle ratio, while not affecting viral particle yield.

Figure 2-1: Second ultracentrifugation improves purity of AAV6 vector

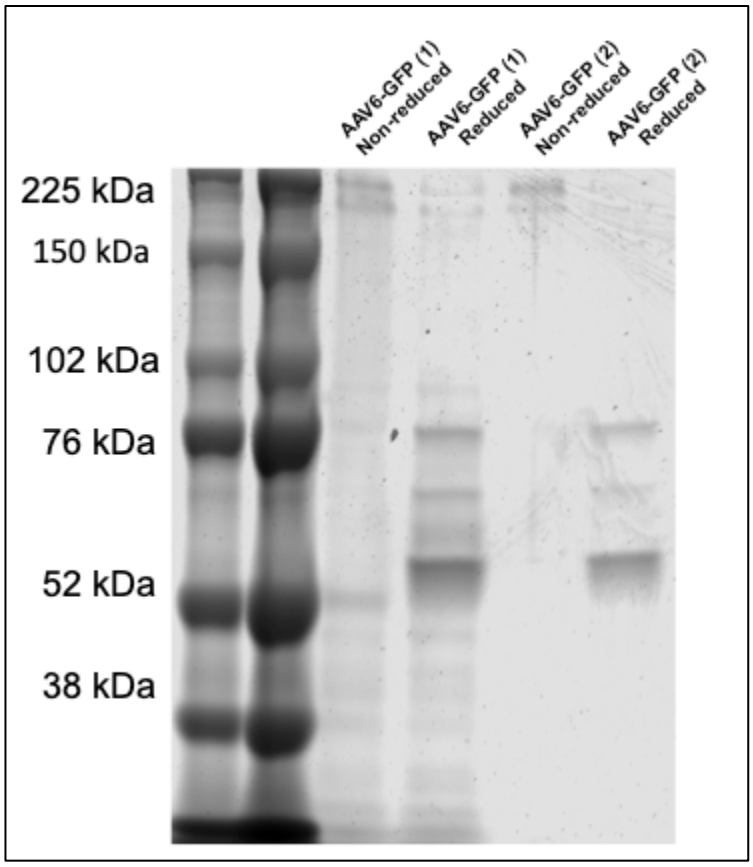


Figure Legend 2-1: Based on SDS page analysis, production of AAV particles using either one (sample 1) or two (sample 2) ultracentrifugation step does not affect successful capsid protein assembly and detection at the corresponding sizes. In addition, adding a second ultracentrifugation resulted in a purer final product, evident by the reduced debris and contaminants in sample 2.

Figure 2-2: Second ultracentrifugation increases the proportion of full capsid particles

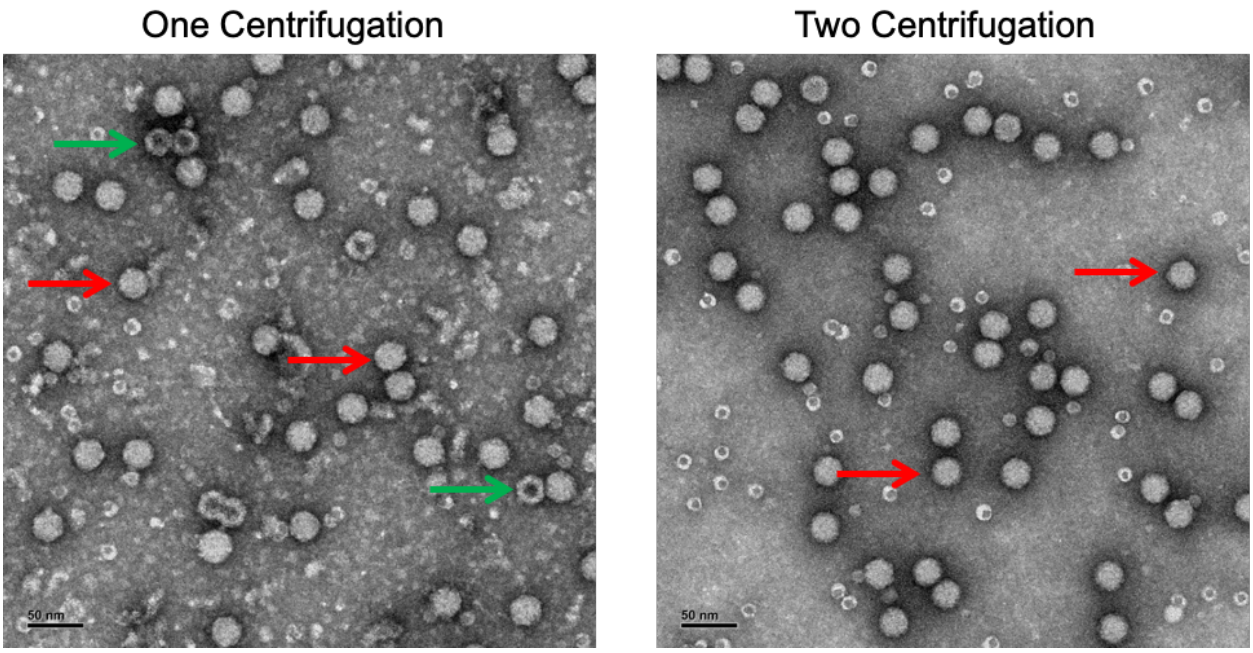


Figure Legend 2-2: Representative electron microscopy images of AAV preparations using one or two ultracentrifugation steps. Red arrows denote examples of full AAV particles, while green arrows denote examples of empty particles. Sample one contains more empty particles, in addition to more contaminants.

Development of Jurkat functional titer

Traditionally, a physical titer is used to characterize viral vector batches, however variations in batch production and differential transduction efficiencies of cell types necessitates an alternative method to measure and compare different batches. Therefore, we developed a standardized functional titer technique using the Jurkat cell line that incorporates transduction efficiency, which allows for a more applicable characterization of AAV batches. The Jurkat cell line is an immortalized T cell line that was isolated from the peripheral blood of a pediatric acute ALL patient, which makes it an ideal cell line for testing engineering techniques for T cells. In contrast to the physical titer, the functional titer is defined as the ratio of transduced cells to the number of viral particles used. To compare the functional titer to the physical titer, two batches of AAV6-MND-CD5 CAR were produced and the physical and functional titers were calculated as stated in the Methods section. Although the physical titer for the first batch was found to be high at 1.01 x 10^{13} vg/mL, the functional titer was calculated to be only 3.4 x 10^{7} TU/mL. A similar trend was found for the second batch, with a physical titer of 2 x 10^{12} vg/mL and a functional titer of only 2.0 x 10^{7} TU/mL. These comparison experiments reveal that the physical titer may not be the best method for characterizing AAV batches, and that the functional titer offers a valuable tool for the direct characterization of the transduction potential for AAV batches and allows for easy comparisons between experiments using different batches. The functional titer, as described in Methods, was used to calculate the multiplicity of infection (MOI) for each transduction.

Microfluidics device improves transduction of T cells but reduces γδ T cell viability

Initial attempts to use AAV to transduce T cells resulted in variable transduction efficiencies and reduced cell viability (Figure 2-3A and 3B). Therefore, we tested the use of a microfluidics device that spatially constrains the viral particles with the cells. To test whether the microfluidics device can improve AAV transduction, we examined the transduction efficiency of an AAV6-CMV-GFP vector (Supplemental Figure 2-1) to engineer the Jurkat cell line using the microfluidics device and compared it to traditional well transductions. Jurkats were transduced at varying MOIs and the GFP expression was detected by flow

cytometry (Figure 2-4A). GFP expression reached about 50% for the traditional well transduction, while the microfluidic device transduction reached over 95%, which shows the device improves the efficiency of AAV transductions. In addition to the increased transduction efficiency, the microfluidics device also resulted in faster expression of GFP, which was seen as early as one day after transduction (Figure 2-4B).

Figure 2-3: AAV6 transduction of γδ T cells results in variable GFP expression and reduced cell viability

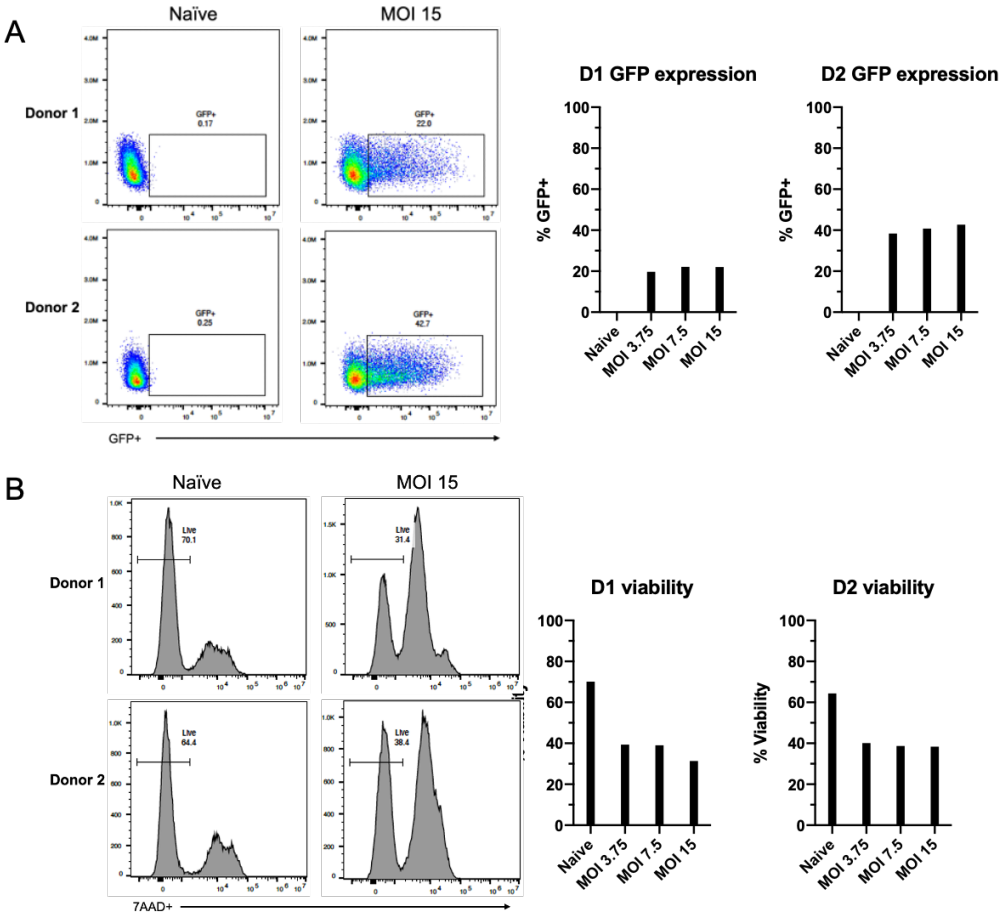


Figure Legend 2-3: GFP expression and cell viability were determined three days after transduction of γδ T cells using traditional well techniques. (A) Initial attempts at using AAV6 to transduce γδ T cells results in variable transduction efficiency. GFP expression ranges from 20-40% with no significant increase as the MOI increases. (B) Cell viability of γδ T cells decreases by about 20-30% after transduction as measured by 7AAD binding.

Figure 2-4: Microfluidics device improves transduction of Jurkat cell line

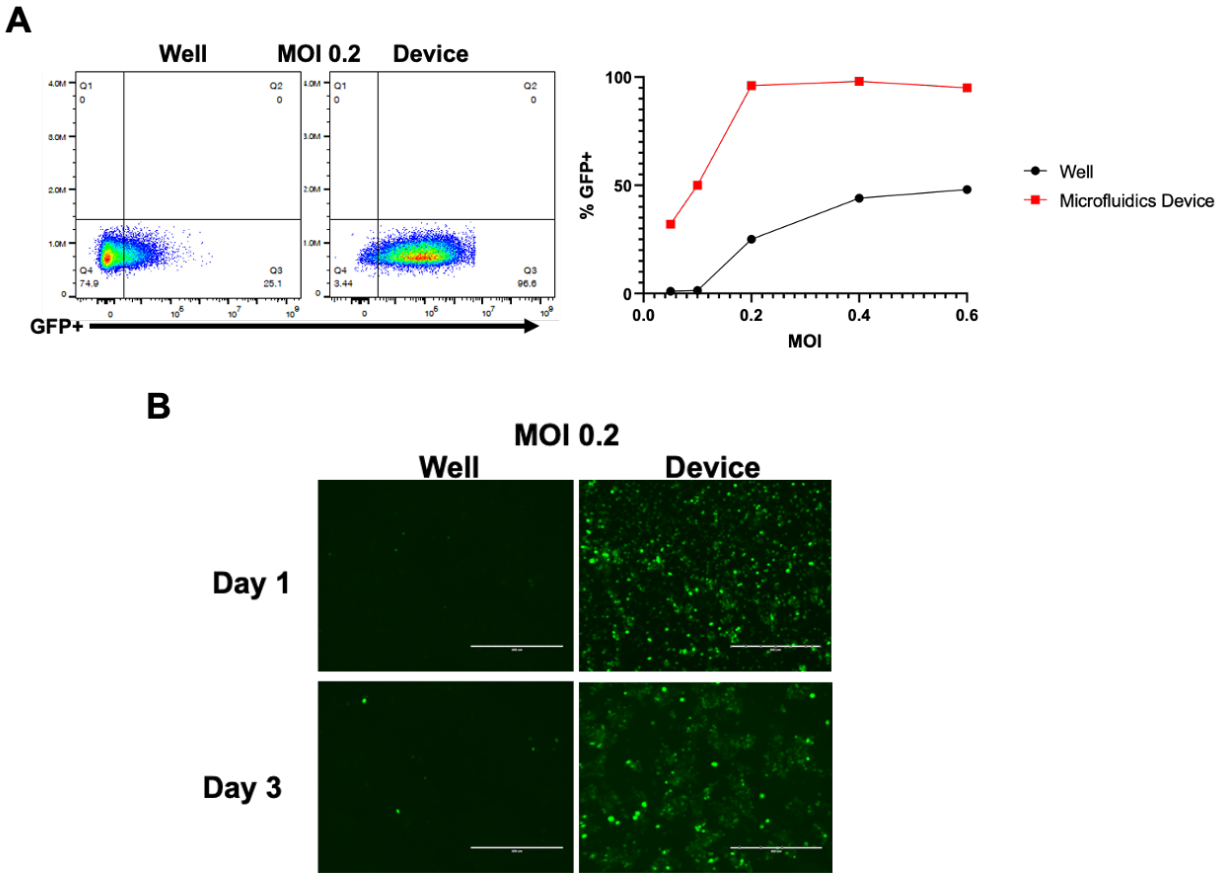


Figure Legend 2-4: (A) Representative flow plots and summary graph comparing traditional well transduction and microfluidic device transduction of Jurkat cells. Three days after transduction, samples from the microfluidics device display increased GFP expression at all MOIs, compared to the well samples. (B) In addition, device transduction leads to faster expression of the transgene, as seen by GFP expression as early as one day after transduction.

Next, we evaluated whether the microfluidics device increases the transduction efficiency of primary γδ T cells. γδ T cells were transduced at MOIs of 0.2 and 0.4 (based on well functional titers) using traditional and device transductions and the GFP expression was again determined by flow cytometry (Figure 2-5A). GFP expression was limited for the well transductions, however increased expression was observed for the device transductions, reaching about 50%. Despite the improved transduction efficiency, γδ T cell viability decreased when using the microfluidic device, with cell viability decreasing up to 20-40% (Figure 2-5B). To determine whether this decrease in viability was caused by the device itself or whether the amount of vector played a role, γδ T cells were transduced in the device at varying concentrations of vector in the total culture volume while keeping the MOI constant at 0.4. As expected, the expression of GFP increased as the vector percent increase because more viral particles are reaching the cells (Supplemental Figure 2-2). However, the higher concentration of vector also resulted in a reduction in cell viability, suggesting increased vector percentages during transduction is harmful to the cells and leads to increased cell death (Figure 2-5C).

Figure 2-5: Microfluidics device improves transduction of γδ T cells but reduces cell viability

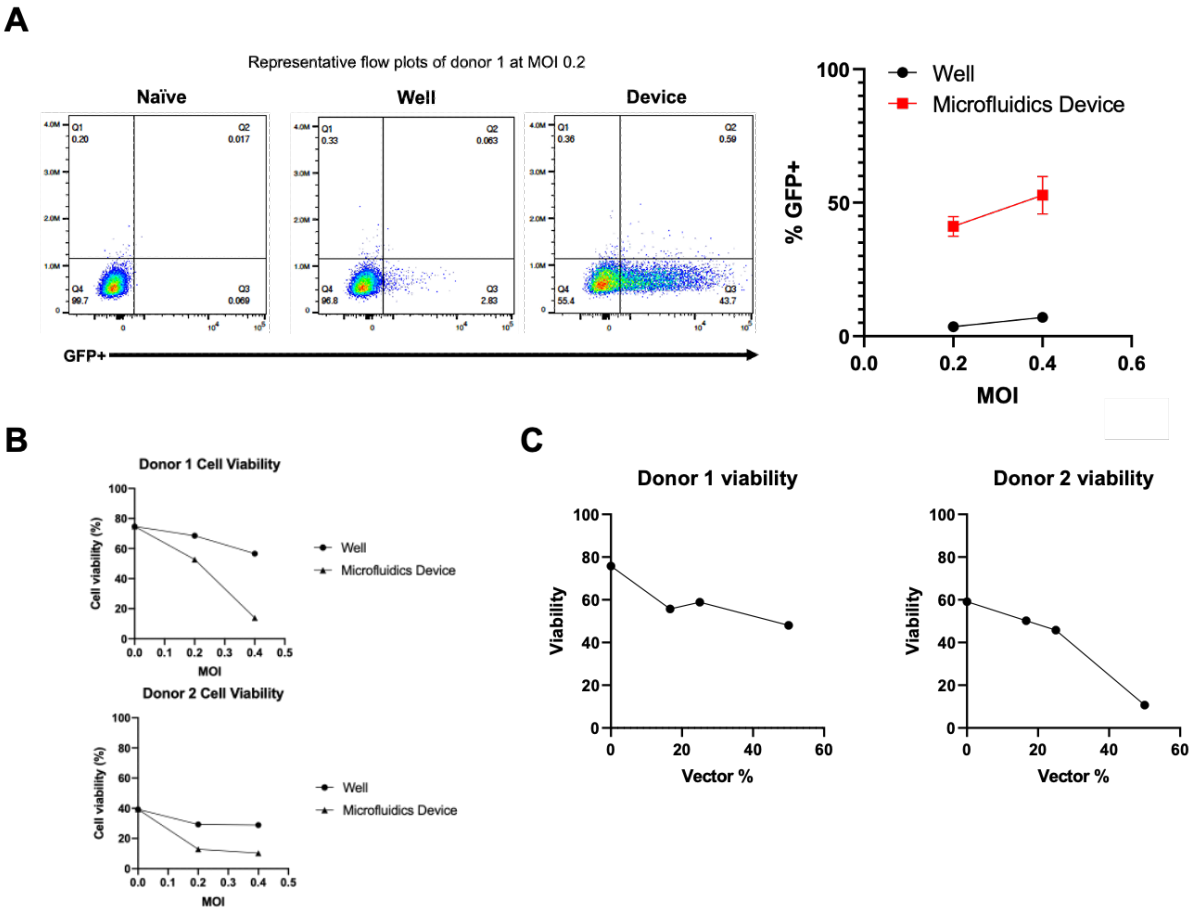


Figure Legend 2-5: (A) γδ T cells were transduced with an AAV6-CMV-GFP vector using the traditional well technique and the microfluidics device. Representative flow plots are shown on the left for one donor at an MOI of 0.2 and a summary graph shown on the right. Increased GFP expression was detected in γδ T cells when transduced using the microfluidics, compared to well transductions. (B) A reduction in cell viability was also observed when using the device, which decreased as the MOI increased. (C) To determine if the percentage of vector within the total culture volume during transduction affects γδ T cell viability, the vector percentage was varied while keeping the MOI constant at 0.4. A slight decreased in viability was observed at 16.7% and 25%, however a larger decrease in viability occurred when the vector percentage reached 50%.

Development of AAV6-MND-CD5 CAR construct leads to limited CAR expression in γδ T cell

After testing AAV transduction of γδ T cells with GFP, we next examined the expression of a functional CD5 CAR. We developed a bicistronic AAV6 GFP-CD5 CAR construct (Supplemental Figure 2-1) that contained an MND promoter, rather than the CMV promoter, as the MND promoter is strong in lymphocytes and other hematopoietic cells and the CMV promoter has been shown to be silenced and downregulated [320-322]. To first confirm successful cloning of the MND-CD5 CAR, HEK293 cell were transfected with plasmid DNA and western blot analysis was performed to confirm protein expression (Figure 2-6A). In addition, GFP expression was confirmed based on microscopy imaging (Supplemental Figure 2-3). Before testing the transduction of γδ T cells, the CD5 CAR construct was tested in Jurkat cells using traditional well transductions, as well as the microfluidic device transduction technique, and flow cytometry was used to determine GFP and CD5 CAR expression (Figure 2-6B). As expected, GFP expression increased as the MOI increased and reached 37% and 84% at an MOI of 0.4 for well and device transductions, respectively. No CD5 CAR expression was observed using the well transduction, and despite a prominent increase in GFP expression using the device, CAR expression remained limited with only about 7% expression. This high transduction efficiency with GFP but low CAR expression can be explained by the downregulation of the CD5 CAR upon cis and trans interactions with the CAR and CD5 on Jurkat cells, which we have previously shown [323, 324]. To confirm this, the same construct was used to transduce a CRISPR-edited Jurkat cell line that does not express CD5. Transduction of the CD5-edited Jurkat cells results in increased levels of the CD5 CAR, compared to the naïve Jurkat cell line (Supplemental Figure 2-4A). Furthermore, we show that the engineering of the naïve Jurkat cell line with the CD5 CAR results in increased activation, evident by increased CD69 expression (Supplemental Figure 2-4B) This suggests that CD5 expression can affect expression of the CD5 CAR in T cells and may be an obstacle that must be addressed. We hypothesized that in order to overcome the downregulation of the CD5 CAR, the transduction efficiency must be high enough to surpass the loss of surface expression. Therefore, we tested the transduction of γδ T cells at the maximum MOI that the device is able to achieve. Even at an MOI of 6 in the device, there was no CAR expression detected, suggesting, while the microfluidics device does

improve the gene transfer of AAV6, it is not able to overcome the downregulation of the CD5 CAR (Figure 2-6C).

Figure 2-6: Transduction of AAV6-MND-CD5 CAR results in limited GFP and CAR expression in Jurkats and γδ T cells

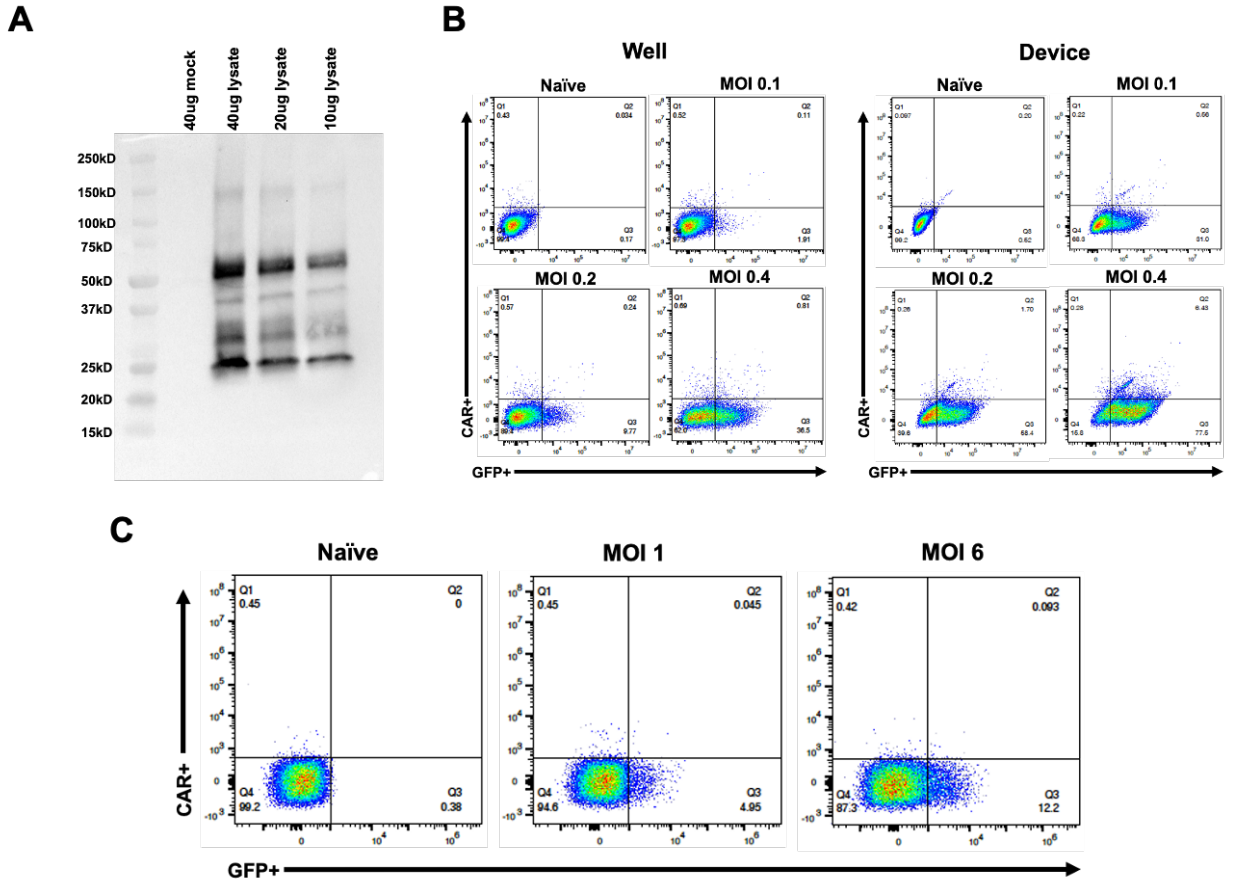


Figure Legend 2-6: HEK293 cells were transfected with plasmid DNA to confirm successful cloning of a bicistronic AAV6- GFP/ CD5 CAR construct containing the MND promoter. (A) Western blot analysis was performed to confirm proper size of the CAR protein. (B) Jurkat cells were transduced with the CD5 CAR construct using traditional well transductions and microfluidic device transductions. The well transduction resulted in limited GFP that reached 37%, while the device transduction resulted in GFP expression reaching 84%. CD5 CAR expression was not observed for the well condition and only limited CAR expression was detected for the device condition. (C) Limited GFP expression and no CD5 CAR expression was detected when transducing γδ T cells using the microfluidics device, even at higher MOIs.

AAV6 mutants improve transduction of γδ T cells but CD5 CAR expression remains low

The development of AAV mutants has been previously shown to improve transduction efficiencies of primary hematopoietic cells. Therefore, we tested whether these vectors are able to improve γδ T cell transduction and overcome the downregulation of the CD5 CAR. Two mutant AAV vectors were generated, one with a serine to valine mutation at position 663 (denoted 663V) and a second with a tyrosine to phenylalanine mutation at positions 705 and 731 (denoted M2). Both vectors were first evaluated for their transduction of the Jurkat cell line using the traditional well and microfluidics device techniques (Figure 2-7A). Well transductions of both the S663V and M2 vectors resulted in no CAR expression and GFP expression reaching about 30% and 40%, respectively. When combining the microfluidics devices and the mutant AAV vectors, GFP expression reached about 80% and CAR expression was detected at all MOIs and reached about 50% for both vectors. In addition, an exponential relationship between GFP and CAR expression was observed when looking across all MOIs (Figure 2-7B). An important observation was also made when analyzing GFP and CAR expression. Once transduction efficiency (i.e. GFP expression) reaches about 50%, CD5 CAR expression is detected, which suggests gene transfer to 50% of cells is sufficient to overcome the downregulation of the CAR. Additionally, an inverse relationship was seen between CD5 CAR and CD5 expression (Figure 2-8). As CD5 CAR expression in Jurkats increased, CD5 expression decreased. These results are consistent with the wildtype AAV6 transductions and further show that the interaction between the CD5 CAR and CD5 causes the downregulation of not only the CAR, but CD5 as well.

Figure 2-7: Jurkat cells transduced with AAV6 mutant vectors express GFP and CD5 CAR

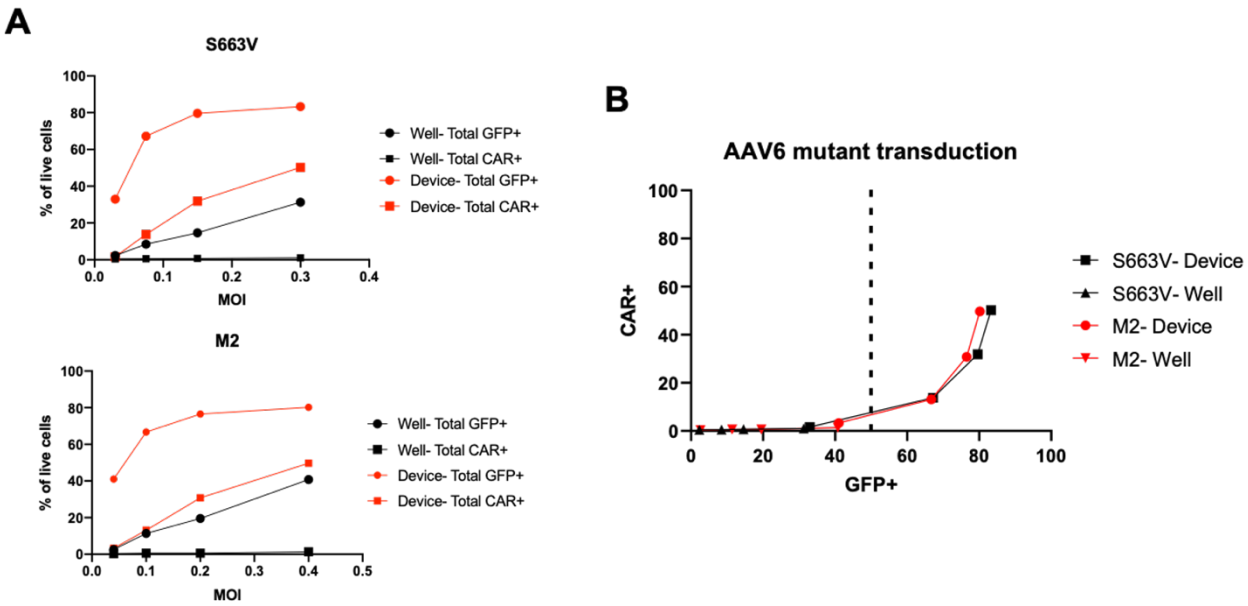


Figure Legend 2-7: Two AAV6 mutants were generated and assessed whether they exhibited increased transduction efficiencies. (A) Jurkat cells were transduced with the mutant vectors using well and device transductions at varying MOIs. Transductions using both mutants resulted in CD5 CAR expression using the device, while no CAR expression was detected when using wells. (B) The GFP-CAR relationship reveals an important threshold for the successful expression of the CAR and shows that transduction efficiencies above 50% overcomes the downregulation of the CD5 CAR.

Figure 2-8: CD5 expression is reduced as CD5 CAR expression increases

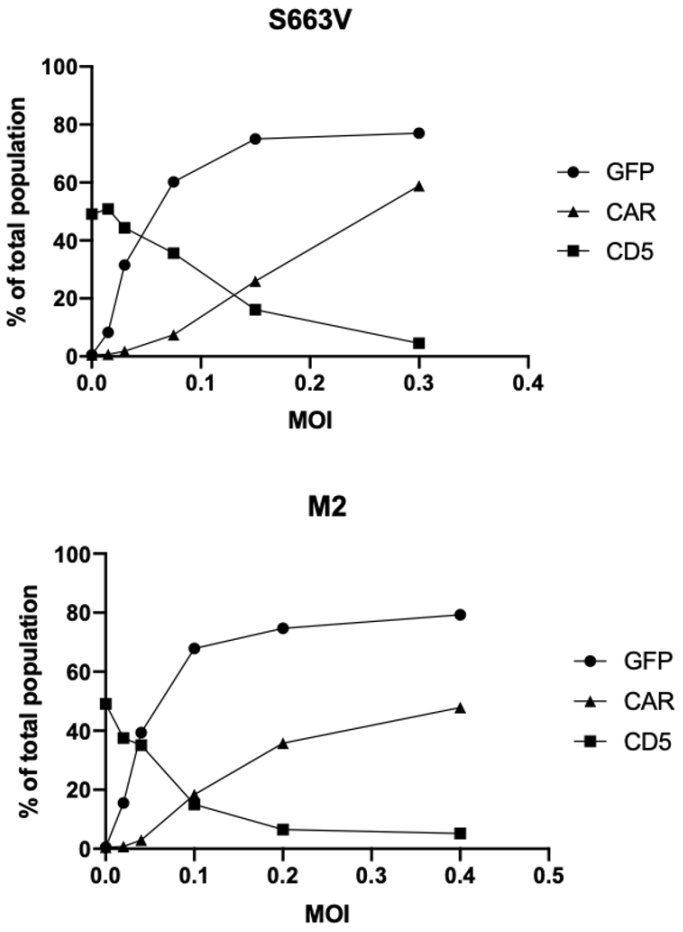


Figure Legend 2-8: Jurkat cells were transduced with the mutant AAV6 vectors at varying MOIs. Flow cytometry analysis reveals an inverse relationship between CD5 CAR expression and CD5 expression. This suggests that the interaction between antigen and CAR not only causes the downregulation of CD5 CAR, but also CD5 surface expression.

Next, we tested whether this engineering technique can be applied to γδ T cells for the expression of the CD5 CAR. γδ T cells were transduced with the two mutant AAV6 vectors in microfluidics devices and GFP and CD5 CAR expression was determined by flow cytometry. Consistent with the wildtype AAV6, expression of GFP in the γδ T cells was limited, with no CD5 CAR expression for all MOIs (Figure 2-9A). In addition, viability remains an issue when using primary γδ T cells in the microfluidics devices, as seen by the increased cell death compared to unmodified γδ T cells (Figure 2-9B). These results show that, despite the promising transduction of Jurkat cells with mutant AAV6 vectors and microfluidics device, γδ T cell transduction using this technique must be improved and further optimized.

Figure 2-9: AAV6 mutant transduction of γδ T cells results in limited transduction efficiency and low cell viability

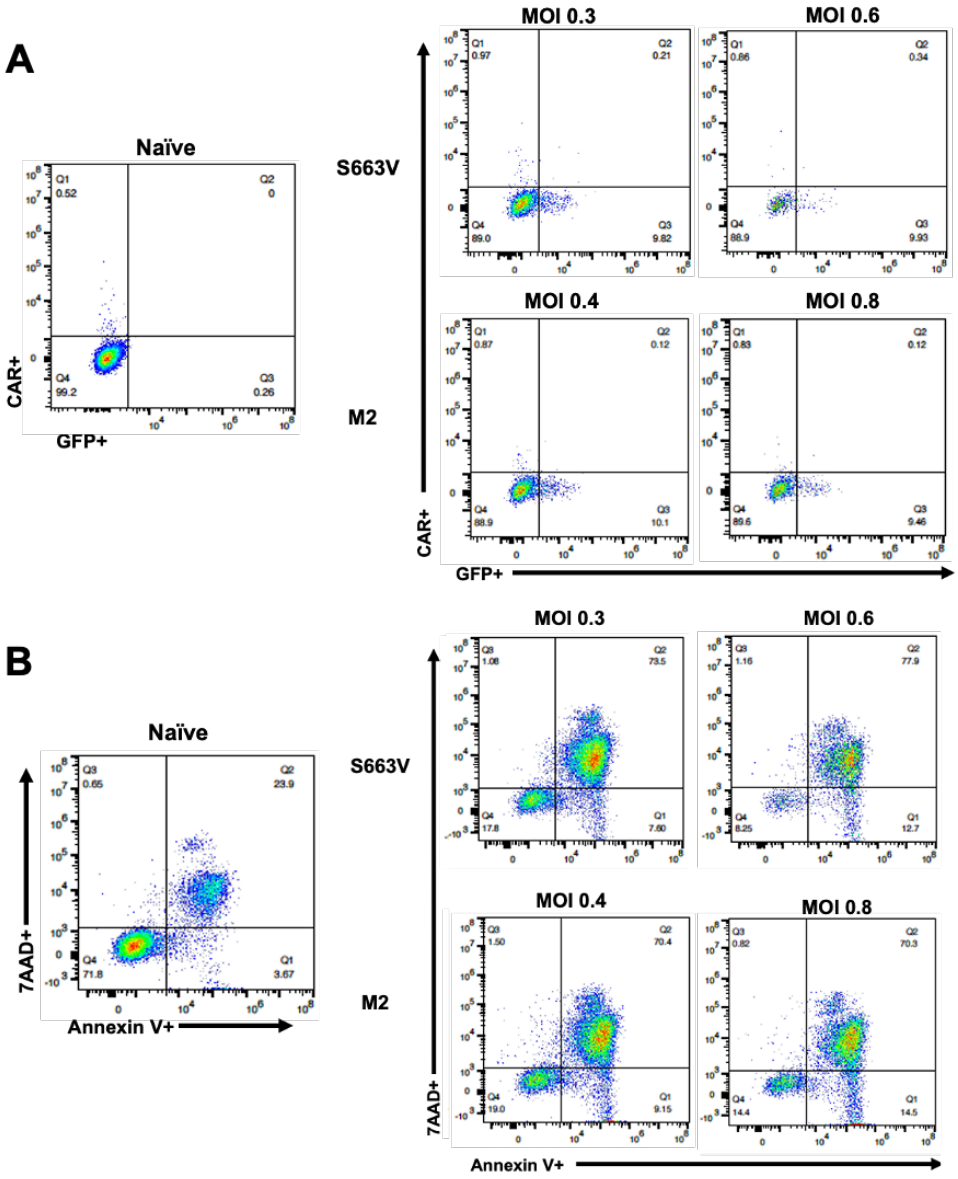


Figure Legend 2-9: (A) AAV6 mutants were used to transduce γδ T cells and GFP and CD5 CAR expression was measured using flow cytometry. GFP expression was limited with only about 10% of cells being GFP positive, while no CD5 CAR expression was detected. (B) An increase in cell viability was observed after γδ T cell transduction using either AAV6 mutant vector, as measured by Annexin V and 7AAD staining.

2.4 Discussion

Gene and cell therapy has become a promising option for many diseases and offers an attractive alternative to current treatments. AAV is one of the primary viral vectors used today and has become a powerful tool for delivering genes to cells. One of the major advantages of using AAV is the ability to transduce many different cell types. The diverse options of serotypes that preferentially transduce different tissues and cells allows for a more directed approach for genetic engineering. This is especially evident by the use of AAV for *in vivo* gene transfer, which has seen great success in preclinical and clinical studies [325]. In the field of cancer therapeutic development, the major focus for this technique has been to target cancer cells *in vivo* by creating oncolytic AAV vectors that either directly cause cancer cell death or make the cancer cells more susceptible to the host immune system [326-328]. In addition, there has also been a focus on targeting T cells *in vivo*, for the expression of CARs to help target cancer [329]. AAV vectors have not only seen success in *in vivo* applications, but also in *ex vivo* applications for the development of cancer immunotherapies. For example, the development of AAV-based cancer vaccines has revolutionized cancer treatments and have allowed for the direct priming of immunocompetent cells that are able to target cancer cells [330, 331]. In addition, adoptive cell therapy (ACT) offers another direction for the development of cancer immunotherapies, with CAR T cell therapy emerging as a promising technique for treating cancer. For CAR T cell therapy, immunocompetent cells are isolated from the patient or healthy donors, expanded and engineered, and then administered to the patient as a cellular therapy. It has seen great clinical success thus far, especially for B-cell malignancies, and has revolutionized approaches for treating cancer patients [332].

Traditionally, αβ T cells have been the cell type of choice for CAR-based therapeutics, however, γδ T cells are quickly becoming a promising option for use in adoptive cell therapy. They are a highly specialized immune cell that has been shown to play important roles in the adaptive and innate immune system. They possess characteristics that are ideal for cancer immunotherapy and adoptive cell therapy and are critical for immune surveillance, fighting infections, and targeting certain cancers [278]. Despite their favorable

characteristics, γδ T cells have traditionally been a difficult cell type to engineer. Unmodified γδ T cells have been tested in clinical trials and have been shown to be safe, however the limited efficacy necessitates strategies to improve they cytotoxic functions [333].

Developing novel engineering strategies for immunocompetent cells, like γδ T cells, to improve their specificity and anti-cancer efficacy is an essential step in the process of generating CAR T cells. Traditionally, AAV has had limited success, however the development of recombinant AAV6 vectors offered a promising engineering method and was found to have a high tropism for not only hematopoietic cells, but also T cells specifically [313]. Although not fully understood, the discovery of specific primary and secondary receptors that are required for AAV6 binding and internalization by target cells was pivotal in explaining why T cells are more susceptible to transduction by this viral vector. One of the major receptors that facilitates AAV6 binding and internalization by target cells are sialic acids, specifically α2,3 and α2,6 N-linked sialic acids [334, 335]. Sialic acids have been found to play an important role in T cell development and function and are attached to the majority of cell surface glycans through various linkages including α2,3 and α2,6 [336]. While no study has directly linked the expression of these viral receptors on T cells with the successful transduction of AAV6, it can be hypothesized that they contribute to the improved transduction efficiencies compared to other serotypes and viral vectors.

Here, we test whether AAV6 is able to engineer γδ T cells to express a CD5 CAR that is able to target T-cell malignancies. T-cell malignancies are a difficult cancer type to target, especially with CAR T cell therapies, and have several obstacles that must be addressed. For example, there are limited tumor-specific targets that can be applied to the development of CARs and on-target-off-tumor effects can lead to adverse side effects [337, 338]. The development of T cell aplasia can occur if the targeted antigen is also expressed on healthy T cells, which can lead to immunodeficiency. The depletion of T cells is caused by the persistence of engineered αβ T cells that can form a memory response against the targeted antigen [339, 340]. Another significant limitation is that the targeted antigen is expressed on the CAR T cells, which can

result in fratricide and depletion of the therapeutic cells [341, 342]. This can also lead to interactions between the antigen and CAR during manufacturing, which can cause downregulation of the CAR [338]. These limitations of the current αβ CAR T cell model must be addressed for the successful application of CAR T cell therapy for T-cell malignancies. We have previously published our efforts to develop a gene engineering platform for targeting T-cell malignancies with γδ T cells, however lentiviral transduction was limited [287, 324]. We also published the development of CD5 non-signaling CARs (NSCARs) that we showed improved γδ T cell killing, however the NSCAR was also downregulated upon interacting with CD5 [324]. Despite the improvement in cytotoxicity, the need for engineering techniques to express a functional CAR remains. Therefore, we hypothesized that the transient engineering of γδ T cells using AAV6 can overcome some of these limitations and create a practical alternative for treating T-cell malignancies.

Initial attempts at transducing γδ T cells with AAV6 using traditional well transductions were unsuccessful and resulted in limited transduction efficiency and poor cell viability. Therefore, we investigated techniques to enhance AAV6 gene transfer into γδ T cells. We improved the purity and quality of the AAV vector by adding an additional ultracentrifugation step during the concentration and purification step. We also utilized two strategies to enhance the transduction efficiency, the use of a microfluidics device that spatially constrains the viral particles to the cells and the use of mutant AAV6 vectors that have been shown to improve transduction of primary cells. We show that the microfluidics device improves transduction efficiencies of the T cell line Jurkat using a bicistronic AAV6-GFP/CD5 CAR vector, compared to traditional well transductions. Despite observing the downregulation of the CD5 CAR, we were able to overcome the phenomenon by expressing enough of the CAR. In fact, during our investigation, we also made an important observation of the relationship between GFP expression and CD5 CAR expression. Once transduction efficiency, which is portrayed as GFP expression, reaches about 50%, we begin to detect CAR expression. This suggests that a transduction efficiency of over 50% may be sufficient to overcome the issues of antigen/CAR interaction-mediated downregulation.

Despite improving transduction of the Jurkat cell line, transduction of primary γδ T cells using the microfluidics device remained limited and resulted in increased cell death. Therefore, we tested whether the combination of AAV6 mutants and the microfluidics device can improve the transduction of γδ T cells and overcome the low gene transfer, however cell yield and cell viability continued to be an issue. In order to reach levels of gene transfer that result in CAR expression in γδ T cells, a large volume of vector is needed. Primary γδ T cells are more susceptible to changes in culture conditions and we theorize that this concept may be the reason for low transduction efficiency and cell viability. A more concentrated AAV batch would allow for less vector volume needed for transduction and may offer a solution to overcome this issue. Overall, we show that AAV6 has the potential to be an effective gene engineering technique for γδ T cells, however more optimization and research is needed to improve this platform.

2.5 Materials and Methods:

Cell lines

The Jurkat cell line was obtained from ATCC. The cells were cultured in RPMI (Corning) media supplemented with 10% FBS and 1% penicillin/streptomycin in a 5% CO_2 incubator.

Expansion and transduction of γδ T cells

γδ T cells were expanded as previously published [287, 343]. Blood was obtained from healthy donors through the Children's Clinical Translational Discovery Core at Emory University. PBMCs were isolated using Ficoll-Paque Plus density centrifugation. PBMCs were cultured in OpTmizer containing OpTmizer supplement, 1% penicillin/streptomycin, and 2mM L-glutamine (complete OpTmizer). Cells were plated at 2 x 10^6 cells/mL and on day 0 and 3 of the expansion, 5μM Zoledronate and 500IU/mL IL-2 was added to the media. On day 6 and 9, 1000IU/mL IL-2 was added to the media. On day 6, an αβ depletion step was performed as previously published [283]. Flow cytometry was performed on day 0, 6, 9, and 12 to monitor γδ T cell expansion. On day 12 of the expansion, γδ T cells were used fresh for experiments. For γδ T cell

transductions, between 100,000 and 500,000 γδ T cells were resuspended in complete OpTmizer and the specified amount of AAV was added for about 16 hours. The next day the media was replaced, and cells were plated at 5 x10^5 cells/mL. Cells were analyzed for successful transduction via flow cytometry. CD5 CAR expression was detected by labelling cells with a CD5-Fc fusion protein (AcroBiosystems) and an anti-IgG Fc secondary antibody (Jackson Immunoresearch Laboratories).

Production of AAV vectors

AAV was produced using the three-plasmid transfection technique. The expression plasmid encoding GFP, the RC6 plasmid, and the pHelper plasmid were transiently transfected into HEK293T cells using calcium chloride. Cells were cultured in DMEM with 10% FBS and 1% pen/strep for 3 days. On day 3, culture media and cells were harvested and centrifuged at 1250rpm for 10 minutes. The supernatant was poured into a new tube and 40% PEG 8000 was added to a final concentration of 8% PEG 8000. The supernatant was then placed on ice for at least 2 hours and centrifuged at 10,700rpm at 4°C for 30 minutes. The pellet was resuspended with lysis buffer (2mM magnesium chloride, 150mM sodium chloride, and 50mM TRIS hydrochloride) and added to the crude lysate after nuclease digestion. The cell pellet was lysed by adding 50mL of lysis buffer containing 0.15M NaCl and 50nM Tris HCl pH8.5. The pellet underwent three freeze/thaw cycles and nucleases, sodium deoxycholate (0.5% final concentration) and benzonase (diluted 10000X) was added. The crude lysate was then digested for 30 minutes at 37°C. Next, 5M NaCl (0.5M final sodium concentration), 4.82M MgCl2 (20mM final), and Salt-Activated Nuclease (50U/mL final) was added to the lysate and was placed in 37C water bath for 30 minutes. The sample was then centrifuged at 10,000g for 45 minutes and the supernatant containing AAV particles was collected and placed into a new ultracentrifugation tube. The sample was concentrated and purified using either one or two iodixanol gradient ultracentrifugation by layering 15% iodixanol, 25% iodixanol, 40% iodixanol, and 54% iodixanol under the sample. The tube was then centrifuged at 58,500rpm at 10°C for 1 hour and 45 minutes. The 54%-40% interphase, containing the AAV particles, was then collected. The sample undergoing two ultracentrifugations was placed in a new ultracentrifugation tube and topped with 30% iodixanol, 40%

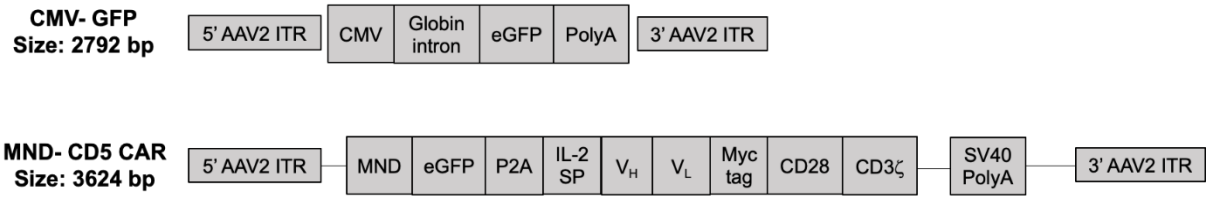
iodixanol, and 54% iodixanol. This sample was centrifuged again and the 54%-40% interphase was collected. Both samples were then concentrated, and buffer exchange was conducted using a 30K Amicon Ultra- 15 centrifugal filter and washing with Pluronic F68 PBS. First, 0.1% Pluronic F68 PBS is placed on the membrane and allowed to incubate for 10 minutes at room temperature. The membrane is centrifuged at 5000g for 4 minutes. Next, 0.01% Pluronic F68 PBS is added and centrifuged again. The sample is then placed on the membrane and washed with 0.01% Pluronic F68 PBS until the iodixanol gradient is completely removed. The sample is then pipetted from the membrane and aliquoted for analysis or transduction. Mutant AAV6 vectors were produced and generously donated by Dr. George Aslanidi's laboratory at the University of Minnesota.

Characterization of AAV vectors

For SDS page analysis, samples were run as non-reduced and reduced using β-ME. A 7.5% SDS page gel was loaded with 10uL of non-reduced and reduced samples and run at 45V for 3 hours. To determine the physical viral titer, real time quantitative PCR was performed. Forward and reverse GFP oligonucleotide primers were used and diluted to 10uM. Real-time PCR was performed in 25ul reaction volumes using viral DNA diluted at 1:2000, 1:5000, and 1:10000. The thermocycler program was set to 10 minutes of pre-incubation at 95°C followed by 40 cycles of 15 seconds at 95°C and one minute at 60C. Standard curves were made using serial dilutions of the expression plasmid. To determine functional titer, Jurkat cells were plated at 100,000 cells/well in a 96-well plate. Cells were either not treated (naïve) or treated with 1uL, 5uL, or 10uL of vector. The next day (day 1), media was replaced with fresh media and cells were re-plated in a 48-well plate. On day 3, cells were collected and analyzed by flow cytometry to determine the number of GFP^+ cells.

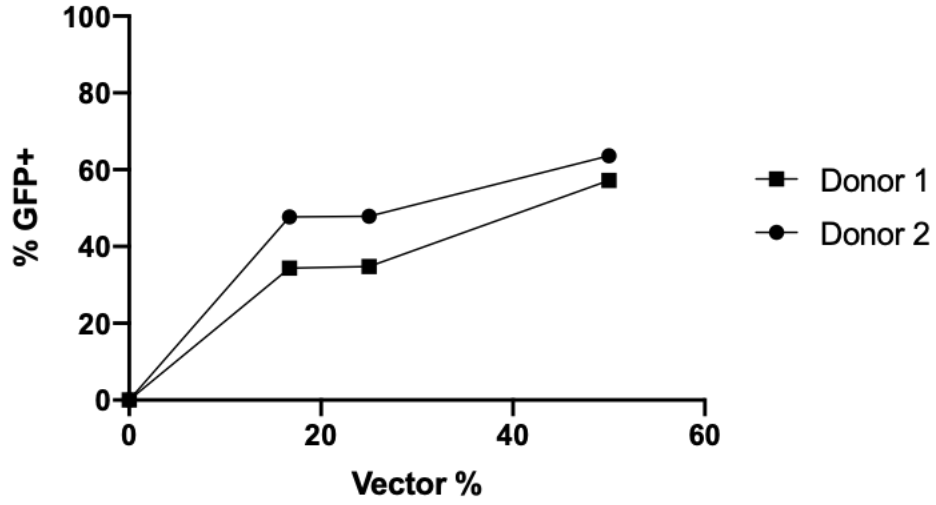
2.6 Supplemental Figures, Tables, and Legends

Supplemental Figure 2-1: Schematic of AAV6 GFP and CD5 CAR vectors



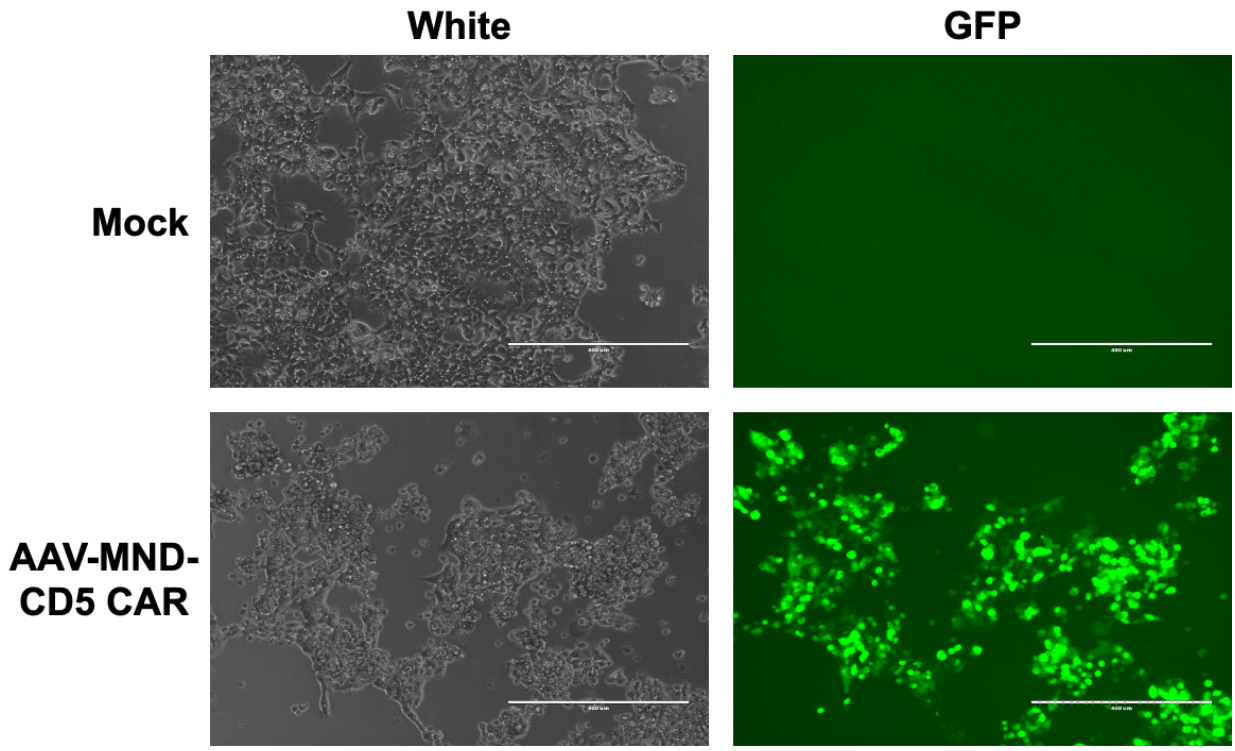
Supplemental Figure Legend 2-1: The AAV6-CMV-GFP construct (top) was used to test transduction efficiencies of the Jurkat cell line and γδ T cells. The construct contains AAV2 ITRs and a globin intron. The bicistronic AAV6-MND-GFP/CD5 CAR construct (bottom) was used to test expression of a functional CAR and also contains AAV2 ITRs. The CAR constructs includes the variable heavy and variable light chains of a CD5-specific scFv, the CD28 transmembrane domain, the CD28 costimulatory domain, and the CD3ς activation domain.

Supplemental Figure 2-2: GFP expression increases with increasing vector percentage of culture volume



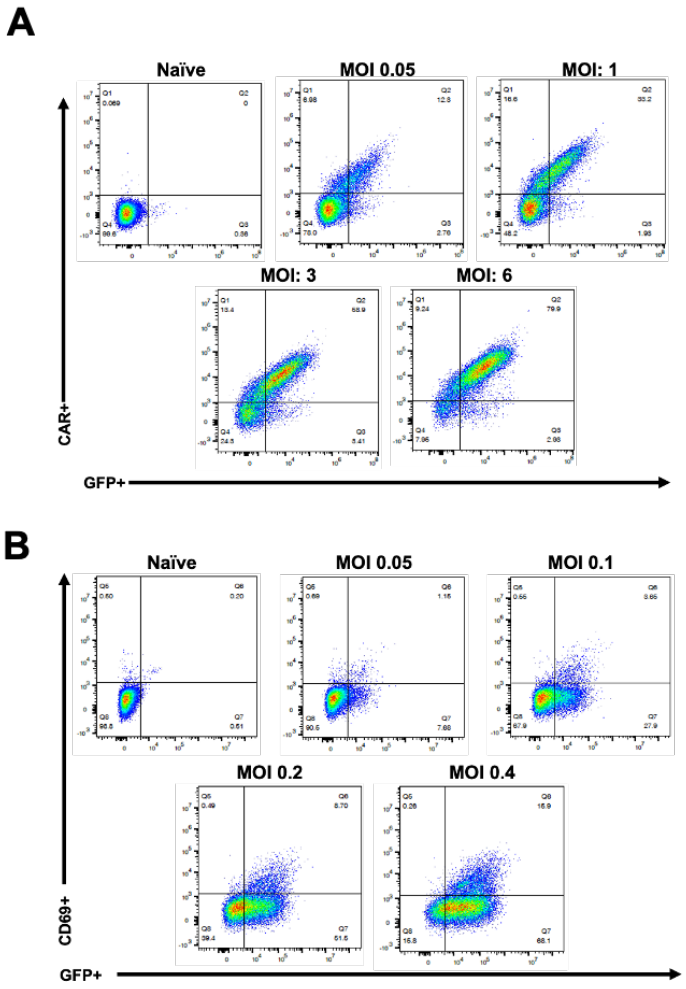
Supplemental Figure Legend 2-2: To test whether vector percentage of total culture volume affects γδ T cell viability, γδ T cells were transduced at a constant MOI and vector volume at different total culture volumes. As expected, GFP expression increased as the vector percentage of total culture volume.

Supplemental Figure 2-3: HEK293 cells express GFP after transfection of newly cloned AAV6 vector



Supplemental Figure Legend 2-3: To test whether the AAV6-MND-CD5 CAR construct is functional, HEK293 cells were transfected with plasmid DNA. GFP expression was confirmed in transfected cells using light microscopy.

Supplemental Figure 2-4: Downregulation of CD5 CAR after interaction with CD5



Supplemental Figure Legend 2-4: CD5-edited Jurkat cells were transduced with the AAV6-MND-CD5 CAR to show that the interaction of CD5 and the CD5 CAR results in downregulation of the CD5 CAR. (A) A linear relationship with GFP and the CD5 CAR is observed in CD5-edited Jurkats, and expression reaches about 80% at an MOI of 6. (B) While CD5 CAR expression is downregulated in naïve Jurkats, the activation of the cells is still evident by CD69 expression and increases with higher MOIs.

Chapter 3

Enhancing the effectiveness of γδ T cells by mRNA transfection of chimeric antigen receptors or bispecific T cell engagers

This research is published in *Molecular Therapy Oncolytics*

Authors: Scott A. Becker[1], Brian G. Petrich[2], Bing Yu[2], Kristopher A. Knight[1], Harrison C. Brown[2], Sunil S. Raikar[3], Christopher B. Doering[1,3], Harold Trent Spencer[1,3]

[1] Molecular and Systems Pharmacology Program, Graduate Division of Biological and Biomedical Sciences, Emory University, Atlanta, GA, USA
[2] Expression Therapeutics, Inc., Tucker, GA, USA
[3] Aflac Cancer and Blood Disorders Center, Department of Pediatrics, Emory University School of Medicine and Children's Healthcare of Atlanta, Atlanta, GA, USA

3.1 Abstract

Adoptive cell therapy (ACT) utilizing γδ T cells is becoming a promising option for the treatment of cancer, because it offers an off-the-shelf allogeneic product that is safe, potent, and clinically effective. Approaches to engineer or enhance immune competent cells for ACT, like expression of chimeric antigen receptors (CARs) or combination treatments with bispecific T cell engagers, have improved the specificity and cytotoxic potential of ACTs and have shown great promise in preclinical and clinical settings. Here, we test whether electroporation of γδ T cells with CAR or secreted bispecific T cell engager (sBite) mRNA is an effective approach to improve the cytotoxicity of γδ T cells. Using a CD19-specific CAR, approximately 60% of γδ T cells are modified after mRNA electroporation and these cells show potent anticancer activity *in vitro* and *in vivo* against two CD19-positive cancer cell lines. In addition, expression and secretion of a CD19 sBite enhances γδ T cell cytotoxicity, both *in vitro* and *in vivo*, and promotes killing of target cells by modified and unmodified γδ T cells. Taken together, we show transient transfection of γδ T cells with CAR or sBite mRNA by electroporation can be an effective treatment platform as a cancer therapeutic.

3.2 Introduction

Immunotherapies are revolutionizing cancer treatment by harnessing the immune system to target cancerous cells. Adoptive cell therapy (ACT) offers a promising direction as an effective cancer therapeutic by using immune competent cells in either an autologous or allogeneic setting [344]. Traditionally, ACT utilizes autologous αβ T cells that are isolated from the patient, engineered to improve their cytotoxicity, and then re-infused into the patient. Although these therapies are effective, off-the-shelf allogeneic products have advanced into clinical testing and have many advantages over autologous strategies [50, 345]. γδ T cells are a small subset of lymphocytes that contributes to the body's innate and adaptive immunity and are involved in immune surveillance, rapid immune response, and modulating other immune cells [244, 257, 346, 347]. γδ T cells are quickly becoming a promising option for ACT because they are non-alloreactive with limited risk of causing graft versus host disease, thus allowing their use in allogeneic settings [348, 349]. They also exhibit several characteristics that make them favorable candidates for use in adoptive cell

therapy. One of the major advantages of γδ T cells is their ability to recognize antigens in an MHC-independent manner, which means they do not require MHC-peptide priming for activation. They recognize several unique ligands and stress markers that direct their killing toward cancer cells, including butyrophilin via phosphoantigen activation [270, 350], Fas [285], heat shock proteins [351, 352], and MHC class I-related molecules MICA, MICB, and ULBPs 1-6 [271, 353]. In addition, they perform antibody-dependent cellular cytotoxicity through expression of CD16 and show promising anti-cancer activity when used in combination with therapeutic antibodies [283, 289, 354]. Importantly, γδ T cells can be expanded *ex vivo* from peripheral blood with a serum-free protocol for clinical use [287, 288, 343].

The development of chimeric antigen receptor (CAR) T cell therapy is among the most promising anti-cancer therapeutics and has improved immunotherapies by allowing for a more targeted treatment approach compared to chemotherapeutics. CAR T cells utilize the specificity of antibodies and the cytotoxic capabilities of T cells to target cancer cells. The most successful application of CAR T cells is the treatment of B-cell malignancies using CD19 CARs with complete remission rates reaching about 60% for children and young adults [355]. Despite its early success, there are obstacles and limitations that must be addressed to improve patient outcomes and safety, including cytokine release syndrome, neurotoxicity, acquired resistance to CAR T cell therapy, and health of the expanded T cell product [356-358]. Additionally, the development of bi-specific antibodies has shown some success in preclinical and clinical studies [359-361]. Bi-specific antibodies are a type of engineered antibody containing two binding regions, allowing for multiple applications including bringing immune cells in close contact with target cells, blocking immune checkpoints, and modulating inflammatory and other signaling pathways [362]. There are two major types of bi-specific antibodies, IgG-like and non-IgG-like, with the major difference being the incorporation of the Fc fragment [363]. Blinatumomab is a type of non-IgG-like bispecific antibody known as a bispecific T cell engager that is specific to CD19. Bispecific T cell engagers are typically comprised of a CD3-specific scFv linked to an scFv specific to a tumor antigen, a design that promotes T cell-cancer cell interactions to improve T cell cytotoxicity, serial killing, and proliferation [364].

Immunotherapies utilizing unmodified or engineered γδ T cells have the potential to be effective cancer treatments. We have previously published our efforts to optimize the expansion and handling of γδ T cells, as well as identify successful donor characteristics to predict a more potent cellular product [343, 365, 366]. These optimizations have resulted in an FDA cleared γδ T cell product candidate for clinical testing against neuroblastoma (NCT05400603). We have also shown that γδ T cell cytotoxicity can be improved by upregulating stress antigens on cancer cells through combination therapy with chemotherapeutics such as temozolomide and bortezomib [367-369]. Another approach to improve γδ T cell cytotoxicity is to genetically engineer these highly potent immune-competent cells. However, engineering γδ T cells has been variable and inefficient [370]. Advances in mRNA design and transfer over the past several decades have allowed for increased stability, higher transfection efficiencies, and rapid expression of proteins [178]. In addition, transient engineering of γδ T cells offers a number of advantages over stable engineering and can reduce some of the risks associated with CAR T cell therapy. For example, in the event of toxicity, treatment can be halted quickly, and specified doses of cells can be administered for individual cases. Also, the use of transient engineering strategies can reduce the risk and duration of cytokine release syndrome because the finite length of expression limits the over-activation and excessive cytokine release of CAR T cells. Here we test whether transient engineering of γδ T cells with CAR or secreted bispecific T cell engager (sBite) mRNA can be an effective cancer treatment platform and an alternative to the traditional stable CAR expression in αβ T cells.

3.3 Results

CD19 CAR expression in electroporated γδ T cells

Several electroporation strategies were tested using the BioRad Gene Pulser Xcell Electroporator or Lonza Nucleofector IIB device. Although both can be optimized for engineering *ex vivo* expanded γδ T cells, here we show optimization of the BioRad Gene Pulser Xcell Electroporator using a bicistronic CD19 CAR-GFP construct (Supplemental Figure 3-1). Successful electroporation was determined by GFP expression (Figure

3-1A). To determine the optimal conditions for electroporation, increasing cell numbers and mRNA concentrations were tested for each individual reaction as described in Methods. Cell yield, which we define as the proportion of live cells remaining 24hrs after electroporation to the starting number of cells used for the electroporation reaction, is an important factor when considering downstream applications. Twenty-four hours after electroporation, reactions containing between 1 x 10^6 and 1 x 10^7 γδ T cells showed increasing cell yield with increasing cell number (Figure 3-1B). To measure transfection efficiency, three parameters were examined: GFP mean fluorescence intensity (MFI), GFP^+ percentage, and CAR^+ percentage (Figure 3-1C & 1D). The MFI for GFP increases with increasing amounts of mRNA per reaction for all cell numbers. Interestingly, the percentage of GFP^+ cells and CAR^+ cells was similar for all reaction conditions and was found to be around 90% and 60%, respectively. Since reactions with 30μg of mRNA did not improve any of the tested parameters compared to 15μg, 15μg of mRNA was used for functional studies. CD19 CAR-expressing γδ T cells from reactions with 5 x 10^6 and 1 x 10^7 were cocultured for 4 hours with 697 cells, a $CD19^+$ B cell leukemia cell line, and the percent cytotoxicity was determined. All effector:target (E:T) ratios tested resulted in the same cytotoxicity, suggesting that varying the number of cells per transfection reaction, while keeping the amount of mRNA constant at 15μg, does not affect the cytotoxicity of the engineered γδ T cells (Figure 3-1E).

Figure 3-1: Optimization of γδ T cell electroporation with bicistronic CD19 CAR-GFP mRNA

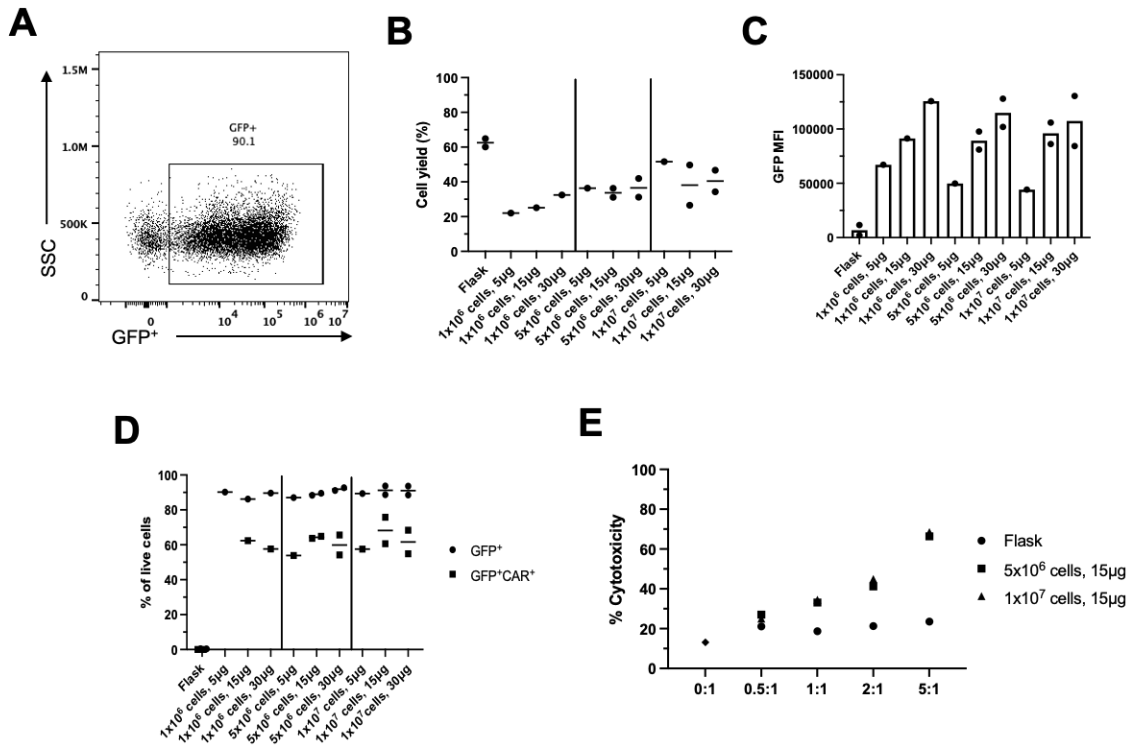


Figure Legend 3-1: (A) γδ T cells express GFP after mRNA electroporation using the BioRad Gene Pulser Xcell Electroporator. γδ T cell electroporation was optimized by testing varying cell numbers and mRNA amounts in each reaction. (B) Cell yield, calculated by determining the proportion of live cells remaining 24hrs after electroporation to the starting number of cells used for the electroporation reaction, was calculated for all reaction conditions and increased as the cell number increased. (C) GFP mean fluorescent intensity (MFI) was determined by flow cytometry and increased with increasing amounts of mRNA. (D) The percentage of live cells expressing GFP and the CD19 CAR was similar for all conditions and was found to be about 90% and 60%, respectively. (E) γδ T cell cytotoxicity was determined by flow cytometry to test the promising electroporation reaction conditions and found no difference when comparing different cell numbers in each reaction.

As γδ T cells are considered candidates for off-the-shelf ACT, a freezing step is anticipated. Therefore, these cells can be genetically engineered either before or after freezing. γδ T cells were electroporated on day 12 of expansion with the CD19 CAR-GFP construct and were examined before freezing and after a freeze/thaw cycle. The GFP^+ percentage was similar (around 90%) prior to freezing and after freeze/thaw whereas the CAR^+ percentage prior to freezing was approximately 60% and decreased to 20-40% after freeze/thaw. (Figure 3-2A). Cell viability was also measured and found that the viability decreased after thawing, compared to after electroporation/before freezing (Supplemental Figure 3-2). To test whether freezing engineered γδ T cells also affected their ability to kill target cells, a cytotoxicity assay was conducted with γδ T cells that were either engineered before freezing or engineered after freezing (Figure 3-2B). Both groups performed similarly at low E:T ratios of 0.5:1 and 1:1. However, differences between the groups were more substantial at the higher E:T ratios of 2:1 and 5:1, with a lower average cytotoxicity of cells engineered before freezing, compared to engineered after freezing. Based on these studies, we found electroporating $5x10^6$ to $1x10^7$ thawed cells with 15μg of mRNA was the optimal conditions for γδ T cell electroporation.

Figure 3-2: Electroporation of γδ T cells before freezing results in lower CAR expression and reduced cytotoxicity

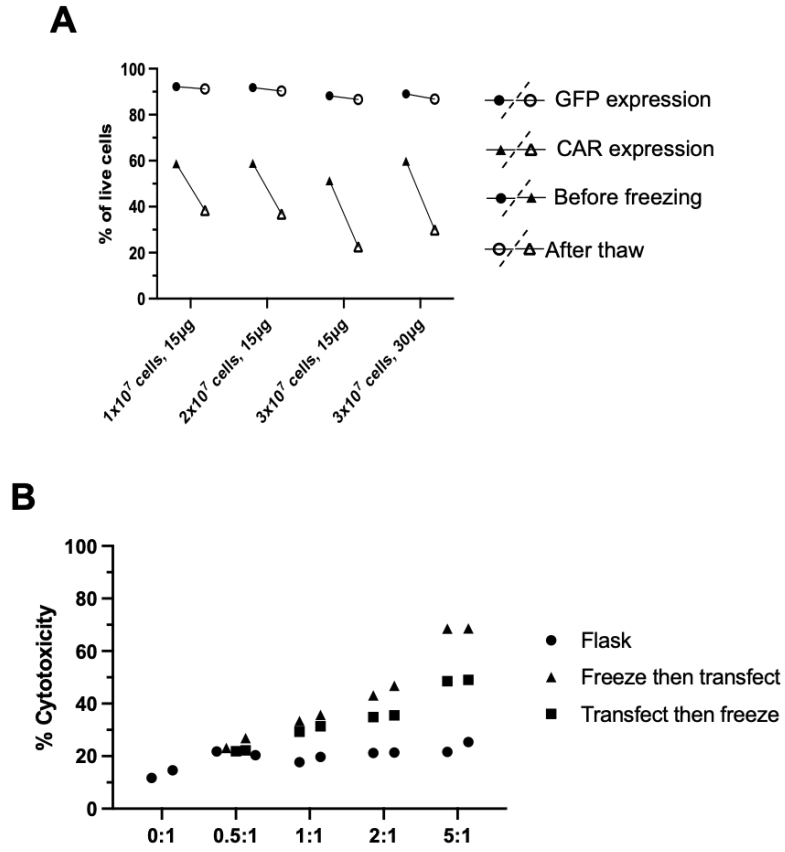


Figure Legend 3-2: γδ T cells were electroporated on Day 12 of expansion and were analyzed before and after freezing. (A) While GFP expression (circles) remained constant at around 90% before and after freezing, the CAR percentage (triangles) decreased from about 60% before freezing to about 30-40% after thawing. Closed data points denote before freezing and open data points denote after thawing. (B) The cytotoxicity of CD19 CAR-expressing γδ T cells before and after freezing was also measured to determine if a freeze/thaw cycle effects the cytotoxicity of the engineered cells. Similar cytotoxicity was observed at low effector to target (E:T) ratios, however there was a reduction at higher E:T ratios for the thawed engineered cells.

Electroporation of CD19 and CD22 CAR mRNA enhances the effectiveness of γδ T cells

We then engineered an mRNA construct that i) did not include GFP and ii) was codon optimized for expression in γδ T cells, as described in Methods. Comparing the codon optimized construct and the initial GFP-containing construct showed they both resulted in similar CAR expression and cytotoxicity against 697 cells (Supplemental Figure 3-3). All subsequent functional experiments were conducted with the codon optimized/non-GFP construct. The efficacy of the engineered cells was then tested using *in vitro* cytotoxicity assays against two B-ALL cell lines, 697 and Nalm6. First, mock electroporated γδ T cells or CD19 CAR-expressing γδ T cells were cocultured with 697 cells for 4hrs at E:T ratios of 0.5:1, 1:1, 2:1, and 5:1 and the percent cytotoxicity was measured by flow cytometry (Figure 3-3A). The percent cytotoxicity of the CD19 CAR-expressing γδ T cells increased with increasing effector cells, reaching 85% at the 5:1 E:T ratio, while the mock electroporated γδ T cells remained constant at <20%. To further examine the effect of engineering γδ T cells, cytotoxicity assays were performed using γδ T cells engineered with a CD22 CAR against the same cell line (Figure 3-3B). Similar to the CD19 CAR-expressing γδ T cells, the cytotoxicity of the CD22 CAR-expressing γδ T cells increased with increasing E:T ratios, reaching 82% at the 5:1 E:T ratio.

To further confirm the efficacy of the engineered γδ T cells, CD19 CAR- and CD22 CAR-expressing γδ T cells were tested against a second B-ALL cell line, Nalm6 (Figure 3-3C). Mock electroporated γδ T cells again had a constant cytotoxicity percentage across all E:T ratios and averaged approximately 6%. In contrast, CD19 CAR- and CD22 CAR-expressing γδ T cells exhibited a dose-dependent increase in cytotoxicity, reaching 76% and 43% at the 5:1 ratio, respectively, demonstrating i) the CD19 CAR-engineered γδ T cells effectively kill B-ALL cell lines *in vitro* and ii) CD19 CAR-engineered γδ T cells are slightly more effective than CD22-based CARs against Nalm6 cells. This difference in cytotoxicity can be explained by lower CD22 expression in Nalm6 cells, compared to the CD19 expression [371].

Figure 3-3: CD19 CAR- and CD22 CAR-expressing γδ T cells enhances cytotoxicity against two B-ALL cell lines

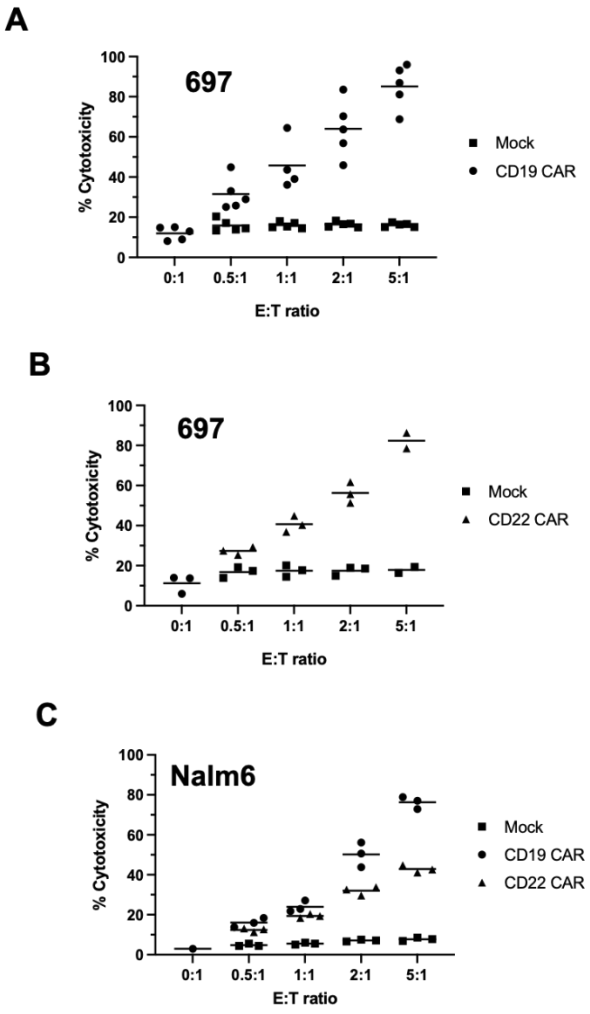


Figure Legend 3-3: Effector and target cells were cocultured at the specified E:T ratio for 4 hours and the percent cytotoxicity was determined by flow cytometry. Target cells were stained with VPD450 to differentiate effector and target cell death. Mock electroporated, CD19 CAR-, and CD22 CAR-expressing γδ T cells were tested against the B-ALL cell lines 697 (A & B) and Nalm6 (C). While the cytotoxicity of mock-electroporated γδ T cells remained constant over all E:T ratios, CD19 CAR- and CD22 CAR-expressing γδ T cells exhibited a dose-dependent increase in cytotoxicity.

Electroporation of sBite mRNA enhances the effectiveness of γδ T cells

Co-administration of γδ T cells with bispecific T cell engagers have shown great promise in preclinical cancer models [372-374]. To test whether γδ T cells engineered to secrete a CD19 bispecific T cell engager would enhance cytotoxicity toward $CD19^+$ tumors, we first developed an mRNA construct using the scFv portion of the CD19 CAR and linked it to an scFv specific to CD3 (Supplemental Figure 3-1). sBite secreted by γδ T cells electroporated with 3-15μg of CD19 sBite mRNA was measured by ELISA (Figure 3-4A). γδ T cells secrete 15 ng/mL of the sBite with as little as 3μg mRNA and reached 80 ng/mL when using our standard 15μg of mRNA. Western blot analysis of media conditioned by sBite mRNA transfected γδ T cells indicated the sBite was of the expected molecular weight and was detected after as little as 4hrs of culture (Supplemental Figure 3-4). To test whether engineering γδ T cells with CD19 sBite mRNA increases their cytotoxic capability, unmodified and sBite-modified γδ T cells were cocultured with several CD19 positive B-ALL and lymphoma cell lines in a cytotoxicity assay. As expected, the unmodified γδ T cells had a modest increase in cytotoxicity with increasing E:T ratios. In contrast, CD19 sBite-modified γδ T cells exhibited increased cytotoxicity for all cell lines and every E:T ratio (Figure 3-4B). Next, we tested the specificity of the CD19 sBite using the 697 cell line and a CRISPR-generated CD19KO 697 cell line. The CD19 sBite-secreting γδ T cells showed greater cytotoxicity toward $CD19^+$ 697 cells compared to unmodified γδ T cells, a difference not observed with CD19KO 697 target cells (Figure 3-4C). Therefore, the CD19 sBite secreted by the γδ T cells enhanced γδ T cell anti-tumor efficacy in a CD19-specific manner.

One of the major advantages of engineering γδ T cells with sBites rather than CARs is that sBites can bind to and activate unmodified T cells. To test this concept, conditioned media was collected from unmodified and CD19 sBite-modified γδ T cells approximately 16 hours after mRNA electroporation. The conditioned media was then mixed with unmodified or sBite-modified γδ T cells and cocultured with 697 cells (Figure 3-4D). As expected, the CD19 sBite-modified cells exhibited increased cytotoxicity regardless of the conditioned media. Notably, mixing sBite-conditioned media with unmodified cells improved their cytotoxicity compared to mixing unmodified cells with unmodified conditioned media. These results

indicate that γδ T cells electroporated with CD19 sBite mRNA secrete CD19 sBite that enhances the cytotoxicity of modified as well as unmodified γδ T cells.

Figure 3-4: γδ T cells express and secrete CD19 sBite after mRNA electroporation

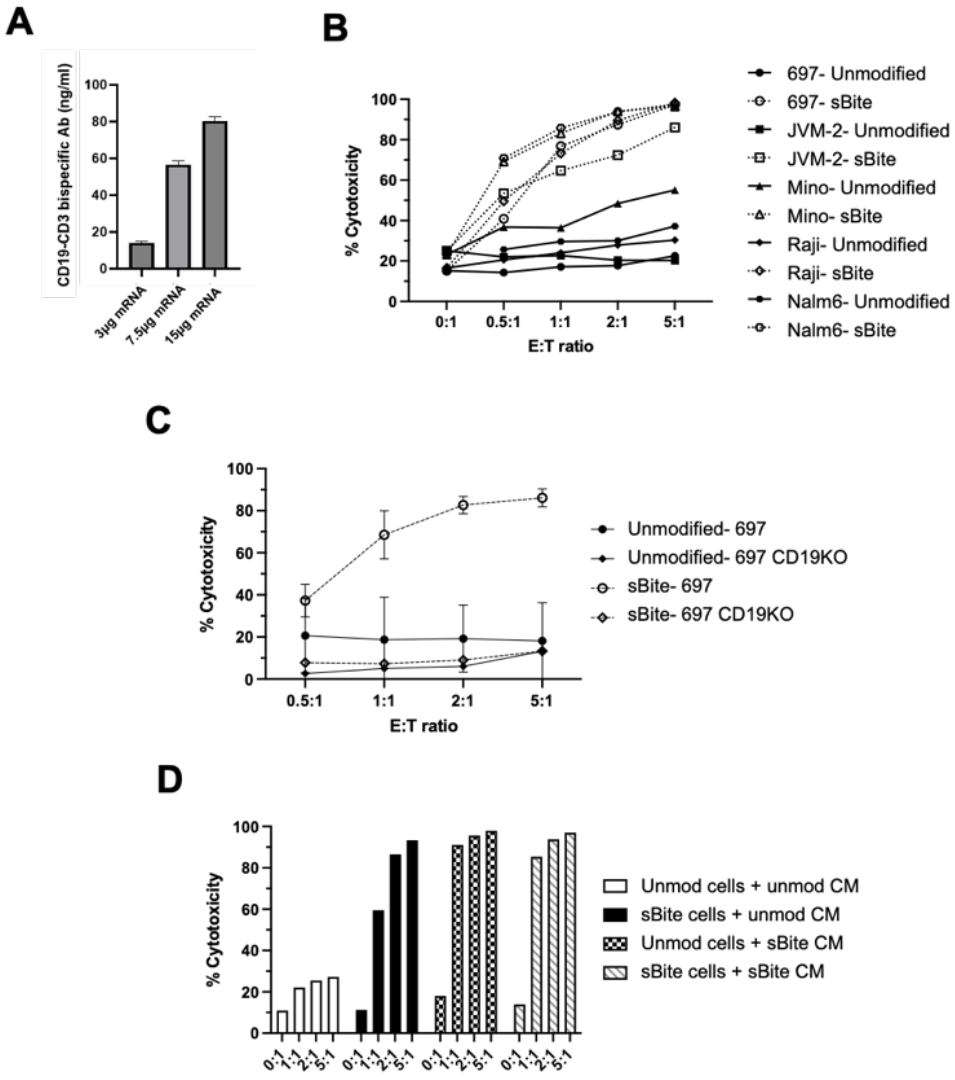


Figure Legend 3-4: γδ T cells express and secrete CD19 sBite after mRNA electroporation. (A) γδ T cells were electroporated with 3μg, 7.5μg, and 15μg of mRNA and the amount of CD19 sBite in the conditioned media was determined using an ELISA. (B) Unmodified and CD19 sBite-modified γδ T cells were cocultured with several CD19+ cancer cells lines to test their cytotoxic capabilities. sBite-modified γδ T cells showed increased cytotoxicity at all E:T ratios and reached about 90% at the 5:1 ratio. (C) A CD19KO 697 cell line was generated using CRISPR to test the specificity of the secreted CD19 sBite. As expected, the sBite-modified γδ T cells showed improved cytotoxicity against the naïve 697 cell line, however no increase in cytotoxicity was seen when the CD19KO 697 cell line was used as target cells. (D) To test whether the CD19 sBite can induce killing of unmodified cells, conditioned media from unmodified and sBite-modified γδ T cells was collected after 16 hours of culture and mixed with either unmodified or sBite-modified γδ T cells. As expected, the sBite-modified cells showed improved cytotoxicity, regardless of the conditioned media. The unmodified cells cultured with the sBite conditioned media showed improved cytotoxicity, compared to unmodified cells with unmodified conditioned media.

In vivo trafficking and growth of 697 cells

The 697 cell line provides a reasonable model for *in vivo* testing of CD19-based CARs and sBites, as CD19 expression is high (data not shown). Although 697 cells expand robustly in NSG mice, we show they i) rapidly leave the blood stream after infusion, ii) home to the bone marrow, and iii) form avascular tumor nodules, especially in the liver (Figure 3-5A). Tissues were collected from NSG mice 3 weeks after intravenous injection with 697 cells. Samples from blood, bone marrow, spleen, and liver were analyzed for the presence of cancer cells using flow cytometry and histopathology. There were substantial numbers of $CD45^{+}CD3^{-}$ populations (i.e. 697 cells) in the bone marrow and a low percentage in the spleen and negligible numbers in the blood (Figure 3-5B). In addition, hematoxylin and eosin (H+E) staining of tissues revealed sheets of neoplastic lymphocytes in the brain, liver, lungs, and kidneys, with avascularized nodules found within the liver (Figure 3-5C). Gross pathological examination found miliary patterns with white foci on the liver (Supplemental Figure 3-5A).

In contrast to 697 growth *in vivo*, flow cytometry analysis of samples from mice administered γδ T cells showed limited $CD45^{+}CD3^{+}$ (i.e. γδ T cells) infiltration in the bone marrow, compared to the blood and spleen (Supplemental Figure 3-5B). Taken together, these results show 697 cells form non-vascularized pockets of cancer cells within a wide range of organs. Once seeded in these peripheral compartments, it may be challenging for engineered γδ T cells to penetrate the 697 tumor nodules. In general, i) cellular therapies require vascularized tumors and ii) γδ T cells do not efficiently migrate to the mouse bone marrow, so it would be predicted that the timing of γδ T cell administration is critical [375, 376]. Also, it can be predicted that early treatment could be effective, but treatments administered after seeding would be less successful, as engineered γδ T cells would be unable to control cancer progression once the cancer cells leave the circulation.

Figure 3-5: γδ T cells are not able to kill cancer cells once they extravasate from circulation

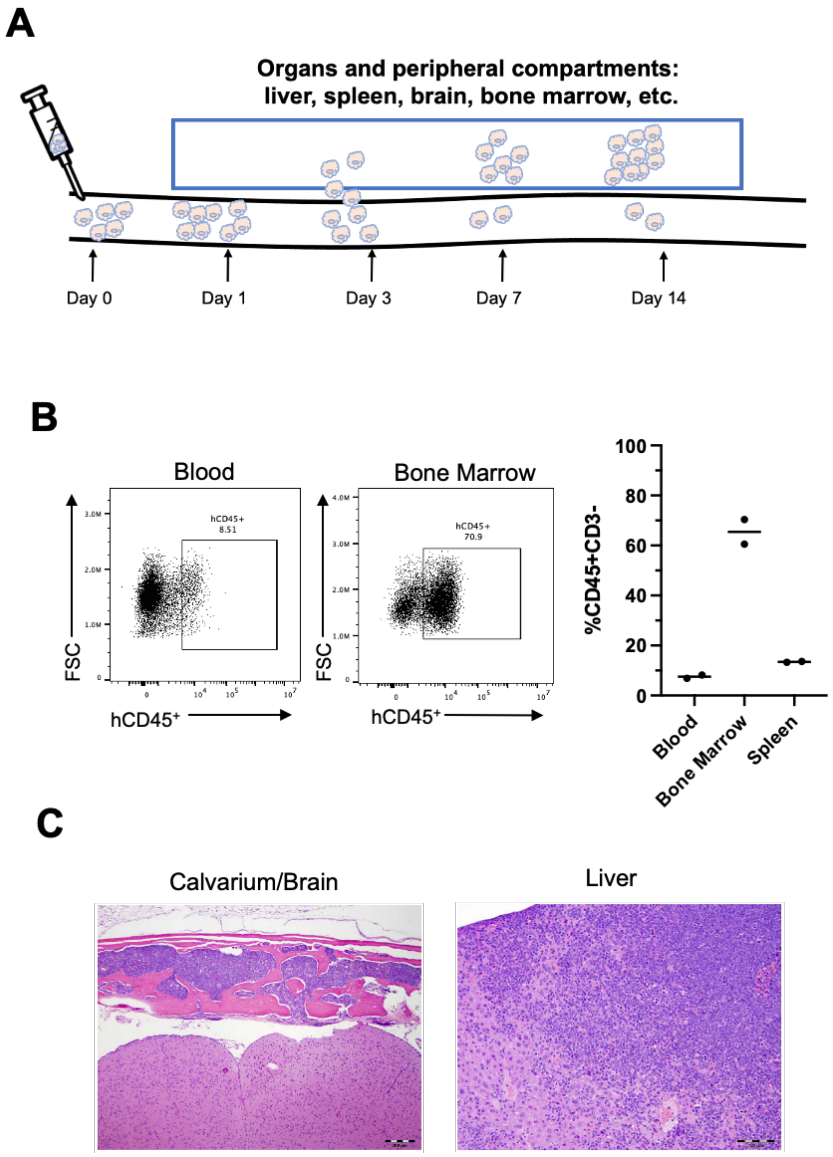


Figure Legend 3-5: (A) In this cancer model, cancer cells are injected i.v. and gradually leave circulation and form non-vascularized nodules in various organs and other compartments, leaving few cancer cells in circulation by day 3 and 7. Based on this model, it can be predicted that the timing for γδ T cell treatment is important in treating mice bearing the 697 cancer cell line. (B) Tissue samples from blood, bone marrow, and spleen were collected 3 weeks after cancer cell injection to detect the presence of cancer cells in each compartment (Left flow plots are representative). A substantial number of $CD45^{+}CD3^{-}$ 697 cells were detected in the bone marrow, while limited numbers were found in the blood and spleen. (C) Representative hematoxylin and eosin (H+E) staining images showing the presence of cancer cells with no vasculature around the cancer cells. Histopathological analysis revealed the presence of cancer cells in the brain, liver, lungs, and kidneys, with avascularized nodules found within the liver.

Engineered γδ T cells reduce tumor burden and improve survival in NSG B-cell leukemia mouse models

To test the efficacy of engineered γδ T cells *in vivo*, the 697 B-ALL mouse model was first used. Luciferase-expressing 697 cells were intravenously injected into the tail vein of NSG mice. The mice were treated twice a week for two weeks with CD19 CAR-expressing γδ T cells starting one day after cancer cell injection. Bioluminescence imaging was performed over the course of the experiment. Treating mice with CD19 CAR-expressing γδ T cells delayed tumor progression and significantly lowered tumor burden, as seen in the bioluminescence images and measured by raw flux values, compared to control mice (Figure 3-6A & 6B). In addition to reducing tumor burden, treating with the CD19 CAR-engineered γδ T cells also improved survival (Figure 3-6C). In contrast, as predicted, treatment of mice 7 days after tumor administration had no effect on overall tumor burden (Supplemental Figure 3-6).

To further test the efficacy of CD19 CAR-engineered γδ T cells and compare CD19 sBite-engineered γδ T cells *in vivo*, a second *in vivo* model was established using luciferase-expressing Nalm6 cells. Mice were treated twice a week for two weeks and started one day after cancer cell injection. Treatments included either unmodified γδ T cells, CD19 CAR-expressing γδ T cells, or CD19 sBite-modified γδ T cells. Prior to administering the engineered cells, CAR expression was about 60%, as determined by flow cytometric detection of CD19Fc binding to γδ T cells (Figure 3-7A). Interestingly, γδ T cells engineered with CD19 sBite mRNA also bound the CD19Fc with about 40% of cells $CD19Fc^{+}$. In addition, the engineered cells were also tested for their cytotoxic capability against the Nalm6 cell line using an *in vitro* cytotoxicity assay at E:T ratios of 1:2 and 2:1, which showed consistent killing with our previous *in vitro* experiments (Figure 3-7B). Mice treated with unmodified γδ T cells exhibited a high tumor burden as early as one to two weeks after cancer cell injection. Mice treated with CD19 CAR- or CD19 sBite-engineered γδ T cells exhibited significantly delayed tumor progression and reduced tumor burden (Figure 3-7C & 7D). A survival benefit was also observed with mice treated with CD19 CAR and CD19 sBite γδ T cells, compared to unmodified γδ T cells (Figure 3-7E). Dual CAR T cells are becoming a promising direction for immunotherapies and have been shown to improve CAR T cell killing and limit acquired resistance as they target two different

antigens on cancer cells. To examine the effectiveness of dual CAR γδ T cells, mice bearing Nalm6 cancer cells were also treated twice a week for two weeks with γδ T cells expressing both the CD19 CAR and CD22 CAR. Compared to the CD19 CAR alone or CD19 sBite, no added benefit was observed by co-expressing CD19/CD22 CARs based on bioluminescence imaging and survival (Supplemental Figure 3-7). The results from these two *in vivo* B-ALL models show engineering γδ T cells with either CD19 CAR or CD19 sBite mRNA effectively delays tumor progression, decreases tumor burden, and improves survival.

Figure 3-6: Engineered γδ T cells expressing a CD19 CAR reduce tumor burden and improve survival in 697 model

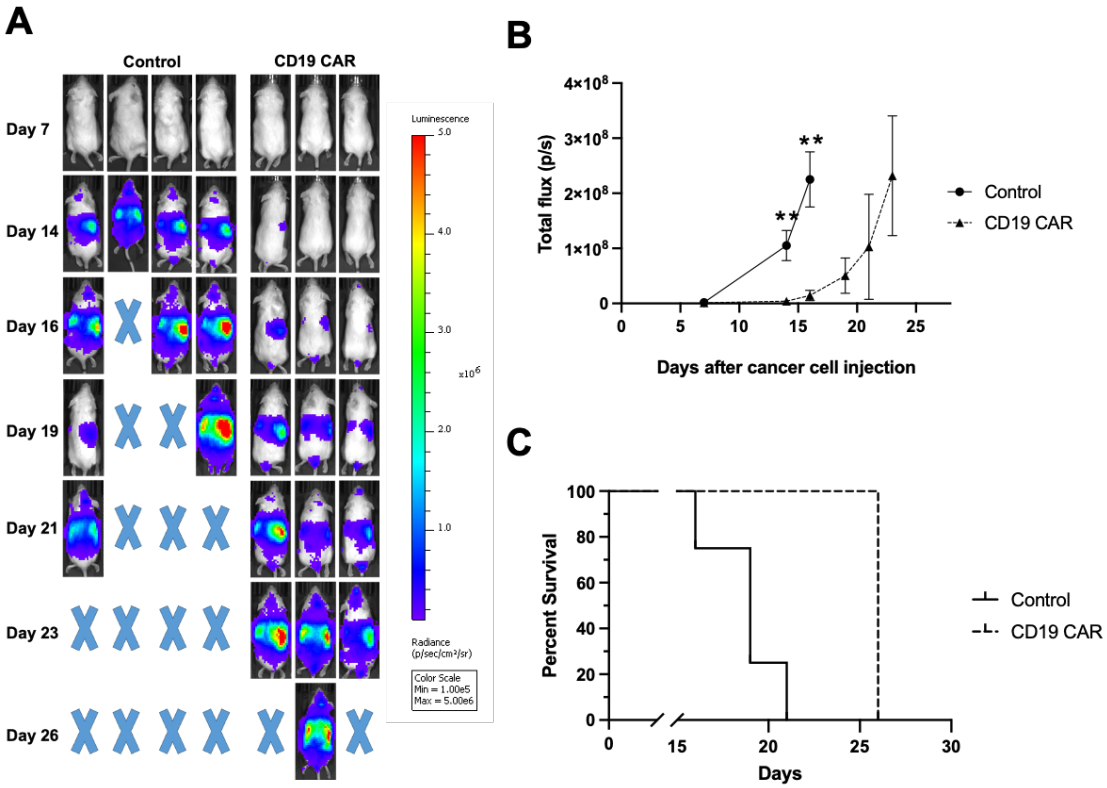


Figure Legend 3-6: (A) NSG mice were injected with 2 x 10^6 luciferase- expressing 697 cells and bioluminescence images were captured during the course of the experiment. Mice treated with CD19 CAR-expressing γδ T cells on day 1 of the experiment showed a reduction in tumor burden compared to control mice. (B) Raw total flux values were calculated and showed delayed tumor progression and significantly reduced tumor burden for mice treated with the CD19 CAR-expressing γδ T cells (triangles), compared to the control mice (circles). Statistics were performed using a 2-tailed Student's t test to compare experimental groups at each given time point. (C) Kaplan-Meier survival curves showed significantly increased survival in mice treated with CD19 CAR-expressing γδ T cells (dashed line), compared to control mice ($p = 0.02$ by log-rank test). Control: n=4; CD19 CAR: n=3; ** = $p<0.01$

Figure 3-7: Engineering γδ T cells with CD19 CAR or sBite mRNA reduce tumor burden and improves survival in Nalm6 model

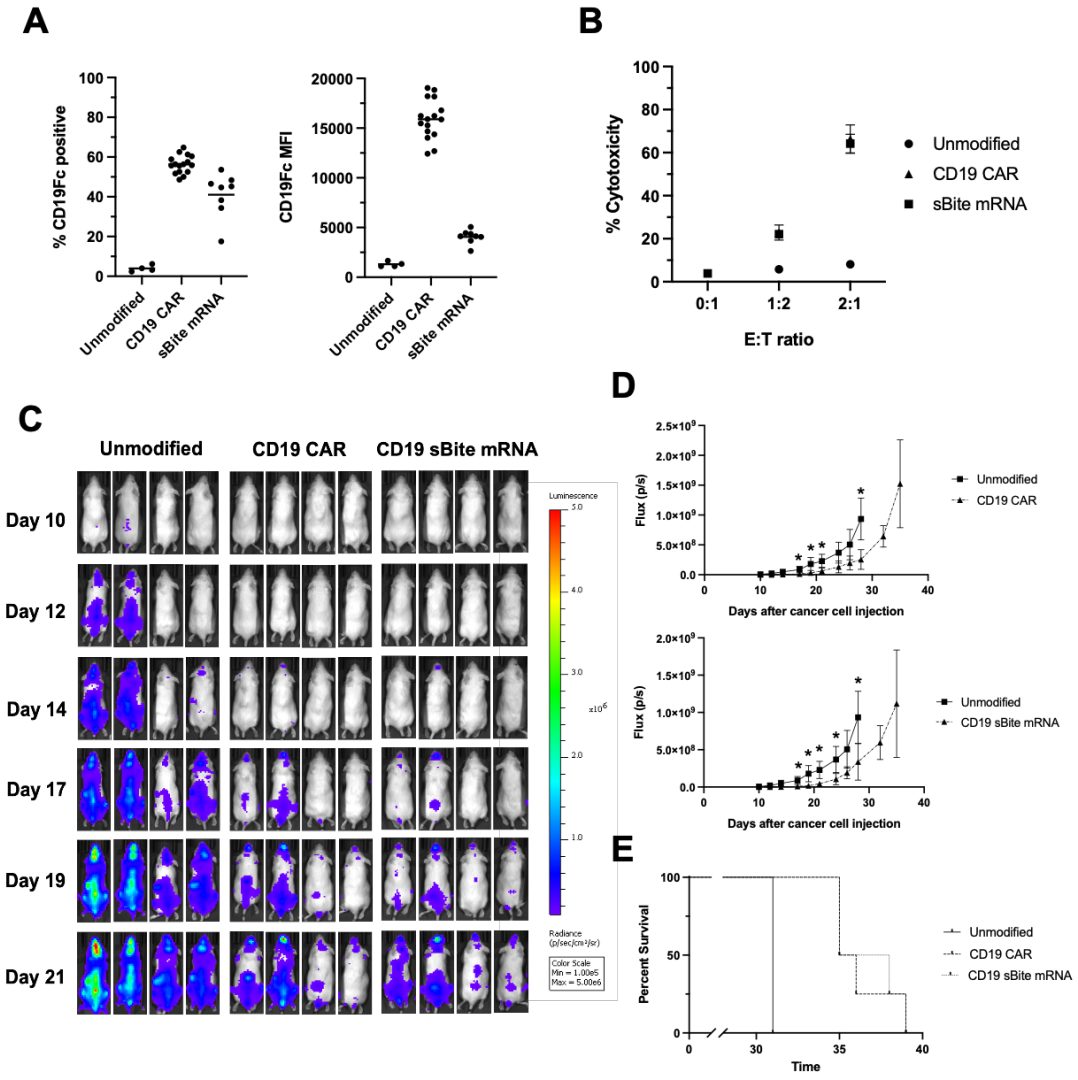


Figure Legend 3-7: NSG mice were injected with 2 x 10^6 luciferase-expressing Nalm6 cells and were treated with either unmodified, CD19 CAR-modified, or CD19 sBite-modified γδ T cells on day 1 of the experiment with a treatment regimen of twice a week for two weeks. (A) Before injection, unmodified or modified γδ T cells were analyzed for CAR expression and MFI using flow cytometry. CAR expression was about 60% for CD19 CAR-expressing γδ T cells and, interestingly, the CD19 sBite-modified γδ T cells bound to the CD19Fc, with an average of about 40% CD19Fc positive (left graph). Despite the CD19 sBite-modified γδ T cell binding to the CD19Fc, the MFI was minimal compared to the CD19 CAR (right graph). (B) The cytotoxicity of the unmodified and modified γδ T cells were also examined before injection at E:T ratios of 1:2 and 2:1. The CD19 CAR- and sBite-modified γδ T cells exhibited increased cytotoxicity compared to the unmodified γδ T cells. (C) Bioluminescent imaging was performed during the experiment and mice treated with unmodified γδ T cells showed a high tumor burden as early as one or two weeks after cancer cell injection. (D) Raw total flux was determined for each image and graphed over time to compare treatment with unmodified γδ T cells and CD19 CAR-expressing (top graph) or CD19 sBite-expressing γδ T cells (bottom graph). Treatment with modified γδ T cells resulted in delayed tumor progression and reduced tumor burden. Statistics were performed using a 2-tailed Student's t test to compare experimental groups at each given time point. (E) Kaplan-Meier survival curves were generated to compare survival for each treatment group to treating with unmodified γδ T cells. Treatment with CD19 CAR- and CD19 sBite-expressing γδ T cells resulted in a significant survival benefit compared to treating with unmodified γδ T cells ($p = 0.01$ for CAR and sBite by log-rank test). n=5; * = $p<0.05$

Increasing the dose and frequency of treatments does not enhance survival

To determine if increasing the frequency and duration of CD19 sBite-engineered γδ T cell administration would further reduce tumor burden and increase the survival benefit, mice were injected with luciferase-expressing Nalm6 cells and treated with 3 doses of CD19 sBite γδ T cells per week for the first two weeks, compared to the previous twice a week for two weeks regimen. In addition, this was followed by two doses per week during week 2 and 3 and finally one dose per week for the final two weeks of treatment (Figure 3-8A). Even with increasing the number of doses in the first two weeks and adding additional doses, the *in vivo* tumor growth was similar compared to the previous Nalm6 experiment (Figure 3-8B & 8C). A slight increase in survival was observed, but the difference was not significant compared to the less aggressive treatment regimen (Figure 3-8D).

Figure 3-8: Longer treatment regimen does not lengthen survival benefit for Nalm6 model

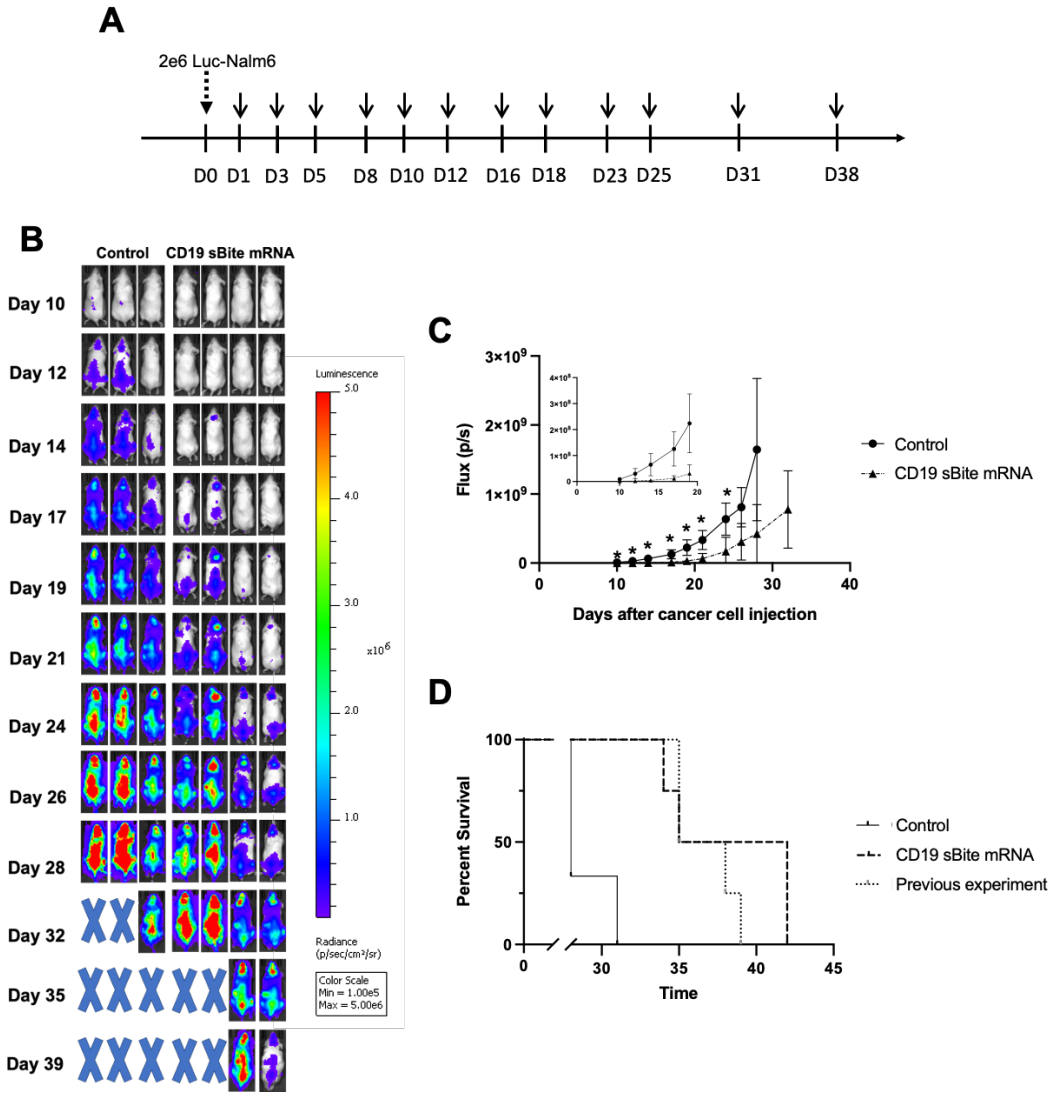


Figure Legend 3-8: Despite being significant, the survival benefit for the previous *in vivo* experiments was not as robust as the *in vitro* data would suggest. (A) To test whether a more extensive treatment regimen of three doses for the first two weeks, two doses for the next two weeks, and one dose for the final two weeks could further improve the survival benefit. (B) NSG mice were injected with 2 x 10^6 luciferase-expressing Nalm6 cells and treated with CD19 sBite-expressing γδ T cells using the more extensive treatment regimen. Bioluminescent images were taken and again showed reduced tumor burden for the sBite-treated group, compared to the control group. (C) Graph of raw total flux shows the more extensive treatment regimen delayed tumor progression and reduced tumor burden, compared to control mice. The inset shows an expansion of the first 20 days of treatment. Statistics were performed using a 2-tailed Student's t test. (D) Kaplan-Meier survival curves were generated for the control group and more extensive treatment regimen of CD19 sBite-expressing γδ T cells. As expected, the more extensive treatment regimen resulted in a significant survival benefit compared to the control group ($p = 0.01$ by log-rank test), however there was no difference in survival when comparing the more extensive treatment regimen to the previous regimen of twice a week for two weeks ($p = 0.39$ by log-rank test). Control: n=3; sBite: n=4; * = $p<0.05$

3.4 Discussion

Developing novel immunotherapies that are effective and safe is a critical step in advancing cancer therapeutics. ACT is among the most promising developments for treating cancer, as these treatment strategies provide the ability to repopulate the patient's immune system with functional and potent anticancer immunocompetent cells. γδ T cells are well-suited for ACT, as they bridge the gap between the innate and adaptive immune system. In fact, based on a large pan-cancer molecular profiling study, γδ T cell infiltration was identified as the best prognostic marker for favorable outcomes [377]. Their ability to detect antigens in an MHC-independent manner gives them advantages over αβ T cells because they are able to be used in allogeneic settings, and they target cancer through endogenous stress markers and phosphoantigen expression that are typically upregulated in cancer cells [378]. In addition to their rapid immune response capabilities, γδ T cells are also involved in recruiting and priming other immune cells that can increase anticancer responses. For these reasons, γδ T cells are a promising ACT platform with great potential to improve cancer therapeutics.

Engineering γδ T cells has traditionally been an important, albeit challenging, step toward developing more effective γδ T cell therapies. Here, we test a therapeutic platform to engineer γδ T cells using mRNA electroporation to improve their cytotoxicity. First, mRNA electroporation of γδ T cells was optimized using a CD19 CAR-GFP construct. Based on the measurement of several parameters, we found rational conditions for modifying γδ T cells and confirmed the engineered cells were functional in cytotoxicity assays against B-ALL cell lines. We then tested whether this platform can be used to engineer γδ T cells to secrete a functional bispecific T cell engager through mRNA electroporation, and indeed showed sBites are secreted and significantly improve γδ T cell cytotoxicity. In addition, sBites can improve the cytotoxicity of unmodified γδ T cells. We then showed, using two B-ALL mouse models, that the engineered γδ T cells are effective at prolonging tumor progression, reducing tumor burden, and improving survival.

mRNA technologies are rapidly improving for a wide range of applications, especially in light of the recent success of covid vaccines, and mRNA electroporation has emerged as a promising option for genetically engineering immune competent cells [379, 380]. The transient nature of electroporation in combination with γδ T cells offers several advantages over stable integrating vectors and may even alleviate some of the limitations of CAR T cell immunotherapies. For example, cytokine release syndrome is a significant obstacle for CAR T cell patients and leads to harmful side effects and early treatment termination. Transient engineering of γδ T cells offers a solution as the transient nature of the modification would limit the risk of the immune system's overactivation [381]. Also, γδ T cells do not form memory responses so their persistence is typically limited to weeks, which means treatment can be halted if treatment-related adverse effects were induced. Another limitation to traditional CAR T cell therapies is the development of acquired resistance through antigen escape. The ability for γδ T cells to detect stressed cells and phosphoantigen expression allows for added cytotoxic capabilities beyond CAR activation. This characteristic of γδ T cells can also be used in the context of combination therapy with chemotherapeutics that have been shown to upregulate some of the stress markers on cancer cells that are detected by γδ T cells [369].

Although this therapeutic platform alleviates many of the side effects and obstacles of traditional CAR T cells, a limitation of the transient engineering technique is the short duration of CAR and sBite expression, which may limit the length of therapeutic efficacy. The duration of expression after mRNA electroporation in hematopoietic cells has been widely studied and has been found to last between 5-7 days, with peak expression occurring after 24-48 hours [187, 191, 209]. Despite being short compared to stable integrating vectors, this is not a problem when using γδ T cells because we have shown that γδ T cell persistence is limited *in vivo* and lasts for about 3 days (data not shown). The short lifespan of expression and limited persistence of γδ T cells would need to be addressed clinically and can be countered by increasing the number of injections.

Currently, the most common engineering platform for immune competent cells is the introduction of complementary DNA to express CARs. However, treatment and combination therapy utilizing bispecific T cell engagers is effective for several cancer indications [382]. They use the specificity of the scFv portion of an antibody to bridge T cells and cancer cells by binding to the CD3ς fragment of T cells and an antigen on cancer cells. This binding activates T cells, increases cytotoxicity, and induces immunocompetent cellular proliferation. There have been some studies investigating the combination of γδ T cells and bispecific T cell engagers, which have shown improvement in cytotoxicity against several types of cancer [372-374, 383, 384]. This study tested a novel technique of expressing sBites in this immune competent cell, instead of co-administration. Having the cells secrete the sBites offers several advantages over co-infusing the cells with recombinant bispecific T cell engager protein. An obstacle for these infused therapies, and other non-IgG-like bispecific antibodies, is their short half-life and need for multiple (indeed sometime continuous) infusions. Engineering γδ T cells using electroporation with sBite mRNA allows for continuous expression as long as the mRNA is within the cells. In addition, having the immune competent cells secrete the sBite allows for a more targeted treatment approach because, in contrast to systemic administration, the sBite is secreted locally where it can be most utilized by immune cells. This concept can be coupled with modulation of chemokine receptor expression on the T cells. For example, modifications to overexpress chemokine receptors on T cells can enhance their migration to the site of the cancer [385-387]. This can be done in a targeted approach as certain cancers are known to express certain chemokines and receptors.

Despite showing signs of success, a limitation of this platform is induction of survival benefits without complete cures. Although we think this is specific to the *in vivo* models, we did thoroughly pursue treatment timing strategies, for example, long durations of treatment and aggressive upfront regimens were tested but provided little improvement. For example, sBite-modified γδ T cells performed similarly in the protracted or extended regimens, showing that increased treatments over longer periods did not improve survival. A priori, this was predicted as the cancer cells quickly leave the circulation and seed in compartments that are

not easily reached by the γδ T cells. For example, γδ T cells are most abundant in the blood followed by the spleen and bone marrow. In contrast, our cancer cell lines are most abundant in the bone marrow with very few in the blood. To investigate this hypothesis, tissue samples from mice bearing 697 cancer were collected and analyzed. We found increased presence of cancer cells in the bone marrow and spleen, compared to the blood. In addition, based on a histopathological examination, neoplastic sheets of lymphocytes were found in many organs including the brain, liver, lungs, and kidneys. No presence of vasculature was found surrounding the cancer cells, which suggests the γδ T cells do not efficiently access these sites. As mentioned above, the expression of specific chemokine receptors on γδ T cells can be used to improve the migratory pathways of the cells *in vivo*. For example, this concept can be utilized to express CXCR4 to enhance the migration of γδ T cells to the bone marrow.

This study investigated the potential of mRNA electroporation as a therapeutic platform to engineer γδ T cells with either CARs or sBites. We utilized CD19 as the target antigen, the most commonly studied target for immunotherapy. However, it can be anticipated this therapeutic platform can be applied to many cancers and may be especially beneficial to those where long-term CAR T persistence is detrimental, such as targeting antigens that are not cancer specific. Overall, these results show γδ T cells can be modified with CAR or sBite mRNA through electroporation and the engineered γδ T cells have improved cytotoxicity against cancer, both *in vitro* and *in vivo*.

3.5 Materials and Methods

Cell lines

The luciferase-expressing 697 cell line was kindly provided by the laboratory of Dr. Douglas Graham (Emory University) and the luciferase-expressing Nalm6 cell line was kindly provided by the laboratory of Dr. Christopher Porter (Emory University). The CD19 knockout 697 cell line was developed at Expression Therapeutics, Inc using CRISPR that is directed by a CD19-directed guide RNA. All cell lines were cultured

in RPMI (Corning) with 10% heat inactivated fetal bovine serum and 1% penicillin/streptomycin at 37°C in a 5% CO2 incubator.

γδ T cell expansion

γδ T cell expansions were performed based on our previously published technique [287, 343]. Briefly, peripheral blood mononuclear cells (PBMCs) were obtained from healthy donor blood through the Children's Clinical Translational Discovery Core at Emory University under the cores approved IRB protocol or ordered directly from AllCells. PBMCs were isolated from fresh blood using Ficoll-Paque Plus density centrifugation. To preferentially expand γδ T cells, PBMCs were cultured in OpTmizer containing OpTmizer supplement, 1% penicillin/streptomycin, and 2 mM L-glutamine (complete OpTmizer). Cells were counted and resuspended at 2 x 10^6 cells/mL in fresh media every 3 days. On day 0 and 3 of expansion, 5μM Zoledronate and 500IU/mL IL-2 were added to the media. On day 6 and 9, 1000IU/mL IL-2 was added to the media. In addition, on day 6 of expansion, an αβ depletion step was performed as previously published [283]. On day 12 of expansion, γδ T cells were either used fresh for experiments or frozen in PBS containing 5% human serum albumin (HSA) and 10% DMSO. Flow cytometry was performed on day 0, 6, 9, and 12 to confirm successful expansion and αβ depletion. Successful expansions resulted in cultures containing about 90% γδ T cells and 10% NK cells (Figure S8).

Construction of mRNA expression vectors

To construct the mRNA expression vectors, plasmid DNA constructs were first cloned with the T7 promoter. The CD19 CAR and CD22 CAR consisted of the variable heavy and variable light regions of the FMC63 and M971-L7 antibodies, respectively. In addition to the scFv portion, the CAR constructs included a CD8 hinge, a CD28 costimulatory and transmembrane domain, and a CD3ς signaling domain. The CD19 sBite plasmid consisted of the scFv portion of the FMC63 antibody for the CD19-specific region and the OKT3 for the CD3-specific region. Codon optimization was performed as previously published

[388]. For mRNA production, DNA plasmids were first linearized, and the mRNA was prepared using the mMessage mMachine T7 Ultra Kit (Life Technologies).

Electroporation

γδ T cells were either electroporated fresh on day 12 of expansion or from thawed cells that were frozen on day 12 of expansion. Cells were thawed in 5% HSA in PBS and were centrifuged at 250g for 10 minutes at room temperature. The cells were cultured at 4 x 10^6/mL for 2 hours in complete OpTmizer media with 1000IU/mL IL-2. Cells were then counted and the appropriate cell number for each reaction was aliquoted, washed twice with PBS, and resuspended in 100uL OptiMEM (Life Technologies). The appropriate amount of mRNA was added to the tube and the mix was transferred to a 4mm cuvette (Fisher Scientific). Electroporations using the BioRad's Gene Pulser Xcell Electroporator were conducted at 500V for 5ms using a square wave. Cells were collected from the cuvette and cultured overnight at 2 x 10^6/mL in complete OpTmizer media with 1000IU/mL IL-2. Flow cytometry was used to confirm and analyze CAR expression after electroporation by labeling cells with a CD19-Fc fusion protein (AcroBiosystems) and an anti-IgG Fc secondary antibody (Jackson Immunoresearch Laboratories).

Cytotoxicity assay

A flow cytometry-based cytotoxicity assay was used to determine the cytotoxic capabilities of effector cells. Target cells were first stained with Violet Proliferation Dye 450 (BD Biosciences), in order to differentiate target cells from effector cells. Effector cells and target cells were cocultured at the specified effector to target ratios for four hours at 37°C in 5% CO2. The cells were then washed and resuspended in Annexin binding buffer (BioLegend) containing the early apoptosis stain Annexin V-APC (BioLegend). Right before analysis, 7AAD (BD Biosciences), a late apoptosis and necrosis marker, was added to differentiate live and dead cells. Cytotoxicity was calculated by adding the 7AAD and Annexin V single positive with the double positive population of target cells.

ELISA and western blot

To measure and detect the presence of the CD19 sBite in culture media, an ELISA and western blot was performed. First, γδ T cells were electroporated and cultured overnight (~16hrs) and the conditioned media was collected. To perform the ELISA, streptavidin-coated plates (Fisher) were coated with biotinylated human CD3ε and CD3δ heterodimer protein with His/Avitag (Acro Biosystems). Conditioned media samples were then added to the plate, with an anti-CD19-anti-CD3 bispecific antibody (BPS Biosciences) used as a standard. Next, a CD19Fc fusion protein (R&D Systems) was added to the plates, followed by horseradish peroxidase (HRP) anti-human Fc (Jackson Labs). Finally, 3,3',5,5'-Tetramethylbenzidine (TMB) substrate solution (Fisher) was added to the plate and the absorbance was measured at 450nm.

To perform the western blot, γδ T cell conditioned media and anti-CD19-anti-CD3 bispecific antibody (BPS Biosciences) standards were prepared under reducing conditions. Next, separation by SDS-PAGE and transfer to a nitrocellulose membrane was performed. The blocked membrane was incubated with an anti-His antibody (R&D Research), followed by an HRP goat anti-mouse IgG secondary antibody (Abcam).

In vivo B-ALL models

All animal studies were conducted in accordance with IACUC regulations. Eight-week-old NOD.Cg-Prkdc^{scid}IL2rg^{tm1Wjl}/SzJ (NSG) mice were purchased from Jackson Laboratory and housed in a pathogen-free facility. To establish the B-cell leukemia models, 2 x 10^6 luciferase-expressing 697 or Nalm6 cells were intravenously (i.v.) injected through the tail vein. Treatments with unmodified or engineered γδ T cells started one day after cancer cell inoculation and followed one of two treatment regimens, twice a week for two weeks or a more extensive treatment regimen as seen in Fig 7A. Each dose included 1 x 10^7 unmodified or engineered γδ T cells. Frozen γδ T cells were thawed and prepared for electroporation as described above. After electroporation, cells were cultured for 2.5 hours in complete OpTmizer with 1000IU/mL IL-2. The cells were then washed twice with PBS, resuspended in fresh PBS at 1 x 10^7

cells/100uL, and administered intravenous. For *in vivo* imaging, mice were anesthetized with 2% inhaled isoflurane and bioluminescence images were taken with the IVIS Spectrum imaging system (PerkinElmer).

Tissue collection and analysis

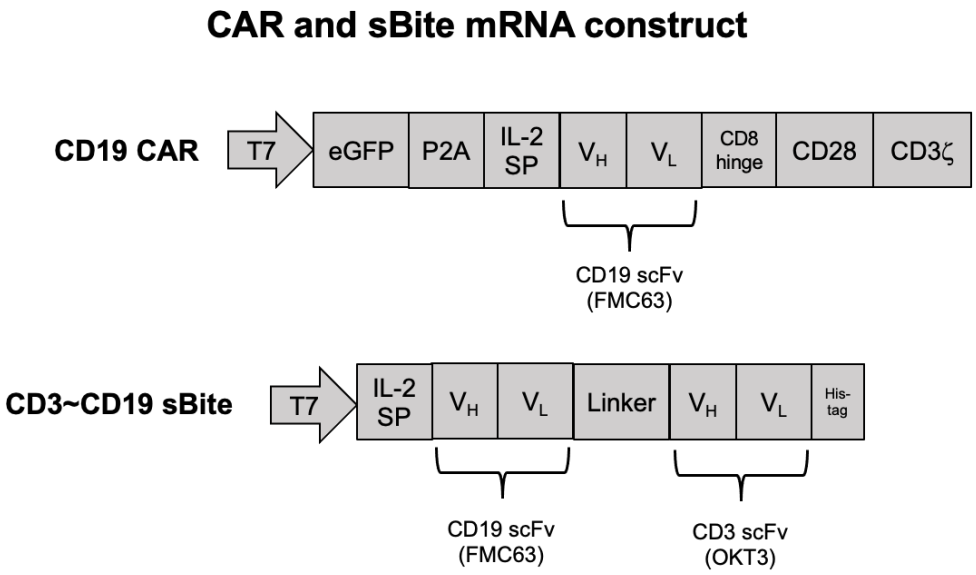
Mouse tissue collection was performed at endpoint of the specified experiment. Mouse blood was collected via submandibular or retro-orbital veins in tubes containing 0.5M EDTA. Samples were centrifuged at 2400g for 15 minutes at 4°C. The plasma layer was discarded, the pellet resuspended in 100uL PBS, and three RBC lysis steps were performed. RBS lysis was conducted by adding 3mL of RBC lysis buffer. The samples were then vortexed and incubated at room temperature for 10 minutes. The samples were centrifuged at 300g for 10 minutes and the supernatant was discarded. Samples were resuspended in 100uL PBS and were stained for flow cytometry. Mouse spleens were processed by first pressing the tissue through a 40μm mesh. The samples were then centrifuged at 300g for 10 minutes at 4°C. One RBS lysis step was performed as described above. Mouse livers were processed as previously published [389]. Briefly, livers were collected and placed in dishes containing PBS. The livers were pressed through a 70μm mesh and then centrifuged at 30g for 3 minutes. The supernatant was collected and centrifuged again at 320g for 5 minutes. The cells were resuspended in 33% Percoll in PBS and centrifuged at 500g for 15 minutes with the break off. The cell pellet was resuspended in RBC lysis buffer and incubated at room temperature for 5 minutes. The samples were centrifuged at 300g for 10 minutes and resuspended in PBS.

Statistical Analysis

All statistics were performed on GraphPad Prism 9. Unpaired two-tailed Student T tests were used for statistical significance. A log rank (Mantel-Cox) test was performed on the Kaplan-Meier survival curves to determine significance between curves. Sample size is shown on graphs as individual data points or specified for that experiment. Error bars represent standard deviation and statistical significance was defined as $p<0.05$, unless otherwise stated.

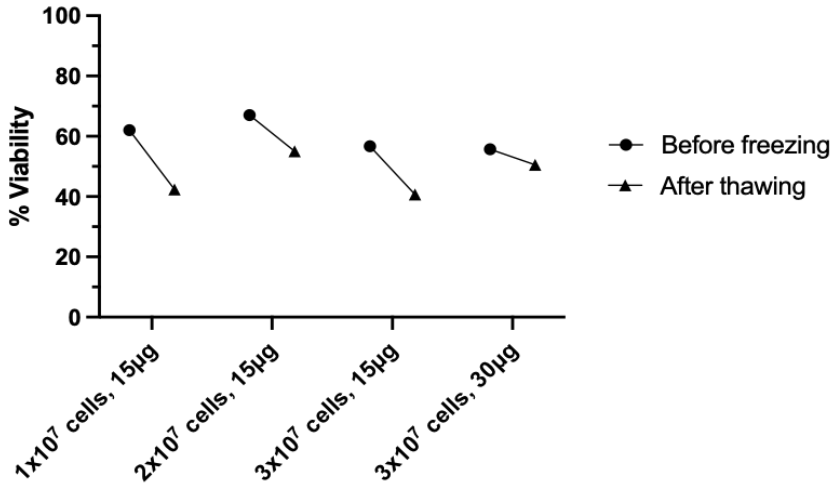
3.6 Supplemental Figures, Tables, and Legends

Supplemental Figure 3-1: Schematic of CD19 CAR and CD19 sBite mRNA constructs



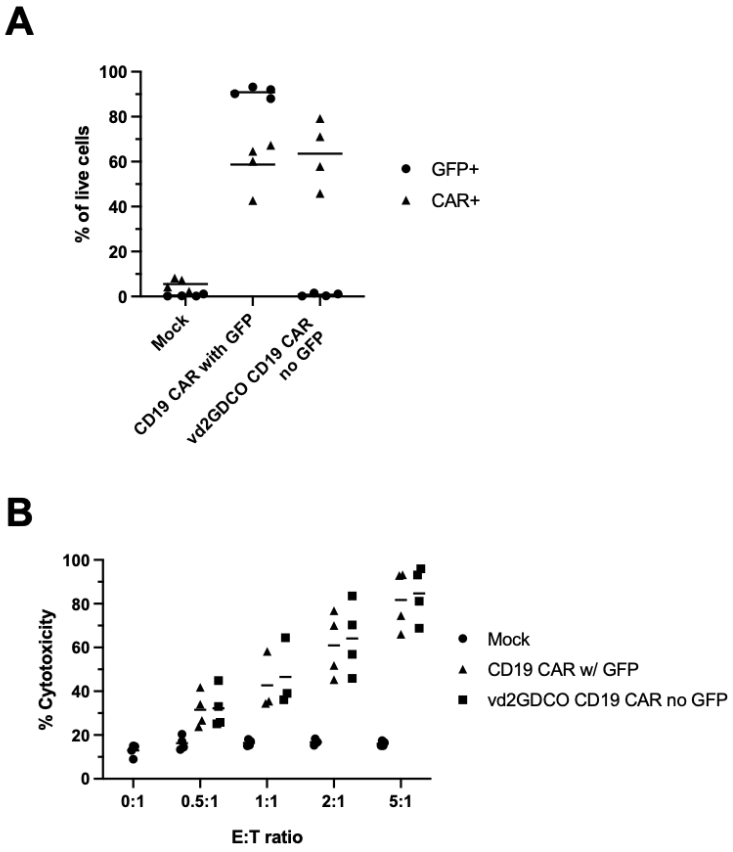
Supplemental Figure Legend 3-1: The bicistronic CD19 CAR-GFP construct (top) was used for the optimization of γδ T cell electroporation. The CD19 CAR contains the scFv portion of the FMC63 antibody, a CD8 hinge region, a CD28 transmembrane and costimulatory domains, and a CD3ς activation domain. The CD19 sBite construct (bottom) consists of the scFv portion of the FMC63 antibody for the CD19-specific region and the OKT3 for the CD3-specific region.

Supplemental Figure 3-2: Freezing engineered cells reduces cell viability



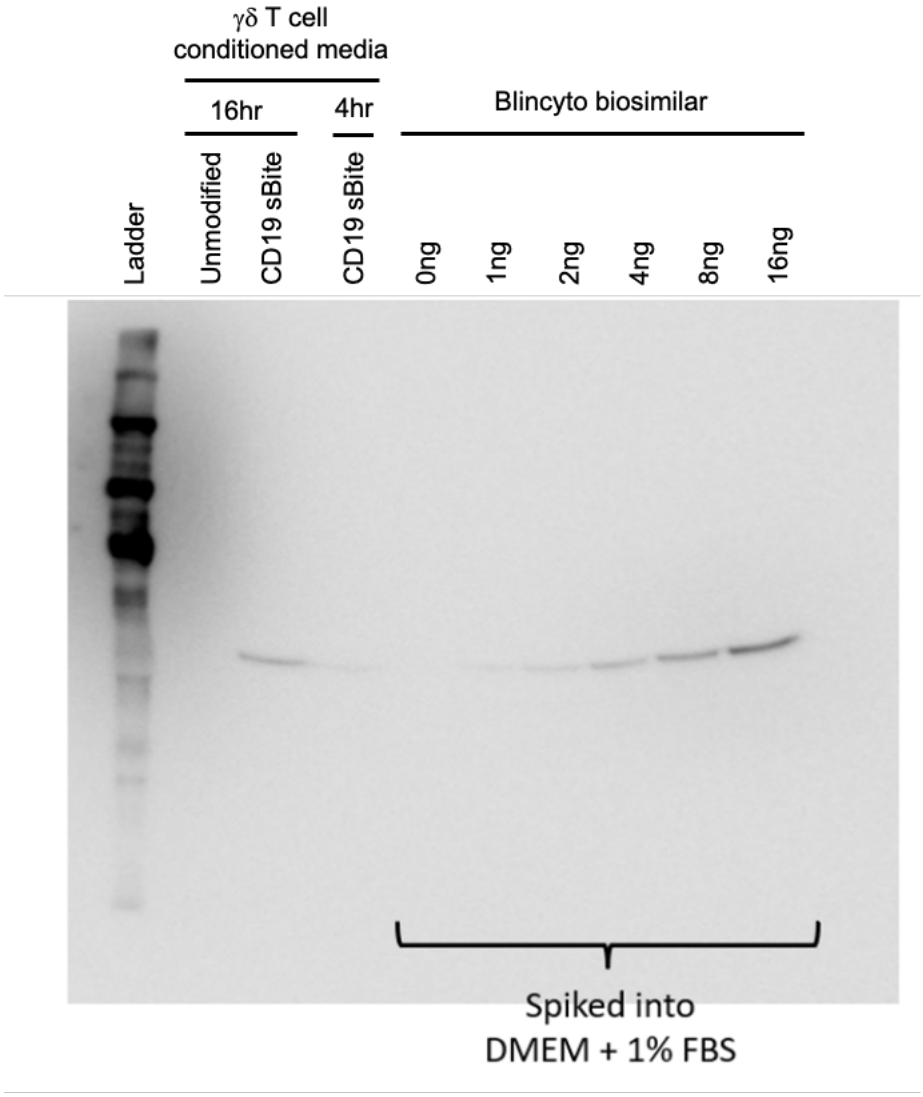
Supplemental Figure Legend 3-2: To determine the effect of freezing engineered γδ T cells, electroporated γδ T cells were analyzed before and after one freeze/thaw cycle. The percent viability was determined by 7AAD staining using flow cytometry. Before freezing, the engineered γδ T cells were about 60% viable for all reaction conditions, however the viability dropped to about 40-45% after thawing. This suggests freezing engineered cells reduces cell viability upon thawing the cells.

Supplemental Figure 3-3: Comparison of newly cloned codon optimized CD19 CAR construct



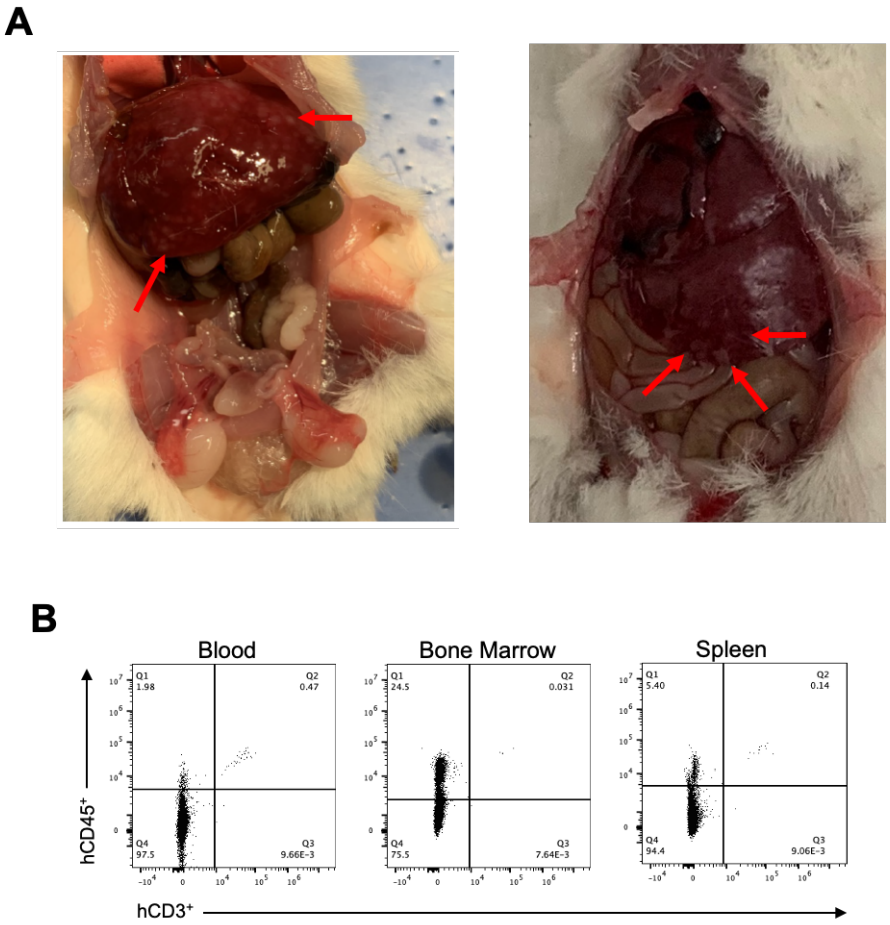
Supplemental Figure Legend 3-3: Once optimization of electroporation was complete, a new CD19 CAR construct was developed that did not contain GFP and was codon optimized for expression in γδ T cells. (A) GFP and CAR expression was determined by flow cytometry to compare the new construct with the bicistronic CD19 CAR-GFP construct. As expected, GFP expression was about 90% for the old construct, with no GFP expression in the new construct. The two constructs resulted in similar CAR expression and averaged around 60%. (B) To test whether there were functional differences between the two constructs, a cytotoxicity assay was performed against 697 cells. Both constructs resulted in improved cytotoxicity compared to mock-electroporated γδ T cells and had similar cytotoxicity when comparing the two constructs at all E:T ratios.

Supplemental Figure 3-4: Western blot analysis of CD19 sBite in γδ T cell conditioned media



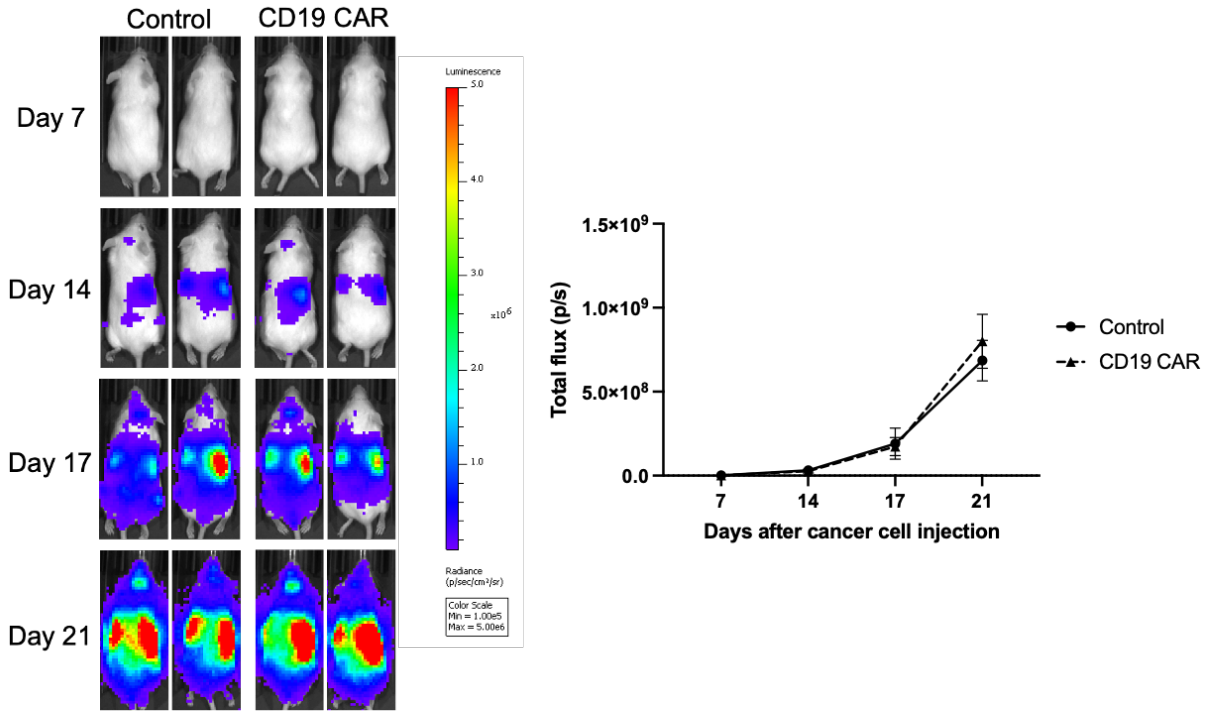
Supplemental Figure Legend 3-4: CD19 sBite-modified γδ T cells were cultured for 4 and 16 hours and the media was collected and compared to a Blincyto biosimilar standard. Bands were visible for the conditioned media at both time points, further showing that γδ T cells are able to express and secrete a CD19 sBite.

Supplemental Figure 3-5: Migration patterns of cancer cells and γδ T cells *in vivo*



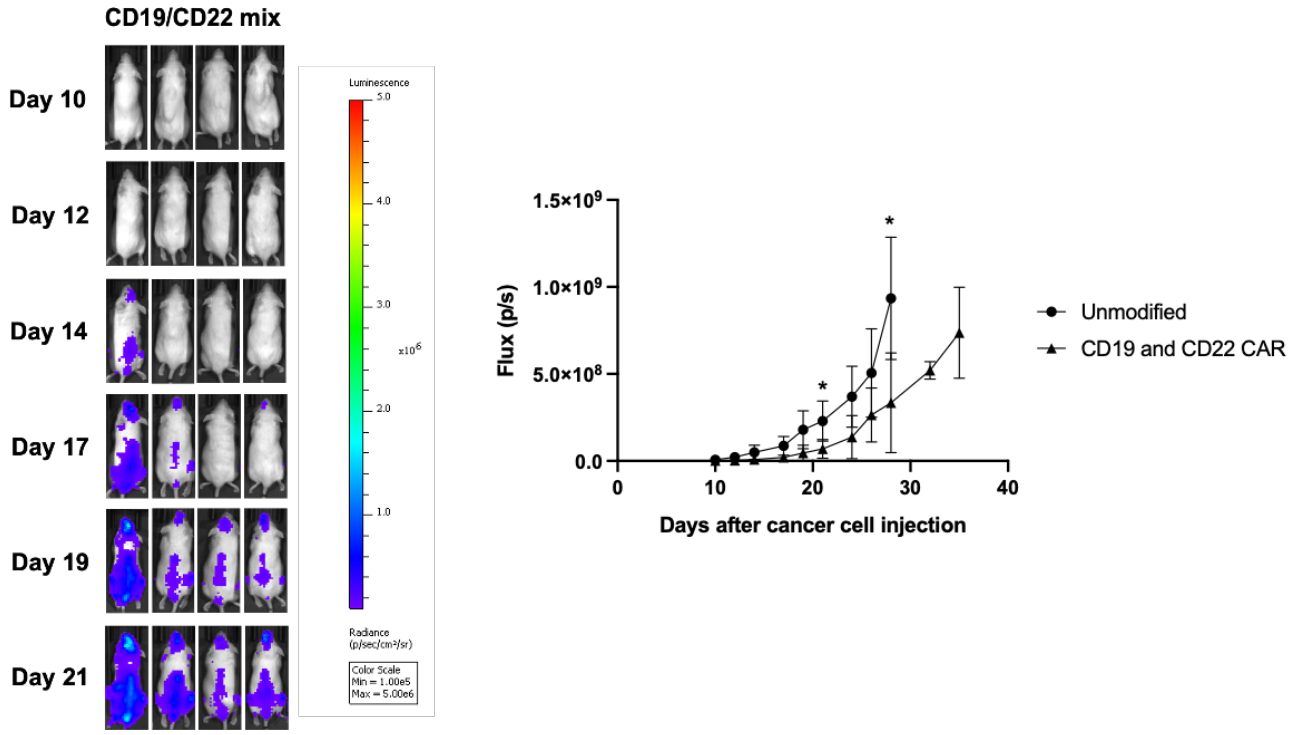
Supplemental Figure Legend 3-5: (A) Gross pathological examination found miliary patterns with white foci (red arrows) within the liver. (B) Mouse tissues were collected from an NSG mouse injected with 2 x 10^6 luciferase-expressing 697 cells and γδ T cells to detect the presence of γδ T cell in different organs. The $CD3^+CD45^+$ γδ T cell (upper right panel in flow plots) were found in the blood and spleen, however limited numbers were found in the bone marrow, suggesting γδ T cells do not efficiently migrate to mouse bone marrow. Taken together, this data shows that γδ T cells do not effectively travel to peripheral compartments where 697 cells form non-vascularized nodules of cancer cells.

Supplemental Figure 3-6: Treatment starting on day 7 is unsuccessful in improving tumor burden



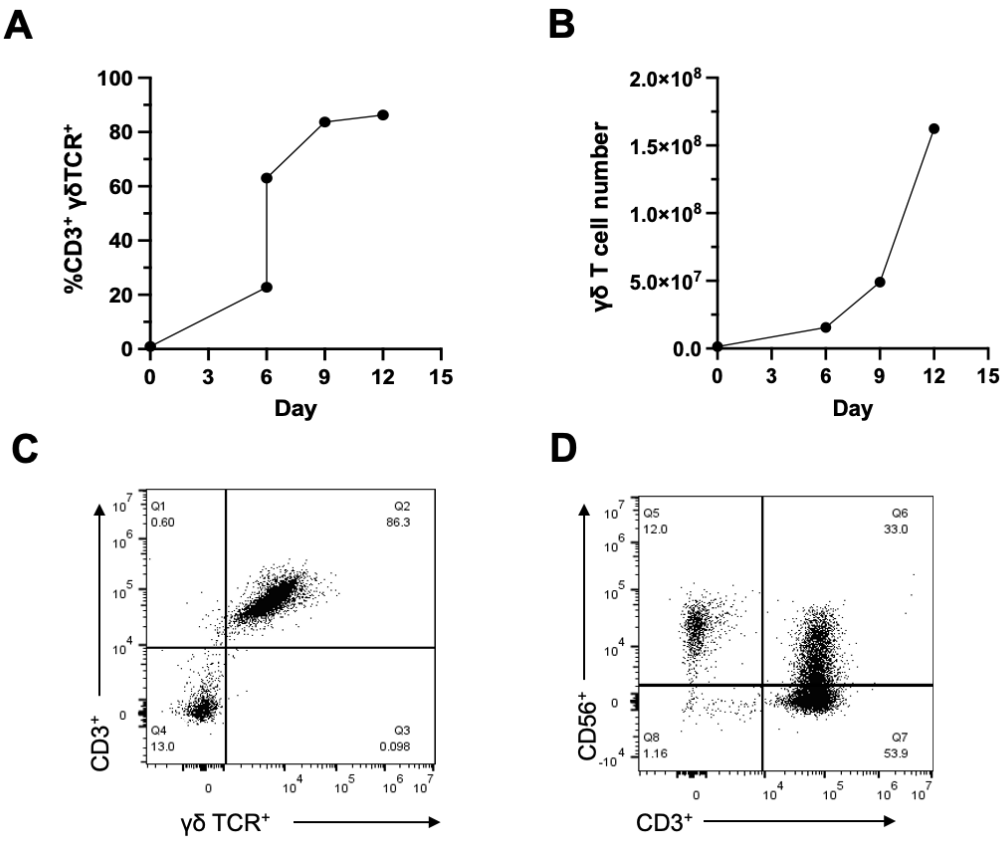
Supplemental Figure Legend 3-6: NSG mice bearing 697 cancer cells were treating with CD19 CAR-expressing γδ T cells on day 7 after cancer cell injection. Based on bioluminescent imaging, mice treated with the engineered γδ T cells had similar tumor burdens throughout the course of the experiment. This suggests that the timing of treatment plays a pivotal role in the success of treatment for this *in vivo* 697 cancer model.

Supplemental Figure 3-7: Co-expressing CD19/CD22 CARs did not further improve efficacy



Supplemental Figure Legend 3-7: NSG mice bearing Nalm6 cancer cells were treated with γδ T cells expressing both CD19 and CD22 CARs. Bioluminescent images (left) and the raw total flux values (right) revealed a slight advantage compared to treating the mice with unmodified γδ T cells, however this advantage was minimal and was less effective at reducing tumor burden compared to the CD19 CAR alone and the CD19 sBite. Statistics were performed using a 2-tailed Student's t test. * = $p<0.05$

Supplemental Figure 3-8: Representative data from characterization of γδ T cell expansion



Supplemental Figure Legend 3-8: (A) The $CD3^{+}$γδ TCR^{+} percentage of the culture was determined by flow cytometry during the course of the expansion. Analysis was performed on day 0, pre- and post- αβ depletion on day 6, day 9, and day 12. (B) The γδ T cell number was also calculated on each day of analysis and showed the expansion results in large numbers of γδ T cells on day 12. (C) Flow plot of day 12 analysis showing the $CD3^{+}$ lymphocytes are predominantly γδ T cells. (D) On day 12 of expansion, the majority of $CD3^{-}$ cells within the culture are CD56+ NK cells, with over a third of the γδ T cells also expressing CD56.

Chapter 4

General Discussion

4.1 Summary of Results

Traditionally, γδ T cells have been a difficult cell type to engineer with variable and limited gene transfer efficiencies. For example, our lab's initial experience with lentiviral transduction of γδ T cells only resulted in about 30% transduction efficiency [287]. These findings are consistent with other groups that have found similar transduction efficiency and have noted that the limited gene transfer of primary cells, especially γδ T cells, may be caused by their increased sensitivity to cell death and limited proliferative capacity [288]. Gammaretroviral vectors have also been tested to engineer γδ T cells, however, similar to lentiviral vectors, limited and variable transgene expression was observed [257]. Therefore, we sought to investigate the use of two promising transient engineering techniques, AAV and electroporation, as a means to engineer γδ T cells to improve their cytotoxic potential to fight cancer.

Chapter 2 highlights our efforts to test whether recombinant AAV6 exhibits increased gene transfer capabilities for γδ T cells, as it has emerged as a promising viral vector for the transduction of αβ T cells [313]. We first developed a protocol using the three-plasmid technique for generating our own recombinant AAV in-house. HEK293T cells were transfected with plasmids encoding the transgene of interest, the Rep/Cap proteins, and genes that are necessary for the assembly of the viral particles (pHelper). After collection of the viral particles from the media and lysed cells, a purification and concentration step is performed via iodixanol density gradient ultracentrifugation. The purification step is essential to not only improve the safety of the viral vector, but can also enhance transduction efficiencies [390]. Many different protein-based contaminants have been found in the batches of AAV. For example, Galectin 3-binding protein was discovered in AAV6 preparations and was found to bind to the AAV6 particles, which can affect vector transduction efficiency and can induce an immune response, reducing the safety profile of the vector [391]. Therefore, we analyzed the purification step of our manufacturing protocol and evaluated the addition of a second ultracentrifugation step on the purity and viral yield. Based on SDS page and electron microscopy, we found that the addition of the second ultracentrifugation step greatly reduced the amount of contaminants and resulted in a purer final product. Furthermore, we show that the added step improves

the quality of the AAV batch by increasing the full-to-empty AAV particle ratio. It has been shown that empty viral particles limit transduction efficiency and increase immune responses and safety risks when administered *in vivo*, and it can be expected that similar effects are seen with *ex vivo* transduction where empty viral particles provide no transduction potential [392, 393]. Next, we sought to determine if the addition of a second ultracentrifugation affected the viral yield after production, as a decrease in yield may affect the potential for clinical applications. However, we found comparable physical titers for AAV batches that underwent one or two ultracentrifuging, suggesting the viral yield was not affected.

Before testing AAV6 transductions, we sought to examine how we characterized each AAV batch. Traditionally, a physical titer based on real-time quantitative PCR is the most common technique to quantitatively characterize batches and is used to depict the amount of vector used for each transduction. There are, however, issues that arise when using the physical titer. There are variations in batch production and differential transduction efficiencies necessitates an alternative technique. A functional titer that describes the concentration of functional viral particles that are able to transduce cells is a valuable technique for characterizing recombinant AAV [394]. Therefore, we developed a functional titer protocol to assess our in-house AAV batches by testing them on the Jurkat cell line. We directly compared the physical and functional titers for two AAV batches and found that the functional titers were 5-6 orders of magnitude smaller compared to the physical titer. This suggests the functional titer may be a more accurate depiction of each AAV batch and may allow for easier comparisons of transduction efficiency between batches.

Once we improved the AAV production process and characterization of AAV batches, we tested whether γδ T cells were able to be engineered using AAV6. Due to the variable transduction and reduced viability of γδ T cells with AAV6 using traditional well techniques, we tested the use of microfluidics devices that we have shown can improve the transduction of primary cell with recombinant vectors [314-316]. Before testing the device transductions on γδ T cells, we evaluated this method on the Jurkat cell line. We show

that, at even low MOIs, the microfluidics device is able to greatly increase transduction efficiency using the AAV6-CMV-GFP vector. In addition, transduction with the device resulted in earlier expression of the transgene, as seen by expression of GFP as early as one day after transduction. Next, we compared device transduction to traditional well transduction for γδ T cells. As expected, there was limited to no transduction when using wells, however transduction efficiency was improved when using the microfluidics device. At MOIs of 0.2 and 0.4, GFP expression was seen in about 40% and 50% of γδ T cells, respectively. Despite the improved gene transfer efficiency, there was also a reduction in cell viability, which decreased by about 20-40%. We hypothesized that this decrease in viability was caused by the high percentage of vector volume within the total volume in the device based on our experience with well transductions. To test this, we transduced γδ T cells at a constant MOI of 0.4 and varied the total culture volume, thus changing the vector volume percentage. As expected, we found that as the vector percentage increased, the cell viability decreased, with the largest decreased observed when the vector percentage reached 50%.

The next step in evaluating this engineering technique was to test whether it is successful in delivery chimeric antigen receptors to T cells. We developed a bicistronic AAV6 vector that expresses GFP and a CD5 CAR that is capable of targeting T-cell malignancies. We replaced the CMV promoter with the MND promoter, as it has been shown to be stronger in lymphocytes [322]. Successful cloning of the construct was confirmed by western blot analysis after transfection of HEK293 cells. Once confirmed, the construct was tested with Jurkats using the microfluidics device and was compared to well transductions. Well transductions led to limited GFP expression reaching 37%, with no CAR expression detected. In contrast, device transductions led to increased GFP expression that peaked at 84%, however CAR expression only increased to 7%. This limited CD5 CAR expression can be explained by the downregulation of the CAR after cis and trans interactions with CD5 on the T cells that we have previously showed in past work [324, 338]. This was also confirmed here by showing increased CD5 CAR expression in a CD5-edited Jurkat cell line. In an attempt to counter this phenomenon, we tested the highest MOI that can be achieved with the

device and examined GFP and CD5 CAR expression in γδ T cells. Despite using an MOI of 6, we still found limited GFP expression and detected no CAR expression.

In an attempt to overcome the downregulation of the CD5 CAR in Jurkats and γδ T cells, we investigated the use of two mutant AAV6 vectors that contained mutations in the viral capsids. These mutant vectors have previously been shown to improve the transduction of hematopoietic cells by enhancing intracellular trafficking of transgenes to the nucleus and preventing degradation [317-319]. First, we tested whether the mutant vectors were able to exhibit increased transduction of the Jurkat cell. We found increased CD5 CAR expression using the mutant vectors in combination with the microfluidics device that reached about 50%. In addition, we detected an inverse relationship between CD5 CAR expression and CD5 expression on the Jurkat cells. This was to be expected because as more CAR molecules were expressed, there were more interactions between the CAR and CD5, which can result in the downregulation of the CAR, as well as CD5. Despite the promising CAR expression in Jurkat cells, we again not only saw limited GFP expression, but also no CD5 CAR expression in γδ T cells. Cell viability was also reduced, which may have contributed to the limited gene transfer efficiency.

Based on the low and variable gene transfer efficiencies and limited expression of the CD5 CAR in γδ T cells using AAV6, we investigated an alternative engineering technique for γδ T cells. With advancements in mRNA technologies, mRNA electroporation has become a promising engineering method that has seen success in transfecting a wide range of cell types [178]. Chapter 3 focuses on the optimization and use of mRNA electroporation to engineer γδ T cells. To properly evaluate the potential for this technique to become a viable engineering platform for γδ T cells, we focused our efforts on the CD19 CAR to target B-cell malignancies. Preliminary experiments using a bicistronic GFP/CD19 CAR mRNA construct revealed high transfection efficiency of over 90%, based on GFP expression. We next sought to optimize the engineering of γδ T cells with electroporation by determining the optimal conditions for each reaction. We tested varying cell numbers and amount of mRNA and based on cell yield, GFP MFI, and GFP and CAR

expression we determined that the ideal conditions was between 5 x 10^6 and 1 x 10^7 cells and 15µg of mRNA. These conditions resulted in a cell yield of about 40-50% and CAR expression of about 60%.

The development of adoptive cell therapies that can be developed as an off-the-shelf product is a valuable feature for cellular cancer treatments. The timing of freezing plays an important role in the manufacturing process, thus, we examined whether freezing before or after engineering was superior. While GFP expression was not affected, we found that freezing engineered γδ T cells resulted in a reduction in CAR expression. This reduction in CAR expression also caused a reduction in cytotoxicity against the B-ALL cell line 697. Based on these results, all subsequent experiments were conducted by engineering thawed, unmodified cells. Once the protocol and gene engineering platform was optimized, we tested the engineered cells for their cytotoxic capabilities. To do this, we examined the cytotoxicity of CD19- and CD22-CAR expressing γδ T cells against 697 cells and Nalm6 cells, another B-ALL cell line. We show that the mock electroporated γδ T cells exhibited minimal killing of both cell lines, however the engineered cells showed increased cytotoxicity against both cell lines at all E:T ratios. The cytotoxicity reached about 80% at the E:T ratio for all conditions, however the CD22 CAR-expressing cells resulted in about 45% cytotoxicity against the Nalm6 cell line at the 5:1 E:T ratio. The lower cytotoxicity can be explained by lower CD22 expression in this cell line, compared to the 697 cell line.

Despite being the most common engineering technique for adoptive cell therapy, we wanted to investigate whether electroporation can be applied to other engineering approaches. Bispecific T cell engagers, a type of bispecific antibody, has become a promising option for treating cancer patients. Traditionally, these T cell engagers are systemically administered or co-administered with immunocompetent cells and have seen success in preclinical and clinical studies, however we investigated whether γδ T cells are able to express and secrete them via mRNA electroporation. The secreted bispecific T cell engager (sBite) is continuously expressed as long as the mRNA is within the cells and offers a more targeted treatment approach. To test whether γδ T cells are able to secrete a functional T cell engager, we electroporated γδ T cells with CD19

sBiTE mRNA. Using an ELISA assay, we were able to detect the sBite in the culture media after 16 hours of culturing post-electroporation and the CD19 sBite-modified γδ T cells exhibited improved cytotoxicity against several CD19-positive cancer cell lines. We confirmed the increased cytotoxicity was specific to CD19 by showing the engineered cells had limited cytotoxicity against a CD19 knockout 697 cell line. Lastly, we tested whether the sBite is able to bind and activate unmodified γδ T cells. We collected conditioned media of sBite-modified γδ T cells and mixed it with unmodified γδ T cells and showed that these cells had increased cytotoxicity against the 697 cell line. Overall, these studies indicate that electroporation of γδ T cells with CD19 sBite mRNA results in successful secretion of the CD19 bispecific T cell engager and increases γδ T cell cytotoxicity of modified and unmodified γδ T cells.

The next step in evaluating whether mRNA electroporation is a viable option for engineering γδ T cells is testing the cells *in vivo*. Despite the promising *in vitro* data, initial attempts at treating mice bearing 697 cells with CD19 CAR-expressing γδ T cells were unsuccessful. In order to investigate the cause of this, we conducted several experiments to characterize the 697 mouse model. We found that the cancer cells quickly leave circulation and seed in peripheral organs and compartments, such as the bone marrow. Further analysis found that the injected γδ T cells mainly stayed within the blood stream, with limited migration to the bone marrow. Therefore, we hypothesized that treatments starting too late (i.e. after cancer cells leave circulation) may not be successful because the engineered γδ T cells would not be able to control cancer progression. We confirmed this by showing treatment with CD19 CAR-expressing γδ T cells starting one day after cancer cell injection not only reduced tumor burden and delayed tumor progression, but also improved survival. Next, to further confirm the efficacy of the CD19 CAR-expressing γδ T cells and test the CD19 sBite-modified γδ T cells *in vivo*, we established a cancer cell model with the Nalm6 cell line. The results from this study again showed that the CD19 CAR-modified γδ T cells reduced tumor and improved survival, and, importantly, revealed that the CD19 sBite-modified γδ T cells had a similar effect.

Despite the promising *in vivo* results and induction of a survival benefit, we only observed temporary responses without complete cures. Although this may be specific to this cancer model, we sought to investigate whether a longer duration of treatment and more aggressive upfront regimen could result in improved responses. Interestingly, treating mice bearing Nalm6 cells with CD19 sBite-modified γδ T cells using the more extensive treatment regimen did not reduce tumor growth or survival, compared to the previous treatment regimen. This may have been related to the extravasation of the cancer cells and would require an even more aggressive treatment regimen after cancer cell injection.

4.2 Implications of Findings

The development of novel therapeutic approaches to treat cancer is essential to improving patient outcomes and safety. Traditional treatments that involve the use of chemotherapeutics result in harmful side effects and toxicities [395]. These drugs have limited specificity for cancer cells, which results in damage to healthy tissue and organs, and many times are unable to fully cure patients. Cancer immunotherapy offers an attractive alternative by harnessing the immune system to fight cancer and allows for a more targeted treatment approach. Advancements and discoveries in the field of immunology have led to several promising directions for cancer immunotherapy including cancer vaccines, immune-modulating agents, and adoptive cell therapy (ACT). ACT involves the isolation and expansion of autologous or allogeneic immunocompetent cells to repopulate the patient's immune system and initiate an anti-cancer immune response. This technique has great potential for developing the next generation of cancer treatments.

While initial attempts for developing ACTs focused on simply infusing immunocompetent cells, scientists quickly discovered the need for engineering the cells to improve their cytotoxic capabilities [2]. One of the most common engineering techniques is the expression of chimeric receptors that are specific to a tumor antigen, which led to the development of CAR T cell therapy. CAR T cell therapy has emerged as a promising alternative for treating cancer and has seen great success in clinical trials [396]. In fact, there are currently six FDA approved CAR T cell therapies on the market. Traditionally, CAR T cell therapy involves

the isolation of autologous αβ T cells and the engineering of these cells with stable integrating vectors. The gold standard for CAR T cell therapy is the CD19 CAR for treating B-cell malignancies, which has resulted in complete remission rates reaching about 70-80% for B-ALL [36]. While CAR T cell therapy has seen great success, there have been several side effects and limitations that were identified during clinical testing. One of the major limitations is the development of on-target/ off-tumor (OTOT) toxicities. There are very few cancer-specific antigens that can be targeted by CAR T cells, which can result in the targeting of healthy tissue that also express the antigen. During the development of CAR T cell therapy for B-cell malignancies, this side effect was acceptable because the depletion of B cells can be countered by the infusion of immunoglobulins. However, for other cancers, especially solid tumors, the targeting of healthy cells can lead to organ toxicities and failure [397]. The formation of memory CAR T cells can exacerbate this issue as the constant targeting of healthy tissue lasts as long as the T cells persist. Another significant side effect of CAR T cell therapy is the development of cytokine release syndrome (CRS). The over activation of the CAR T cells, as well as other cell types within the immune system, can lead to an influx of cytokines and cause a harmful inflammatory response. Additionally, like most cancer treatments, therapy resistance has emerged as a hurdle for CAR T cell therapy in the form of antigen escape. Cancer cells can develop mechanisms to limit antigen expression that renders CAR T cell therapy ineffective. These limitations must be addressed to not only improve the safety of this treatment option, but also improve the efficacy and effectiveness.

To reduce the risks associated with traditional CAR T cell therapy, transient engineering techniques with γδ T cells may be an effective solution. γδ T cells are becoming a promising cell type for adoptive cell therapy and offer an attractive alternative to the traditional αβ T cell. They have characteristics and functions in both the adaptive and innate immune systems and undergo MHC-independent antigen recognition [292]. The ability to mount an immune response without the need for priming allows for rapid detection and killing of infected or cancerous cells. In addition, they are non-alloreactive and have a limited risk of causing graft-versus-host disease, which allows them to be developed as an allogeneic product [398]. Our lab has

extensive experience with these cells and have published our efforts on optimizing the expansion and handling of γδ T cells [287, 343, 365, 366, 369]. These endeavors have been instrumental in advancing the use of γδ T cells toward the development of cancer immunotherapies, however another critical step is improving their efficacy by genetic modification. Lentiviral vectors have become one of the most widely used engineering technique for engineering cells for ACT, however it has seen limited success for γδ T cells. These cells have traditionally been difficult to engineer, which necessitates alternative engineering strategies beyond the traditional lentiviral vectors.

The use of transient engineering, especially with γδ T cells, offers solutions to many of the issues and limitations seen in traditional CAR T cell therapy. This method limits the risk of OTOT toxicities because the treatment can be halted quickly and specified doses can be administered for individual cases, which is not possible with stable expression of CAR T cells. In addition, transient expression can reduce the risk of cytokine release syndrome because it reduces the over-activation and proliferation of the cells. It also eliminates the concern of developing memory CAR T cells that can result in the constant targeting of the antigen, which in the case of solid tumors can lead to harmful OTOT side effects. Therefore, the goal of this dissertation was to develop a successful engineering platform for γδ T cells via transient gene transfer methods. We investigated the use of AAV and mRNA electroporation, two promising transient engineering techniques that have seen success for other hematopoietic cells [307, 379, 380].

Leukemia is the most common form of pediatric cancer, accounting for about 20-25% of all cases [399]. T cell acute lymphoblastic leukemia (T-ALL), a distinct hematological cancer, is characterized by abnormal production of malignant precursor T lymphoid cells originating at different points in lymphocyte development [400]. Traditionally, combination chemotherapy has been the most common form of treatment for T-ALL and has seen some success in the clinic, however relapse remains a significant problem [401-403]. Relapse or refractory T-ALL is associated with a poor prognosis and a survival rate of about 20% [404, 405]. Due to the high occurrence of pediatric T-ALL and increased risk of relapse, novel treatments

must be developed to improve outcomes. Therefore, out initial attempts at engineering γδ T cell focused on targeting T-cell malignancies through the expression of a CD5 CAR. There are, however, added obstacles that must be overcome for the use of CAR T cell therapy to treat T-cell malignancies, which has been previously characterized by our lab [338]. The major obstacle is the down regulation of the CD5 CAR upon interacting with CD5 on T cells. We hypothesized that increased gene transfer using AAV6 may be able to overcome this phenomenon and result in sufficient numbers of engineered γδ T cells. Despite observing increased transduction efficiencies with AAV6 in combination with microfluidics devices or when using mutant AAV6 vectors, we ultimately were not able to overcome the downregulation of the CD5 CAR in γδ T cells. We also observed a significant decrease in cell viability, which may have limited the successful gene transfer of the CAR into γδ T cells. For this reason, we focused on the optimization of mRNA electroporation to engineer γδ T cells. We tested the mRNA electroporation platform with a CD19 CAR, in order to properly examine this gene transfer technique without the added hurdles of expressing a CD5 CAR in T cells.

mRNA electroporation resulted in very high gene transfer efficiency for γδ T cells and CAR expression reaching 60-70%. This high transfection efficiency led to engineered γδ T cells that exhibited improved cytotoxicity *in vitro* against two B-ALL cell lines and even reached about 80-90% at an E:T ratio of 5:1. We confirmed the efficacy of the engineered cells in an *in vivo* 697 cell model and found that the cells prolonged tumor progression, reduced tumor burden, and improved survival. In doing so, we also characterized this cancer model and found that the cancer cells quickly leave circulation, which may be a limitation when testing the anti-cancer effectiveness of cellular therapies with the cell line. Overall, we show a practical and functional engineering platform for γδ T cell to express a CD19 CAR, as well as a functioning CD22 CAR. Although we tested this platform for the treatment of B-cell malignancies, we expect this engineering technique can be applied to all cancer types and may even overcome the limitations of targeting T-cell malignancies. In addition, while electroporation has the potential to become a technique for the development of therapeutics, it also offers a promising high-throughput screening platform. The

protocol that was developed here is a fast and simple method that can be used to quickly test target candidates for TCRs, CARs, bispecific T cell engagers, and other immunotherapy modalities. In fact, this concept has already been used by at least one group to examine the potential of specific TCRs to target cancer cell lines [204]. It allows for easy assessment of whether a construct is functional and whether it can be developed into an effective cancer treatment.

Another promising technique for enhancing the immune response to fight cancer is the use of bispecific antibodies, specifically bispecific T cell engagers. Traditionally, these T cell engagers are administered systemically, in combination with immunocompetent cells. In fact, there have been studies investigating the combination of bispecific T cell engagers with γδ T cells for several cancer types including chronic lymphocytic leukemia, acute myeloid leukemia, and pancreatic cancer [373, 374, 383, 384]. Here, however, we test whether the T cell engagers are able to be expressed and secreted by the immunocompetent cells. One limitation of the systemic administration is the short half-life and need for continuous infusions. This novel technique would allow for continuous expression for the duration that the mRNA remains in the cells. In addition, it also allows for a more targeted treatment approach because they are secreted locally where the immune cells can be directly activated. This approach offers an exciting avenue for developing bispecific antibody-based therapeutics and may improve upon the already promising results. Overall, the data presented in this dissertation shows that the transient engineering of γδ T cells with CARs or sBites offers a promising alternative to the stable engineering in αβ T cells for the treatment of pediatric cancer.

4.3 Limitations and Future Directions

The objective of this dissertation was to investigate the efficacy of a transient engineering platform for γδ T cells with the goal of not only showing this technique can be effective at treating cancer, but also limit some of the issues with traditional ACT and CAR T cell therapy. While we show that engineering γδ T cells with electroporation improved their cytotoxicity *in vitro* and *in vivo,* being able to provide direct evidence that this platform reduces the risk of side effects and improves the safety profile remains difficult.

The need to use immunodeficient mice, like NSG mice, for cancer research limits the possibility of investigating the effects of therapeutics on the immune system. For example, there are limited models that can effectively depict cytokine release syndrome in mice because the entire immune system plays a role in its progression. The development of humanized mice models is an intriguing approach that would better predict the success of immunotherapies [406, 407]. Several techniques to create humanized mice models have been tested, including the engraftment of PBMCs and the engraftment of hematopoietic stem and progenitor cells (HSPCs). The advantage of using HSPCs is the repopulation of not only T cells but also B cells, macrophages dendritic cells, and NK cells [408, 409]. Testing transient engineering techniques using these humanized models would allow for direct comparisons of safety profiles between engineering platforms, as well as better characterize cancer immunotherapies before clinical testing.

Although we show great promise for electroporation as an engineering technique for γδ T cells, the results from Chapter 2 reveal limited efficiency and variability for AAV. We hypothesized that the success of AAV6 with αβ T cells may translate to γδ T cells, especially due to the expression of the receptors that are necessary for AAV6 transduction. Our initial goal was to test the engineering of γδ T cells to treat T-cell malignancies, by using AAV to engineer the cells with a CD5 CAR. As mentioned previously, there are additional hurdles that must be overcome for creating CAR T cell therapies for T-cell malignancies. We experienced some of these limitations including the downregulation of the CD5 CAR, as well as the downregulation of CD5. Despite this, we found several limitations to AAV6 transduction of γδ T cells, including poor and variable transduction efficiency based on GFP expression and a reduction in cell viability that must be addressed. There are, however, techniques that can be explored to overcome the issue of low gene transfer and reduced viability. For example, a method to produce a more concentrated AAV batch can limit the amount of vector used during transduction, thus reducing the cell death caused by high vector percentages in the culture media. In addition, improvements to the microfluidics device design can limit the loss of cells during collection and may improve cell viability of the engineered cells.

While we were unable to successfully develop a dependable AAV engineering platform, we observed promising gene transfer efficiency for some donors, suggesting donor variability may play a role in transduction efficiency for primary γδ T cells. Our lab has previously shown donor variability exists during expansion of γδ T cells from PBMCs and have identified donor characteristics that may predict successful expansion [287, 343]. We found that young, healthy non-smoking donors who exercise at least three times a week are more likely to have successful γδ T cell expansions. A similar phenomenon may exist for transduction efficiency. There may be certain characteristics of the donor or the γδ T cells that make them more susceptible to AAV transduction. Additional research is needed to determine whether there are donor-specific characteristics and what those characteristics may be to allow for increased gene transfer efficiency using AAV.

Once we established an effective transient engineering platform for γδ T cells with mRNA electroporation and showed that the engineered cells had impressive anti-cancer capabilities *in vitro*, we experienced several obstacles when testing the cells *in vivo*. During the development of the 697 cancer model, we made several observations that highlighted the limitations of using cancer cell lines to test cancer therapeutics. We found that the 697 cells quickly leave circulation and seed in different organs and compartments, such as the bone marrow. While this migration is specific for this cell model, it can be hypothesized that other cell lines either have similar or completely different migratory patterns. This observation not only emphasizes the importance of choosing a model that best tests the specific hypothesis or goal of the study, but also the importance of using multiple cancer cell models for therapeutic testing. Additionally, this observation highlights the importance of investigating the migration of the therapeutic cells. The modulation of T cell homing has become a popular field of research and holds great promise in improving ACT, especially for solid tumors where T cell homing is a limitation. The expression of specific chemokine and chemokine receptors has the potential to preferentially target certain tissues or organs where cancer may be more abundant [385]. The combination of engineering γδ T cells to improve their cytotoxicity, as

well as their tumor homing capabilities offers an attractive approach for improving their use as a cancer immunotherapy.

In order to show proof-of-concept that transient engineering, specifically electroporation, is a viable option for engineering γδ T cells, we utilized CD19 as the targeted antigen. CD19 is the most commonly studied target for cancer immunotherapy and has been extensively studied for the treatment of B-cell malignancies, which makes it the ideal target for testing novel therapeutics and engineering platforms. However, we hypothesize that the technique developed and optimized in this dissertation can be applied to other cancer types as well. The transient method described here can be especially beneficial to cancer types that have limited tumor-specific targets and are more susceptible to the side effects of current immunotherapies. Current efforts are being made to apply this platform to other common pediatric cancers, including AML, neuroblastoma, and osteosarcoma.

4.4 Conclusions

This dissertation investigated the use of transient engineering techniques to improve the cytotoxicity of γδ T cells by genetic modification. We tested two engineering techniques, AAV as a viral vector and electroporation as a nonviral technique. Despite limited transduction efficiency and increased cell death using AAV6 transduction, mRNA electroporation offered a promising engineering platform for γδ T cells. After optimization, we show electroporation resulted in substantial transfection efficiency for the expression of CARs and the engineered cells exhibited enhanced cytotoxicity *in vitro* against several cancer cell lines. We then applied this platform for the expression of a secreted bispecific T cell engager, which we show improved the cytotoxicity of unmodified and modified γδ T cells and is specific to the targeted antigen. In an *in vivo* cancer model, we also show these engineered cells not only prolonged tumor progression and reduced tumor burden, but also improved survival. Overall, the work here reveals a promising technique for engineering γδ T cells that can improve their potential to be an effective cancer treatment.

References

1. Starnes CO. 1992. Coley's toxins in perspective. *Nature* 357:11-2
2. Dobosz P, Dzieciątkowski T. 2019. The Intriguing History of Cancer Immunotherapy. *Front Immunol* 10:2965
3. Rosenberg SA, Lotze MT, Muul LM, Leitman S, Chang AE, et al. 1985. Observations on the systemic administration of autologous lymphokine-activated killer cells and recombinant interleukin-2 to patients with metastatic cancer. *N Engl J Med* 313:1485-92
4. Rosenberg SA, Spiess P, Lafreniere R. 1986. A new approach to the adoptive immunotherapy of cancer with tumor-infiltrating lymphocytes. *Science* 233:1318-21
5. Rosenberg SA, Packard BS, Aebersold PM, Solomon D, Topalian SL, et al. 1988. Use of tumor-infiltrating lymphocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma. A preliminary report. *N Engl J Med* 319:1676-80
6. Rosenberg SA, Restifo NP. 2015. Adoptive cell transfer as personalized immunotherapy for human cancer. *Science* 348:62-8
7. Rosenberg SA, Yannelli JR, Yang JC, Topalian SL, Schwartzentruber DJ, et al. 1994. Treatment of patients with metastatic melanoma with autologous tumor-infiltrating lymphocytes and interleukin 2. *J Natl Cancer Inst* 86:1159-66
8. Dudley ME, Wunderlich JR, Robbins PF, Yang JC, Hwu P, et al. 2002. Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. *Science* 298:850-4
9. Rosenberg SA, Yang JC, Sherry RM, Kammula US, Hughes MS, et al. 2011. Durable complete responses in heavily pretreated patients with metastatic melanoma using T-cell transfer immunotherapy. *Clin Cancer Res* 17:4550-7
10. Radvanyi LG, Bernatchez C, Zhang M, Fox PS, Miller P, et al. 2012. Specific lymphocyte subsets predict response to adoptive cell therapy using expanded autologous tumor-infiltrating lymphocytes in metastatic melanoma patients. *Clin Cancer Res* 18:6758-70
11. Jin J, Sabatino M, Somerville R, Wilson JR, Dudley ME, et al. 2012. Simplified method of the growth of human tumor infiltrating lymphocytes in gas-permeable flasks to numbers needed for patient treatment. *J Immunother* 35:283-92
12. Lin B, Du L, Li H, Zhu X, Cui L, Li X. 2020. Tumor-infiltrating lymphocytes: Warriors fight against tumors powerfully. *Biomed Pharmacother* 132:110873
13. Dembić Z, Haas W, Weiss S, McCubrey J, Kiefer H, et al. 1986. Transfer of specificity by murine alpha and beta T-cell receptor genes. *Nature* 320:232-8
14. Clay TM, Custer MC, Sachs J, Hwu P, Rosenberg SA, Nishimura MI. 1999. Efficient transfer of a tumor antigen-reactive TCR to human peripheral blood lymphocytes confers anti-tumor reactivity. *J Immunol* 163:507-13
15. Sadelain M, Rivière I, Brentjens R. 2003. Targeting tumours with genetically enhanced T lymphocytes. *Nat Rev Cancer* 3:35-45
16. Gross G, Waks T, Eshhar Z. 1989. Expression of immunoglobulin-T-cell receptor chimeric molecules as functional receptors with antibody-type specificity. *Proc Natl Acad Sci U S A* 86:10024-8
17. Eshhar Z, Waks T, Gross G, Schindler DG. 1993. Specific activation and targeting of cytotoxic lymphocytes through chimeric single chains consisting of antibody-binding domains and the gamma or zeta subunits of the immunoglobulin and T-cell receptors. *Proc Natl Acad Sci U S A* 90:720-4
18. Brocker T, Peter A, Traunecker A, Karjalainen K. 1993. New simplified molecular design for functional T cell receptor. *Eur J Immunol* 23:1435-9
19. Figueroa JA, Reidy A, Mirandola L, Trotter K, Suvorava N, et al. 2015. Chimeric antigen receptor engineering: a right step in the evolution of adoptive cellular immunotherapy. *Int Rev Immunol* 34:154-87

20. Branella GM, Spencer HT. 2021. Natural Receptor- and Ligand-Based Chimeric Antigen Receptors: Strategies Using Natural Ligands and Receptors for Targeted Cell Killing. *Cells* 11
21. Guest RD, Hawkins RE, Kirillova N, Cheadle EJ, Arnold J, et al. 2005. The role of extracellular spacer regions in the optimal design of chimeric immune receptors: evaluation of four different scFvs and antigens. *J Immunother* 28:203-11
22. Bridgeman JS, Hawkins RE, Bagley S, Blaylock M, Holland M, Gilham DE. 2010. The optimal antigen response of chimeric antigen receptors harboring the CD3zeta transmembrane domain is dependent upon incorporation of the receptor into the endogenous TCR/CD3 complex. *J Immunol* 184:6938-49
23. Shirasu N, Kuroki M. 2012. Functional design of chimeric T-cell antigen receptors for adoptive immunotherapy of cancer: architecture and outcomes. *Anticancer Res* 32:2377-83
24. Brocker T, Karjalainen K. 1995. Signals through T cell receptor-zeta chain alone are insufficient to prime resting T lymphocytes. *J Exp Med* 181:1653-9
25. Park JR, Digiusto DL, Slovak M, Wright C, Naranjo A, et al. 2007. Adoptive transfer of chimeric antigen receptor re-directed cytolytic T lymphocyte clones in patients with neuroblastoma. *Mol Ther* 15:825-33
26. Till BG, Jensen MC, Wang J, Chen EY, Wood BL, et al. 2008. Adoptive immunotherapy for indolent non-Hodgkin lymphoma and mantle cell lymphoma using genetically modified autologous CD20-specific T cells. *Blood* 112:2261-71
27. Kowolik CM, Topp MS, Gonzalez S, Pfeiffer T, Olivares S, et al. 2006. CD28 costimulation provided through a CD19-specific chimeric antigen receptor enhances in vivo persistence and antitumor efficacy of adoptively transferred T cells. *Cancer Res* 66:10995-1004
28. Long AH, Haso WM, Shern JF, Wanhainen KM, Murgai M, et al. 2015. 4-1BB costimulation ameliorates T cell exhaustion induced by tonic signaling of chimeric antigen receptors. *Nat Med* 21:581-90
29. Cappell KM, Kochenderfer JN. 2021. A comparison of chimeric antigen receptors containing CD28 versus 4-1BB costimulatory domains. *Nat Rev Clin Oncol* 18:715-27
30. Lamers CH, Sleijfer S, Vulto AG, Kruit WH, Kliffen M, et al. 2006. Treatment of metastatic renal cell carcinoma with autologous T-lymphocytes genetically retargeted against carbonic anhydrase IX: first clinical experience. *J Clin Oncol* 24:e20-2
31. Kershaw MH, Westwood JA, Parker LL, Wang G, Eshhar Z, et al. 2006. A phase I study on adoptive immunotherapy using gene-modified T cells for ovarian cancer. *Clin Cancer Res* 12:6106-15
32. Jensen MC, Popplewell L, Cooper LJ, DiGiusto D, Kalos M, et al. 2010. Antitransgene rejection responses contribute to attenuated persistence of adoptively transferred CD20/CD19-specific chimeric antigen receptor redirected T cells in humans. *Biol Blood Marrow Transplant* 16:1245-56
33. Kochenderfer JN, Wilson WH, Janik JE, Dudley ME, Stetler-Stevenson M, et al. 2010. Eradication of B-lineage cells and regression of lymphoma in a patient treated with autologous T cells genetically engineered to recognize CD19. *Blood* 116:4099-102
34. Gill S, Maus MV, Porter DL. 2016. Chimeric antigen receptor T cell therapy: 25years in the making. *Blood Rev* 30:157-67
35. Park JH, Geyer MB, Brentjens RJ. 2016. CD19-targeted CAR T-cell therapeutics for hematologic malignancies: interpreting clinical outcomes to date. *Blood* 127:3312-20
36. Martino M, Alati C, Canale FA, Musuraca G, Martinelli G, Cerchione C. 2021. A Review of Clinical Outcomes of CAR T-Cell Therapies for B-Acute Lymphoblastic Leukemia. *Int J Mol Sci* 22
37. Maude SL, Laetsch TW, Buechner J, Rives S, Boyer M, et al. 2018. Tisagenlecleucel in Children and Young Adults with B-Cell Lymphoblastic Leukemia. *N Engl J Med* 378:439-48
38. Schuster SJ, Bishop MR, Tam CS, Waller EK, Borchmann P, et al. 2019. Tisagenlecleucel in Adult Relapsed or Refractory Diffuse Large B-Cell Lymphoma. *N Engl J Med* 380:45-56

39. Neelapu SS, Locke FL, Bartlett NL, Lekakis LJ, Miklos DB, et al. 2017. Axicabtagene Ciloleucel CAR T-Cell Therapy in Refractory Large B-Cell Lymphoma. *N Engl J Med* 377:2531-44
40. Beatty GL, O'Hara MH, Lacey SF, Torigian DA, Nazimuddin F, et al. 2018. Activity of Mesothelin-Specific Chimeric Antigen Receptor T Cells Against Pancreatic Carcinoma Metastases in a Phase 1 Trial. *Gastroenterology* 155:29-32
41. Haas AR, Tanyi JL, O'Hara MH, Gladney WL, Lacey SF, et al. 2019. Phase I Study of Lentiviral-Transduced Chimeric Antigen Receptor-Modified T Cells Recognizing Mesothelin in Advanced Solid Cancers. *Mol Ther* 27:1919-29
42. Qi C, Gong J, Li J, Liu D, Qin Y, et al. 2022. Claudin18.2-specific CAR T cells in gastrointestinal cancers: phase 1 trial interim results. *Nat Med* 28:1189-98
43. Koneru M, O'Cearbhaill R, Pendharkar S, Spriggs DR, Brentjens RJ. 2015. A phase I clinical trial of adoptive T cell therapy using IL-12 secreting MUC-16(ecto) directed chimeric antigen receptors for recurrent ovarian cancer. *J Transl Med* 13:102
44. Zhang C, Wang Z, Yang Z, Wang M, Li S, et al. 2017. Phase I Escalating-Dose Trial of CAR-T Therapy Targeting CEA(+) Metastatic Colorectal Cancers. *Mol Ther* 25:1248-58
45. Heczey A, Courtney AN, Montalbano A, Robinson S, Liu K, et al. 2020. Anti-GD2 CAR-NKT cells in patients with relapsed or refractory neuroblastoma: an interim analysis. *Nat Med* 26:1686-90
46. Ceppi F, Rivers J, Annesley C, Pinto N, Park JR, et al. 2018. Lymphocyte apheresis for chimeric antigen receptor T-cell manufacturing in children and young adults with leukemia and neuroblastoma. *Transfusion* 58:1414-20
47. Schaft N. 2020. The Landscape of CAR-T Cell Clinical Trials against Solid Tumors-A Comprehensive Overview. *Cancers (Basel)* 12
48. Köhl U, Arsenieva S, Holzinger A, Abken H. 2018. CAR T Cells in Trials: Recent Achievements and Challenges that Remain in the Production of Modified T Cells for Clinical Applications. *Hum Gene Ther* 29:559-68
49. Zhao J, Lin Q, Song Y, Liu D. 2018. Universal CARs, universal T cells, and universal CAR T cells. *J Hematol Oncol* 11:132
50. Depil S, Duchateau P, Grupp SA, Mufti G, Poirot L. 2020. 'Off-the-shelf' allogeneic CAR T cells: development and challenges. *Nat Rev Drug Discov* 19:185-99
51. Lin JK, Muffly LS, Spinner MA, Barnes JI, Owens DK, Goldhaber-Fiebert JD. 2019. Cost Effectiveness of Chimeric Antigen Receptor T-Cell Therapy in Multiply Relapsed or Refractory Adult Large B-Cell Lymphoma. *J Clin Oncol* 37:2105-19
52. Singh N, Perazzelli J, Grupp SA, Barrett DM. 2016. Early memory phenotypes drive T cell proliferation in patients with pediatric malignancies. *Sci Transl Med* 8:320ra3
53. Caldwell KJ, Gottschalk S, Talleur AC. 2020. Allogeneic CAR Cell Therapy-More Than a Pipe Dream. *Front Immunol* 11:618427
54. Chang YJ, Zhao XY, Huang XJ. 2018. Strategies for Enhancing and Preserving Anti-leukemia Effects Without Aggravating Graft-Versus-Host Disease. *Front Immunol* 9:3041
55. Klein J, Sato A. 2000. The HLA system. First of two parts. *N Engl J Med* 343:702-9
56. Sheldon S, Poulton K. 2006. HLA typing and its influence on organ transplantation. *Methods Mol Biol* 333:157-74
57. Petersdorf EW, Hansen JA, Martin PJ, Woolfrey A, Malkki M, et al. 2001. Major-histocompatibility-complex class I alleles and antigens in hematopoietic-cell transplantation. *N Engl J Med* 345:1794-800
58. Ho VT, Kim HT, Liney D, Milford E, Gribben J, et al. 2006. HLA-C mismatch is associated with inferior survival after unrelated donor non-myeloablative hematopoietic stem cell transplantation. *Bone Marrow Transplant* 37:845-50
59. Petersdorf EW, Kollman C, Hurley CK, Dupont B, Nademanee A, et al. 2001. Effect of HLA class II gene disparity on clinical outcome in unrelated donor hematopoietic cell transplantation

for chronic myeloid leukemia: the US National Marrow Donor Program Experience. *Blood* 98:2922-9
60. Lee SJ, Klein J, Haagenson M, Baxter-Lowe LA, Confer DL, et al. 2007. High-resolution donor-recipient HLA matching contributes to the success of unrelated donor marrow transplantation. *Blood* 110:4576-83
61. Gragert L, Eapen M, Williams E, Freeman J, Spellman S, et al. 2014. HLA match likelihoods for hematopoietic stem-cell grafts in the U.S. registry. *N Engl J Med* 371:339-48
62. Torikai H, Reik A, Liu PQ, Zhou Y, Zhang L, et al. 2012. A foundation for universal T-cell based immunotherapy: T cells engineered to express a CD19-specific chimeric-antigen-receptor and eliminate expression of endogenous TCR. *Blood* 119:5697-705
63. Berdien B, Mock U, Atanackovic D, Fehse B. 2014. TALEN-mediated editing of endogenous T-cell receptors facilitates efficient reprogramming of T lymphocytes by lentiviral gene transfer. *Gene Ther* 21:539-48
64. Boissel S, Jarjour J, Astrakhan A, Adey A, Gouble A, et al. 2014. megaTALs: a rare-cleaving nuclease architecture for therapeutic genome engineering. *Nucleic Acids Res* 42:2591-601
65. Eyquem J, Mansilla-Soto J, Giavridis T, van der Stegen SJ, Hamieh M, et al. 2017. Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection. *Nature* 543:113-7
66. Osborn MJ, Webber BR, Knipping F, Lonetree CL, Tennis N, et al. 2016. Evaluation of TCR Gene Editing Achieved by TALENs, CRISPR/Cas9, and megaTAL Nucleases. *Mol Ther* 24:570-81
67. Kagoya Y, Guo T, Yeung B, Saso K, Anczurowski M, et al. 2020. Genetic Ablation of HLA Class I, Class II, and the T-cell Receptor Enables Allogeneic T Cells to Be Used for Adoptive T-cell Therapy. *Cancer Immunol Res* 8:926-36
68. Ren J, Liu X, Fang C, Jiang S, June CH, Zhao Y. 2017. Multiplex Genome Editing to Generate Universal CAR T Cells Resistant to PD1 Inhibition. *Clin Cancer Res* 23:2255-66
69. 2020. First Allogeneic CAR T-Cell Therapy Impresses in Relapsed/Refractory Lymphoma. *Oncologist* 25 Suppl 1:S4-s5
70. Benjamin R, Jain N, Maus MV, Boissel N, Graham C, et al. 2022. UCART19, a first-in-class allogeneic anti-CD19 chimeric antigen receptor T-cell therapy for adults with relapsed or refractory B-cell acute lymphoblastic leukaemia (CALM): a phase 1, dose-escalation trial. *Lancet Haematol* 9:e833-e43
71. Shah BD, Jacobson C, Solomon SR, Jain N, Johnson MC, et al. 2021. Allogeneic CAR-T PBCAR0191 with Intensified Lymphodepletion Is Highly Active in Patients with Relapsed/Refractory B-Cell Malignancies. *Blood* 138:302-
72. Jain N, Roboz GJ, Konopleva M, Liu H, Jabbour E, et al. 2020. Preliminary Results of Balli-01: A Phase I Study of UCART22 (allogeneic engineered T-cells expressing anti-CD22 Chimeric Antigen Receptor) in Adult Patients with Relapsed or Refractory (R/R) CD22+ B-Cell Acute Lymphoblastic Leukemia (B-ALL). *Blood* 136:7-8
73. Harrer DC, Dörrie J, Schaft N. 2018. Chimeric Antigen Receptors in Different Cell Types: New Vehicles Join the Race. *Hum Gene Ther* 29:547-58
74. Ali AK, Tarannum M, Romee R. 2021. Is Adoptive Cellular Therapy With Non-T-Cell Immune Effectors the Future? *Cancer J* 27:168-75
75. Patel S, Burga RA, Powell AB, Chorvinsky EA, Hoq N, et al. 2019. Beyond CAR T Cells: Other Cell-Based Immunotherapeutic Strategies Against Cancer. *Front Oncol* 9:196
76. Mehta RS, Rezvani K. 2018. Chimeric Antigen Receptor Expressing Natural Killer Cells for the Immunotherapy of Cancer. *Front Immunol* 9:283
77. Rozenbaum M, Meir A, Aharony Y, Itzhaki O, Schachter J, et al. 2020. Gamma-Delta CAR-T Cells Show CAR-Directed and Independent Activity Against Leukemia. *Front Immunol* 11:1347
78. Richman SA, Nunez-Cruz S, Moghimi B, Li LZ, Gershenson ZT, et al. 2018. High-Affinity GD2-Specific CAR T Cells Induce Fatal Encephalitis in a Preclinical Neuroblastoma Model. *Cancer Immunol Res* 6:36-46

79. Thistlethwaite FC, Gilham DE, Guest RD, Rothwell DG, Pillai M, et al. 2017. The clinical efficacy of first-generation carcinoembryonic antigen (CEACAM5)-specific CAR T cells is limited by poor persistence and transient pre-conditioning-dependent respiratory toxicity. *Cancer Immunol Immunother* 66:1425-36
80. Hill JA, Giralt S, Torgerson TR, Lazarus HM. 2019. CAR-T - and a side order of IgG, to go? - Immunoglobulin replacement in patients receiving CAR-T cell therapy. *Blood Rev* 38:100596
81. Flugel CL, Majzner RG, Krenciute G, Dotti G, Riddell SR, et al. 2023. Overcoming on-target, off-tumour toxicity of CAR T cell therapy for solid tumours. *Nat Rev Clin Oncol* 20:49-62
82. Castellarin M, Sands C, Da T, Scholler J, Graham K, et al. 2020. A rational mouse model to detect on-target, off-tumor CAR T cell toxicity. *JCI Insight* 5
83. Liao Q, Liu Z, Zhu C, He H, Feng M, et al. 2022. Rapid generation of a mouse model for evaluating on-target normal tissue toxicity of human CAR-T cells using replication-defective recombinant adenovirus. *J Adv Res*
84. Sterner RC, Sterner RM. 2021. CAR-T cell therapy: current limitations and potential strategies. *Blood Cancer J* 11:69
85. Steentoft C, Migliorini D, King TR, Mandel U, June CH, Posey AD, Jr. 2018. Glycan-directed CAR-T cells. *Glycobiology* 28:656-69
86. Hege KM, Bergsland EK, Fisher GA, Nemunaitis JJ, Warren RS, et al. 2017. Safety, tumor trafficking and immunogenicity of chimeric antigen receptor (CAR)-T cells specific for TAG-72 in colorectal cancer. *J Immunother Cancer* 5:22
87. Ritchie DS, Neeson PJ, Khot A, Peinert S, Tai T, et al. 2013. Persistence and efficacy of second generation CAR T cell against the LeY antigen in acute myeloid leukemia. *Mol Ther* 21:2122-9
88. Louis CU, Savoldo B, Dotti G, Pule M, Yvon E, et al. 2011. Antitumor activity and long-term fate of chimeric antigen receptor-positive T cells in patients with neuroblastoma. *Blood* 118:6050-6
89. Garrido G, Tikhomirov IA, Rabasa A, Yang E, Gracia E, et al. 2011. Bivalent binding by intermediate affinity of nimotuzumab: a contribution to explain antibody clinical profile. *Cancer Biol Ther* 11:373-82
90. Caruso HG, Hurton LV, Najjar A, Rushworth D, Ang S, et al. 2015. Tuning Sensitivity of CAR to EGFR Density Limits Recognition of Normal Tissue While Maintaining Potent Antitumor Activity. *Cancer Res* 75:3505-18
91. Pardoll DM. 2012. The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer* 12:252-64
92. Fedorov VD, Themeli M, Sadelain M. 2013. PD-1- and CTLA-4-based inhibitory chimeric antigen receptors (iCARs) divert off-target immunotherapy responses. *Sci Transl Med* 5:215ra172
93. Chatenoud L, Ferran C, Legendre C, Thouard I, Merite S, et al. 1990. In vivo cell activation following OKT3 administration. Systemic cytokine release and modulation by corticosteroids. *Transplantation* 49:697-702
94. Miao L, Zhang Z, Ren Z, Li Y. 2021. Reactions Related to CAR-T Cell Therapy. *Front Immunol* 12:663201
95. Hu Y, Wu Z, Luo Y, Shi J, Yu J, et al. 2017. Potent Anti-leukemia Activities of Chimeric Antigen Receptor-Modified T Cells against CD19 in Chinese Patients with Relapsed/Refractory Acute Lymphocytic Leukemia. *Clin Cancer Res* 23:3297-306
96. Lee DW, Kochenderfer JN, Stetler-Stevenson M, Cui YK, Delbrook C, et al. 2015. T cells expressing CD19 chimeric antigen receptors for acute lymphoblastic leukaemia in children and young adults: a phase 1 dose-escalation trial. *Lancet* 385:517-28
97. Norelli M, Camisa B, Barbiera G, Falcone L, Purevdorj A, et al. 2018. Monocyte-derived IL-1 and IL-6 are differentially required for cytokine-release syndrome and neurotoxicity due to CAR T cells. *Nat Med* 24:739-48

98. Giavridis T, van der Stegen SJC, Eyquem J, Hamieh M, Piersigilli A, Sadelain M. 2018. CAR T cell-induced cytokine release syndrome is mediated by macrophages and abated by IL-1 blockade. *Nat Med* 24:731-8
99. Morris EC, Neelapu SS, Giavridis T, Sadelain M. 2022. Cytokine release syndrome and associated neurotoxicity in cancer immunotherapy. *Nat Rev Immunol* 22:85-96
100. Xiao X, Huang S, Chen S, Wang Y, Sun Q, et al. 2021. Mechanisms of cytokine release syndrome and neurotoxicity of CAR T-cell therapy and associated prevention and management strategies. *J Exp Clin Cancer Res* 40:367
101. Brudno JN, Kochenderfer JN. 2019. Recent advances in CAR T-cell toxicity: Mechanisms, manifestations and management. *Blood Rev* 34:45-55
102. Kochenderfer JN, Dudley ME, Feldman SA, Wilson WH, Spaner DE, et al. 2012. B-cell depletion and remissions of malignancy along with cytokine-associated toxicity in a clinical trial of anti-CD19 chimeric-antigen-receptor-transduced T cells. *Blood* 119:2709-20
103. Lee DW, Gardner R, Porter DL, Louis CU, Ahmed N, et al. 2014. Current concepts in the diagnosis and management of cytokine release syndrome. *Blood* 124:188-95
104. Morgan RA, Yang JC, Kitano M, Dudley ME, Laurencot CM, Rosenberg SA. 2010. Case report of a serious adverse event following the administration of T cells transduced with a chimeric antigen receptor recognizing ERBB2. *Mol Ther* 18:843-51
105. Pepys MB, Hirschfield GM. 2003. C-reactive protein: a critical update. *J Clin Invest* 111:1805-12
106. Davila ML, Riviere I, Wang X, Bartido S, Park J, et al. 2014. Efficacy and toxicity management of 19-28z CAR T cell therapy in B cell acute lymphoblastic leukemia. *Sci Transl Med* 6:224ra25
107. Hay KA, Hanafi LA, Li D, Gust J, Liles WC, et al. 2017. Kinetics and biomarkers of severe cytokine release syndrome after CD19 chimeric antigen receptor-modified T-cell therapy. *Blood* 130:2295-306
108. Shah NN, Fry TJ. 2019. Mechanisms of resistance to CAR T cell therapy. *Nat Rev Clin Oncol* 16:372-85
109. Majzner RG, Mackall CL. 2018. Tumor Antigen Escape from CAR T-cell Therapy. *Cancer Discov* 8:1219-26
110. Brudno JN, Maric I, Hartman SD, Rose JJ, Wang M, et al. 2018. T Cells Genetically Modified to Express an Anti-B-Cell Maturation Antigen Chimeric Antigen Receptor Cause Remissions of Poor-Prognosis Relapsed Multiple Myeloma. *J Clin Oncol* 36:2267-80
111. Cohen AD, Garfall AL, Stadtmauer EA, Melenhorst JJ, Lacey SF, et al. 2019. B cell maturation antigen-specific CAR T cells are clinically active in multiple myeloma. *J Clin Invest* 129:2210-21
112. Brown CE, Alizadeh D, Starr R, Weng L, Wagner JR, et al. 2016. Regression of Glioblastoma after Chimeric Antigen Receptor T-Cell Therapy. *N Engl J Med* 375:2561-9
113. Fischer J, Paret C, El Malki K, Alt F, Wingerter A, et al. 2017. CD19 Isoforms Enabling Resistance to CART-19 Immunotherapy Are Expressed in B-ALL Patients at Initial Diagnosis. *J Immunother* 40:187-95
114. Xu X, Sun Q, Liang X, Chen Z, Zhang X, et al. 2019. Mechanisms of Relapse After CD19 CAR T-Cell Therapy for Acute Lymphoblastic Leukemia and Its Prevention and Treatment Strategies. *Front Immunol* 10:2664
115. Sotillo E, Barrett DM, Black KL, Bagashev A, Oldridge D, et al. 2015. Convergence of Acquired Mutations and Alternative Splicing of CD19 Enables Resistance to CART-19 Immunotherapy. *Cancer Discov* 5:1282-95
116. Liao W, Kohler ME, Fry T, Ernst P. 2021. Does lineage plasticity enable escape from CAR-T cell therapy? Lessons from MLL-r leukemia. *Exp Hematol* 100:1-11
117. Gardner R, Wu D, Cherian S, Fang M, Hanafi LA, et al. 2016. Acquisition of a CD19-negative myeloid phenotype allows immune escape of MLL-rearranged B-ALL from CD19 CAR-T-cell therapy. *Blood* 127:2406-10

118. Oberley MJ, Gaynon PS, Bhojwani D, Pulsipher MA, Gardner RA, et al. 2018. Myeloid lineage switch following chimeric antigen receptor T-cell therapy in a patient with TCF3-ZNF384 fusion-positive B-lymphoblastic leukemia. *Pediatr Blood Cancer* 65:e27265
119. Ruella M, Maus MV. 2016. Catch me if you can: Leukemia Escape after CD19-Directed T Cell Immunotherapies. *Comput Struct Biotechnol J* 14:357-62
120. Hegde M, Mukherjee M, Grada Z, Pignata A, Landi D, et al. 2016. Tandem CAR T cells targeting HER2 and IL13Rα2 mitigate tumor antigen escape. *J Clin Invest* 126:3036-52
121. Han X, Wang Y, Wei J, Han W. 2019. Multi-antigen-targeted chimeric antigen receptor T cells for cancer therapy. *J Hematol Oncol* 12:128
122. Hegde M, Corder A, Chow KK, Mukherjee M, Ashoori A, et al. 2013. Combinational targeting offsets antigen escape and enhances effector functions of adoptively transferred T cells in glioblastoma. *Mol Ther* 21:2087-101
123. Wilkie S, van Schalkwyk MC, Hobbs S, Davies DM, van der Stegen SJ, et al. 2012. Dual targeting of ErbB2 and MUC1 in breast cancer using chimeric antigen receptors engineered to provide complementary signaling. *J Clin Immunol* 32:1059-70
124. Wang N, Hu X, Cao W, Li C, Xiao Y, et al. 2020. Efficacy and safety of CAR19/22 T-cell cocktail therapy in patients with refractory/relapsed B-cell malignancies. *Blood* 135:17-27
125. Huebner RJ, Rowe WP, Ward TG, Parrott RH, Bell JA. 1954. Adenoidal-pharyngeal-conjunctival agents: a newly recognized group of common viruses of the respiratory system. *N Engl J Med* 251:1077-86
126. Douglas JT. 2007. Adenoviral vectors for gene therapy. *Mol Biotechnol* 36:71-80
127. Bergelson JM, Cunningham JA, Droguett G, Kurt-Jones EA, Krithivas A, et al. 1997. Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. *Science* 275:1320-3
128. Li E, Stupack D, Bokoch GM, Nemerow GR. 1998. Adenovirus endocytosis requires actin cytoskeleton reorganization mediated by Rho family GTPases. *J Virol* 72:8806-12
129. Gaden F, Franqueville L, Magnusson MK, Hong SS, Merten MD, et al. 2004. Gene transduction and cell entry pathway of fiber-modified adenovirus type 5 vectors carrying novel endocytic peptide ligands selected on human tracheal glandular cells. *J Virol* 78:7227-47
130. Bremner KH, Scherer J, Yi J, Vershinin M, Gross SP, Vallee RB. 2009. Adenovirus transport via direct interaction of cytoplasmic dynein with the viral capsid hexon subunit. *Cell Host Microbe* 6:523-35
131. Rekosh DM, Russell WC, Bellet AJ, Robinson AJ. 1977. Identification of a protein linked to the ends of adenovirus DNA. *Cell* 11:283-95
132. Gingeras TR, Sciaky D, Gelinas RE, Bing-Dong J, Yen CE, et al. 1982. Nucleotide sequences from the adenovirus-2 genome. *J Biol Chem* 257:13475-91
133. Akusjärvi G. 1993. Proteins with transcription regulatory properties encoded by human adenoviruses. *Trends Microbiol* 1:163-70
134. McGrory WJ, Bautista DS, Graham FL. 1988. A simple technique for the rescue of early region I mutations into infectious human adenovirus type 5. *Virology* 163:614-7
135. Yang Y, Nunes FA, Berencsi K, Furth EE, Gönczöl E, Wilson JM. 1994. Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy. *Proc Natl Acad Sci U S A* 91:4407-11
136. Engelhardt JF, Ye X, Doranz B, Wilson JM. 1994. Ablation of E2A in recombinant adenoviruses improves transgene persistence and decreases inflammatory response in mouse liver. *Proc Natl Acad Sci U S A* 91:6196-200
137. Gorziglia MI, Kadan MJ, Yei S, Lim J, Lee GM, et al. 1996. Elimination of both E1 and E2 from adenovirus vectors further improves prospects for in vivo human gene therapy. *J Virol* 70:4173-8
138. Amalfitano A, Hauser MA, Hu H, Serra D, Begy CR, Chamberlain JS. 1998. Production and characterization of improved adenovirus vectors with the E1, E2b, and E3 genes deleted. *J Virol* 72:926-33
139. Wang Q, Finer MH. 1996. Second-generation adenovirus vectors. *Nat Med* 2:714-6

140. Alemany R, Dai Y, Lou YC, Sethi E, Prokopenko E, et al. 1997. Complementation of helper-dependent adenoviral vectors: size effects and titer fluctuations. *J Virol Methods* 68:147-59
141. Mitani K, Graham FL, Caskey CT, Kochanek S. 1995. Rescue, propagation, and partial purification of a helper virus-dependent adenovirus vector. *Proc Natl Acad Sci U S A* 92:3854-8
142. Parks RJ, Chen L, Anton M, Sankar U, Rudnicki MA, Graham FL. 1996. A helper-dependent adenovirus vector system: removal of helper virus by Cre-mediated excision of the viral packaging signal. *Proc Natl Acad Sci U S A* 93:13565-70
143. Hillgenberg M, Tönnies H, Strauss M. 2001. Chromosomal integration pattern of a helper-dependent minimal adenovirus vector with a selectable marker inserted into a 27.4-kilobase genomic stuffer. *J Virol* 75:9896-908
144. Harui A, Suzuki S, Kochanek S, Mitani K. 1999. Frequency and stability of chromosomal integration of adenovirus vectors. *J Virol* 73:6141-6
145. Stephen SL, Sivanandam VG, Kochanek S. 2008. Homologous and heterologous recombination between adenovirus vector DNA and chromosomal DNA. *J Gene Med* 10:1176-89
146. Yu D, Jin C, Ramachandran M, Xu J, Nilsson B, et al. 2013. Adenovirus serotype 5 vectors with Tat-PTD modified hexon and serotype 35 fiber show greatly enhanced transduction capacity of primary cell cultures. *PLoS One* 8:e54952
147. Schroers R, Hildebrandt Y, Hasenkamp J, Glass B, Lieber A, et al. 2004. Gene transfer into human T lymphocytes and natural killer cells by Ad5/F35 chimeric adenoviral vectors. *Exp Hematol* 32:536-46
148. Lv Y, Xiao FJ, Wang Y, Zou XH, Wang H, et al. 2019. Efficient gene transfer into T lymphocytes by fiber-modified human adenovirus 5. *BMC Biotechnol* 19:23
149. Atchison RW, Casto BC, Hammon WM. 1965. ADENOVIRUS-ASSOCIATED DEFECTIVE VIRUS PARTICLES. *Science* 149:754-6
150. Hoggan MD, Blacklow NR, Rowe WP. 1966. Studies of small DNA viruses found in various adenovirus preparations: physical, biological, and immunological characteristics. *Proc Natl Acad Sci U S A* 55:1467-74
151. Rose JA, Berns KI, Hoggan MD, Koczot FJ. 1969. Evidence for a single-stranded adenovirus-associated virus genome: formation of a DNA density hybrid on release of viral DNA. *Proc Natl Acad Sci U S A* 64:863-9
152. Lusby E, Fife KH, Berns KI. 1980. Nucleotide sequence of the inverted terminal repetition in adeno-associated virus DNA. *J Virol* 34:402-9
153. Carter BJ, Khoury G, Denhardt DT. 1975. Physical map and strand polarity of specific fragments of adenovirus-associated virus DNA produced by endonuclease R-EcoRI. *J Virol* 16:559-68
154. Carter BJ, Khoury G, Rose JA. 1972. Adenovirus-associated virus multiplication. IX. Extent of transcription of the viral genome in vivo. *J Virol* 10:1118-25
155. Hauswirth WW, Berns KI. 1977. Origin and termination of adeno-associated virus DNA replication. *Virology* 78:488-99
156. Berns KI, Pinkerton TC, Thomas GF, Hoggan MD. 1975. Detection of adeno-associated virus (AAV)-specific nucleotide sequences in DNA isolated from latently infected Detroit 6 cells. *Virology* 68:556-60
157. Mietzsch M, Broecker F, Reinhardt A, Seeberger PH, Heilbronn R. 2014. Differential adeno-associated virus serotype-specific interaction patterns with synthetic heparins and other glycans. *J Virol* 88:2991-3003
158. Zincarelli C, Soltys S, Rengo G, Rabinowitz JE. 2008. Analysis of AAV serotypes 1-9 mediated gene expression and tropism in mice after systemic injection. *Mol Ther* 16:1073-80
159. Samulski RJ, Berns KI, Tan M, Muzyczka N. 1982. Cloning of adeno-associated virus into pBR322: rescue of intact virus from the recombinant plasmid in human cells. *Proc Natl Acad Sci U S A* 79:2077-81
160. Laughlin CA, Tratschin JD, Coon H, Carter BJ. 1983. Cloning of infectious adeno-associated virus genomes in bacterial plasmids. *Gene* 23:65-73

161. Tratschin JD, West MH, Sandbank T, Carter BJ. 1984. A human parvovirus, adeno-associated virus, as a eucaryotic vector: transient expression and encapsidation of the procaryotic gene for chloramphenicol acetyltransferase. *Mol Cell Biol* 4:2072-81
162. Carter BJ, Koczot FJ, Garrison J, Rose JA, Dolin R. 1973. Separate helper functions provided by adenovirus for adenovirus-associated virus multiplication. *Nat New Biol* 244:71-3
163. Samulski RJ, Chang LS, Shenk T. 1989. Helper-free stocks of recombinant adeno-associated viruses: normal integration does not require viral gene expression. *J Virol* 63:3822-8
164. Xiao X, Li J, Samulski RJ. 1998. Production of high-titer recombinant adeno-associated virus vectors in the absence of helper adenovirus. *J Virol* 72:2224-32
165. Kaplitt MG, Xiao X, Samulski RJ, Li J, Ojamaa K, et al. 1996. Long-term gene transfer in porcine myocardium after coronary infusion of an adeno-associated virus vector. *Ann Thorac Surg* 62:1669-76
166. Kimura T, Ferran B, Tsukahara Y, Shang Q, Desai S, et al. 2019. Production of adeno-associated virus vectors for in vitro and in vivo applications. *Sci Rep* 9:13601
167. Grieger JC, Soltys SM, Samulski RJ. 2016. Production of Recombinant Adeno-associated Virus Vectors Using Suspension HEK293 Cells and Continuous Harvest of Vector From the Culture Media for GMP FIX and FLT1 Clinical Vector. *Mol Ther* 24:287-97
168. McCarty DM, Monahan PE, Samulski RJ. 2001. Self-complementary recombinant adeno-associated virus (scAAV) vectors promote efficient transduction independently of DNA synthesis. *Gene Ther* 8:1248-54
169. Neumann E, Schaefer-Ridder M, Wang Y, Hofschneider PH. 1982. Gene transfer into mouse lyoma cells by electroporation in high electric fields. *Embo j* 1:841-5
170. Crowley JM. 1973. Electrical breakdown of bimolecular lipid membranes as an electromechanical instability. *Biophys J* 13:711-24
171. Sugár IP. 1979. A theory of the electric field-induced phase transition of phospholipid bilayers. *Biochim Biophys Acta* 556:72-85
172. Cruzeiro-Hansson L, Mouritsen OG. 1988. Passive ion permeability of lipid membranes modelled via lipid-domain interfacial area. *Biochim Biophys Acta* 944:63-72
173. Abidor IG, Arakelyan VB, Chernomordik LV, Chizmadzhev YA, Pastushenko VF, Tarasevich MP. 1979. Electric breakdown of bilayer lipid membranes: I. The main experimental facts and their qualitative discussion. *Journal of Electroanalytical Chemistry and Interfacial Electrochemistry* 104:37-52
174. Barnett A, Weaver JC. 1991. Electroporation: a unified, quantitative theory of reversible electrical breakdown and mechanical rupture in artificial planar bilayer membranes. *Journal of Electroanalytical Chemistry and Interfacial Electrochemistry* 320:163-82
175. Freeman SA, Wang MA, Weaver JC. 1994. Theory of electroporation of planar bilayer membranes: predictions of the aqueous area, change in capacitance, and pore-pore separation. *Biophys J* 67:42-56
176. Spugnini EP, Arancia G, Porrello A, Colone M, Formisano G, et al. 2007. Ultrastructural modifications of cell membranes induced by "electroporation" on melanoma xenografts. *Microsc Res Tech* 70:1041-50
177. Kotnik T, Kramar P, Pucihar G, Miklavcic D, Tarek M. 2012. Cell membrane electroporation-Part 1: The phenomenon. *IEEE Electrical Insulation Magazine* 28:14-23
178. Tavernier G, Andries O, Demeester J, Sanders NN, De Smedt SC, Rejman J. 2011. mRNA as gene therapeutic: how to control protein expression. *J Control Release* 150:238-47
179. Gallie DR. 1991. The cap and poly(A) tail function synergistically to regulate mRNA translational efficiency. *Genes Dev* 5:2108-16
180. Peng J, Murray EL, Schoenberg DR. 2008. In vivo and in vitro analysis of poly(A) length effects on mRNA translation. *Methods Mol Biol* 419:215-30

181. Mockey M, Gonçalves C, Dupuy FP, Lemoine FM, Pichon C, Midoux P. 2006. mRNA transfection of dendritic cells: synergistic effect of ARCA mRNA capping with Poly(A) chains in cis and in trans for a high protein expression level. *Biochem Biophys Res Commun* 340:1062-8
182. Yamamoto A, Kormann M, Rosenecker J, Rudolph C. 2009. Current prospects for mRNA gene delivery. *Eur J Pharm Biopharm* 71:484-9
183. Gao M, Zhang Q, Feng XH, Liu J. 2021. Synthetic modified messenger RNA for therapeutic applications. *Acta Biomater* 131:1-15
184. Chicaybam L, Barcelos C, Peixoto B, Carneiro M, Limia CG, et al. 2016. An Efficient Electroporation Protocol for the Genetic Modification of Mammalian Cells. *Front Bioeng Biotechnol* 4:99
185. Michiels A, Tuyaerts S, Bonehill A, Corthals J, Breckpot K, et al. 2005. Electroporation of immature and mature dendritic cells: implications for dendritic cell-based vaccines. *Gene Ther* 12:772-82
186. Javorovic M, Pohla H, Frankenberger B, Wölfel T, Schendel DJ. 2005. RNA transfer by electroporation into mature dendritic cells leading to reactivation of effector-memory cytotoxic T lymphocytes: a quantitative analysis. *Mol Ther* 12:734-43
187. Van Tendeloo VF, Ponsaerts P, Lardon F, Nijs G, Lenjou M, et al. 2001. Highly efficient gene delivery by mRNA electroporation in human hematopoietic cells: superiority to lipofection and passive pulsing of mRNA and to electroporation of plasmid cDNA for tumor antigen loading of dendritic cells. *Blood* 98:49-56
188. Zhang Z, Qiu S, Zhang X, Chen W. 2018. Optimized DNA electroporation for primary human T cell engineering. *BMC Biotechnol* 18:4
189. Zhao Y, Zheng Z, Cohen CJ, Gattinoni L, Palmer DC, et al. 2006. High-efficiency transfection of primary human and mouse T lymphocytes using RNA electroporation. *Mol Ther* 13:151-9
190. Ang WX, Ng YY, Xiao L, Chen C, Li Z, et al. 2020. Electroporation of NKG2D RNA CAR Improves Vγ9Vδ2 T Cell Responses against Human Solid Tumor Xenografts. *Mol Ther Oncolytics* 17:421-30
191. Harrer DC, Simon B, Fujii SI, Shimizu K, Uslu U, et al. 2017. RNA-transfection of γ/δ T cells with a chimeric antigen receptor or an α/β T-cell receptor: a safer alternative to genetically engineered α/β T cells for the immunotherapy of melanoma. *BMC Cancer* 17:551
192. Li L, Allen C, Shivakumar R, Peshwa MV. 2013. Large volume flow electroporation of mRNA: clinical scale process. *Methods Mol Biol* 969:127-38
193. Ingegnere T, Mariotti FR, Pelosi A, Quintarelli C, De Angelis B, et al. 2019. Human CAR NK Cells: A New Non-viral Method Allowing High Efficient Transfection and Strong Tumor Cell Killing. *Front Immunol* 10:957
194. Xu X, Gao D, Wang P, Chen J, Ruan J, et al. 2018. Efficient homology-directed gene editing by CRISPR/Cas9 in human stem and primary cells using tube electroporation. *Sci Rep* 8:11649
195. Lissandrello CA, Santos JA, Hsi P, Welch M, Mott VL, et al. 2020. High-throughput continuous-flow microfluidic electroporation of mRNA into primary human T cells for applications in cellular therapy manufacturing. *Sci Rep* 10:18045
196. Wiesinger M, März J, Kummer M, Schuler G, Dörrie J, et al. 2019. Clinical-Scale Production of CAR-T Cells for the Treatment of Melanoma Patients by mRNA Transfection of a CSPG4-Specific CAR under Full GMP Compliance. *Cancers (Basel)* 11
197. Bonehill A, Van Nuffel AM, Corthals J, Tuyaerts S, Heirman C, et al. 2009. Single-step antigen loading and activation of dendritic cells by mRNA electroporation for the purpose of therapeutic vaccination in melanoma patients. *Clin Cancer Res* 15:3366-75
198. Lesterhuis WJ, De Vries IJ, Schreibelt G, Schuurhuis DH, Aarntzen EH, et al. 2010. Immunogenicity of dendritic cells pulsed with CEA peptide or transfected with CEA mRNA for vaccination of colorectal cancer patients. *Anticancer Res* 30:5091-7

199. Wilgenhof S, Van Nuffel AM, Corthals J, Heirman C, Tuyaerts S, et al. 2011. Therapeutic vaccination with an autologous mRNA electroporated dendritic cell vaccine in patients with advanced melanoma. *J Immunother* 34:448-56
200. Lu Y, Xue J, Deng T, Zhou X, Yu K, et al. 2020. Safety and feasibility of CRISPR-edited T cells in patients with refractory non-small-cell lung cancer. *Nat Med* 26:732-40
201. Zhang J, Hu Y, Yang J, Li W, Zhang M, et al. 2022. Non-viral, specifically targeted CAR-T cells achieve high safety and efficacy in B-NHL. *Nature* 609:369-74
202. Bell MP, Huntoon CJ, Graham D, McKean DJ. 2001. The analysis of costimulatory receptor signaling cascades in normal T lymphocytes using in vitro gene transfer and reporter gene analysis. *Nat Med* 7:1155-8
203. Van Tendeloo VF, Willems R, Ponsaerts P, Lenjou M, Nijs G, et al. 2000. High-level transgene expression in primary human T lymphocytes and adult bone marrow CD34+ cells via electroporation-mediated gene delivery. *Gene Ther* 7:1431-7
204. Zhao Y, Zheng Z, Robbins PF, Khong HT, Rosenberg SA, Morgan RA. 2005. Primary human lymphocytes transduced with NY-ESO-1 antigen-specific TCR genes recognize and kill diverse human tumor cell lines. *J Immunol* 174:4415-23
205. Schaft N, Dörrie J, Müller I, Beck V, Baumann S, et al. 2006. A new way to generate cytolytic tumor-specific T cells: electroporation of RNA coding for a T cell receptor into T lymphocytes. *Cancer Immunol Immunother* 55:1132-41
206. Koh S, Shimasaki N, Suwanarusk R, Ho ZZ, Chia A, et al. 2013. A practical approach to immunotherapy of hepatocellular carcinoma using T cells redirected against hepatitis B virus. *Mol Ther Nucleic Acids* 2:e114
207. Mensali N, Myhre MR, Dillard P, Pollmann S, Gaudernack G, et al. 2019. Preclinical assessment of transiently TCR redirected T cells for solid tumour immunotherapy. *Cancer Immunol Immunother* 68:1235-43
208. Bendle GM, Linnemann C, Hooijkaas AI, Bies L, de Witte MA, et al. 2010. Lethal graft-versus-host disease in mouse models of T cell receptor gene therapy. *Nat Med* 16:565-70, 1p following 70
209. Campillo-Davo D, Fujiki F, Van den Bergh JMJ, De Reu H, Smits E, et al. 2018. Efficient and Non-genotoxic RNA-Based Engineering of Human T Cells Using Tumor-Specific T Cell Receptors With Minimal TCR Mispairing. *Front Immunol* 9:2503
210. Almåsbak H, Rian E, Hoel HJ, Pulè M, Wälchli S, et al. 2011. Transiently redirected T cells for adoptive transfer. *Cytotherapy* 13:629-40
211. Barrett DM, Zhao Y, Liu X, Jiang S, Carpenito C, et al. 2011. Treatment of advanced leukemia in mice with mRNA engineered T cells. *Hum Gene Ther* 22:1575-86
212. Rabinovich PM, Komarovskaya ME, Wrzesinski SH, Alderman JL, Budak-Alpdogan T, et al. 2009. Chimeric receptor mRNA transfection as a tool to generate antineoplastic lymphocytes. *Hum Gene Ther* 20:51-61
213. Yoon SH, Lee JM, Cho HI, Kim EK, Kim HS, et al. 2009. Adoptive immunotherapy using human peripheral blood lymphocytes transferred with RNA encoding Her-2/neu-specific chimeric immune receptor in ovarian cancer xenograft model. *Cancer Gene Ther* 16:489-97
214. Zhao Y, Moon E, Carpenito C, Paulos CM, Liu X, et al. 2010. Multiple injections of electroporated autologous T cells expressing a chimeric antigen receptor mediate regression of human disseminated tumor. *Cancer Res* 70:9053-61
215. Birkholz K, Hombach A, Krug C, Reuter S, Kershaw M, et al. 2009. Transfer of mRNA encoding recombinant immunoreceptors reprograms CD4+ and CD8+ T cells for use in the adoptive immunotherapy of cancer. *Gene Ther* 16:596-604
216. Barrett DM, Liu X, Jiang S, June CH, Grupp SA, Zhao Y. 2013. Regimen-specific effects of RNA-modified chimeric antigen receptor T cells in mice with advanced leukemia. *Hum Gene Ther* 24:717-27

217. Wang D, Zhou Q, Qiu X, Liu X, Zhang C. 2022. Optimizing rAAV6 transduction of primary T cells for the generation of anti-CD19 AAV-CAR-T cells. *Biomed Pharmacother* 150:113027
218. Svoboda J, Rheingold SR, Gill SI, Grupp SA, Lacey SF, et al. 2018. Nonviral RNA chimeric antigen receptor-modified T cells in patients with Hodgkin lymphoma. *Blood* 132:1022-6
219. Beatty GL, Haas AR, Maus MV, Torigian DA, Soulen MC, et al. 2014. Mesothelin-specific chimeric antigen receptor mRNA-engineered T cells induce anti-tumor activity in solid malignancies. *Cancer Immunol Res* 2:112-20
220. Maus MV, Haas AR, Beatty GL, Albelda SM, Levine BL, et al. 2013. T cells expressing chimeric antigen receptors can cause anaphylaxis in humans. *Cancer Immunol Res* 1:26-31
221. Tchou J, Zhao Y, Levine BL, Zhang PJ, Davis MM, et al. 2017. Safety and Efficacy of Intratumoral Injections of Chimeric Antigen Receptor (CAR) T Cells in Metastatic Breast Cancer. *Cancer Immunol Res* 5:1152-61
222. Boissel L, Tuncer HH, Betancur M, Wolfberg A, Klingemann H. 2008. Umbilical cord mesenchymal stem cells increase expansion of cord blood natural killer cells. *Biol Blood Marrow Transplant* 14:1031-8
223. Boissel L, Betancur M, Lu W, Wels WS, Marino T, et al. 2012. Comparison of mRNA and lentiviral based transfection of natural killer cells with chimeric antigen receptors recognizing lymphoid antigens. *Leuk Lymphoma* 53:958-65
224. Li L, Liu LN, Feller S, Allen C, Shivakumar R, et al. 2010. Expression of chimeric antigen receptors in natural killer cells with a regulatory-compliant non-viral method. *Cancer Gene Ther* 17:147-54
225. Simon B, Wiesinger M, März J, Wistuba-Hamprecht K, Weide B, et al. 2018. The Generation of CAR-Transfected Natural Killer T Cells for the Immunotherapy of Melanoma. *Int J Mol Sci* 19
226. Yoon SH, Lee JM, Woo SJ, Park MJ, Park JS, et al. 2009. Transfer of Her-2/neu specificity into cytokine-induced killer (CIK) cells with RNA encoding chimeric immune receptor (CIR). *J Clin Immunol* 29:806-14
227. Motzer RJ, Rakhit A, Schwartz LH, Olencki T, Malone TM, et al. 1998. Phase I trial of subcutaneous recombinant human interleukin-12 in patients with advanced renal cell carcinoma. *Clin Cancer Res* 4:1183-91
228. Lenzi R, Edwards R, June C, Seiden MV, Garcia ME, et al. 2007. Phase II study of intraperitoneal recombinant interleukin-12 (rhIL-12) in patients with peritoneal carcinomatosis (residual disease < 1 cm) associated with ovarian cancer or primary peritoneal carcinoma. *J Transl Med* 5:66
229. Conlon KC, Lugli E, Welles HC, Rosenberg SA, Fojo AT, et al. 2015. Redistribution, hyperproliferation, activation of natural killer cells and CD8 T cells, and cytokine production during first-in-human clinical trial of recombinant human interleukin-15 in patients with cancer. *J Clin Oncol* 33:74-82
230. Lee JM, Yoon SH, Kim HS, Kim SY, Sohn HJ, et al. 2010. Direct and indirect antitumor effects by human peripheral blood lymphocytes expressing both chimeric immune receptor and interleukin-2 in ovarian cancer xenograft model. *Cancer Gene Ther* 17:742-50
231. Etxeberria I, Bolaños E, Quetglas JI, Gros A, Villanueva A, et al. 2019. Intratumor Adoptive Transfer of IL-12 mRNA Transiently Engineered Antitumor CD8(+) T Cells. *Cancer Cell* 36:613-29.e7
232. Olivera I, Bolaños E, Gonzalez-Gomariz J, Hervas-Stubbs S, Mariño KV, et al. 2023. mRNAs encoding IL-12 and a decoy-resistant variant of IL-18 synergize to engineer T cells for efficacious intratumoral adoptive immunotherapy. *Cell Rep Med* 4:100978
233. You CX, Liu Y, Shi M, Cao M, Luo RC, Hermonat PL. 2010. Comparison of AAV/IL-7 autocrine (T cell) versus paracrine (DC) gene delivery for enhancing CTL stimulation and function. *Cancer Immunol Immunother* 59:779-87

234. Rowley J, Monie A, Hung CF, Wu TC. 2009. Expression of IL-15RA or an IL-15/IL-15RA fusion on CD8+ T cells modifies adoptively transferred T-cell function in cis. *Eur J Immunol* 39:491-506
235. Weinstein-Marom H, Pato A, Levin N, Susid K, Itzhaki O, et al. 2016. Membrane-attached Cytokines Expressed by mRNA Electroporation Act as Potent T-Cell Adjuvants. *J Immunother* 39:60-70
236. Medzhitov R. 2001. Toll-like receptors and innate immunity. *Nat Rev Immunol* 1:135-45
237. Pato A, Eisenberg G, Machlenkin A, Margalit A, Cafri G, et al. 2015. Messenger RNA encoding constitutively active Toll-like receptor 4 enhances effector functions of human T cells. *Clin Exp Immunol* 182:220-9
238. Elgueta R, Benson MJ, de Vries VC, Wasiuk A, Guo Y, Noelle RJ. 2009. Molecular mechanism and function of CD40/CD40L engagement in the immune system. *Immunol Rev* 229:152-72
239. Levin N, Weinstein-Marom H, Pato A, Itzhaki O, Besser MJ, et al. 2018. Potent Activation of Human T Cells by mRNA Encoding Constitutively Active CD40. *J Immunol* 201:2959-68
240. Mitchell DA, Karikari I, Cui X, Xie W, Schmittling R, Sampson JH. 2008. Selective modification of antigen-specific T cells by RNA electroporation. *Hum Gene Ther* 19:511-21
241. Carlsten M, Levy E, Karambelkar A, Li L, Reger R, et al. 2016. Efficient mRNA-Based Genetic Engineering of Human NK Cells with High-Affinity CD16 and CCR7 Augments Rituximab-Induced ADCC against Lymphoma and Targets NK Cell Migration toward the Lymph Node-Associated Chemokine CCL19. *Front Immunol* 7:105
242. Ng YY, Tay JCK, Wang S. 2020. CXCR1 Expression to Improve Anti-Cancer Efficacy of Intravenously Injected CAR-NK Cells in Mice with Peritoneal Xenografts. *Mol Ther Oncolytics* 16:75-85
243. Wu YL, Ding YP, Tanaka Y, Shen LW, Wei CH, et al. 2014. γδ T cells and their potential for immunotherapy. *Int J Biol Sci* 10:119-35
244. Ribot JC, Lopes N, Silva-Santos B. 2021. γδ T cells in tissue physiology and surveillance. *Nat Rev Immunol* 21:221-32
245. Witherden DA, Johnson MD, Havran WL. 2018. Coreceptors and Their Ligands in Epithelial γδ T Cell Biology. *Front Immunol* 9:731
246. Davey MS, Willcox CR, Joyce SP, Ladell K, Kasatskaya SA, et al. 2017. Clonal selection in the human Vδ1 T cell repertoire indicates γδ TCR-dependent adaptive immune surveillance. *Nat Commun* 8:14760
247. Déchanet J, Merville P, Lim A, Retière C, Pitard V, et al. 1999. Implication of gammadelta T cells in the human immune response to cytomegalovirus. *J Clin Invest* 103:1437-49
248. Couzi L, Pitard V, Netzer S, Garrigue I, Lafon ME, et al. 2009. Common features of gammadelta T cells and CD8(+) alphabeta T cells responding to human cytomegalovirus infection in kidney transplant recipients. *J Infect Dis* 200:1415-24
249. Farnault L, Gertner-Dardenne J, Gondois-Rey F, Michel G, Chambost H, et al. 2013. Clinical evidence implicating gamma-delta T cells in EBV control following cord blood transplantation. *Bone Marrow Transplant* 48:1478-9
250. Correia DV, Fogli M, Hudspeth K, da Silva MG, Mavilio D, Silva-Santos B. 2011. Differentiation of human peripheral blood Vδ1+ T cells expressing the natural cytotoxicity receptor NKp30 for recognition of lymphoid leukemia cells. *Blood* 118:992-1001
251. Mikulak J, Oriolo F, Bruni E, Roberto A, Colombo FS, et al. 2019. NKp46-expressing human gut-resident intraepithelial Vδ1 T cell subpopulation exhibits high antitumor activity against colorectal cancer. *JCI Insight* 4
252. Davey MS, Willcox CR, Hunter S, Oo YH, Willcox BE. 2018. Vδ2(+) T Cells-Two Subsets for the Price of One. *Front Immunol* 9:2106
253. Davey MS, Willcox CR, Hunter S, Kasatskaya SA, Remmerswaal EBM, et al. 2018. The human Vδ2(+) T-cell compartment comprises distinct innate-like Vγ9(+) and adaptive Vγ9(-) subsets. *Nat Commun* 9:1760

254. Tanaka Y, Sano S, Nieves E, De Libero G, Rosa D, et al. 1994. Nonpeptide ligands for human gamma delta T cells. *Proc Natl Acad Sci U S A* 91:8175-9
255. Costa G, Loizon S, Guenot M, Mocan I, Halary F, et al. 2011. Control of Plasmodium falciparum erythrocytic cycle: γδ T cells target the red blood cell-invasive merozoites. *Blood* 118:6952-62
256. Silva-Santos B, Serre K, Norell H. 2015. γδ T cells in cancer. *Nat Rev Immunol* 15:683-91
257. Capsomidis A, Benthall G, Van Acker HH, Fisher J, Kramer AM, et al. 2018. Chimeric Antigen Receptor-Engineered Human Gamma Delta T Cells: Enhanced Cytotoxicity with Retention of Cross Presentation. *Mol Ther* 26:354-65
258. Davey MS, Morgan MP, Liuzzi AR, Tyler CJ, Khan MWA, et al. 2014. Microbe-specific unconventional T cells induce human neutrophil differentiation into antigen cross-presenting cells. *J Immunol* 193:3704-16
259. Tyler CJ, Doherty DG, Moser B, Eberl M. 2015. Human Vγ9/Vδ2 T cells: Innate adaptors of the immune system. *Cell Immunol* 296:10-21
260. Kenna T, Golden-Mason L, Norris S, Hegarty JE, O'Farrelly C, Doherty DG. 2004. Distinct subpopulations of gamma delta T cells are present in normal and tumor-bearing human liver. *Clin Immunol* 113:56-63
261. Dunne MR, Elliott L, Hussey S, Mahmud N, Kelly J, et al. 2013. Persistent changes in circulating and intestinal γδ T cell subsets, invariant natural killer T cells and mucosal-associated invariant T cells in children and adults with coeliac disease. *PLoS One* 8:e76008
262. Knight A, Madrigal AJ, Grace S, Sivakumaran J, Kottaridis P, et al. 2010. The role of Vδ2-negative γδ T cells during cytomegalovirus reactivation in recipients of allogeneic stem cell transplantation. *Blood* 116:2164-72
263. Kabelitz D, Hinz T, Dobmeyer T, Mentzel U, Marx S, et al. 1997. Clonal expansion of Vgamma3/Vdelta3-expressing gammadelta T cells in an HIV-1/2-negative patient with CD4 T-cell deficiency. *Br J Haematol* 96:266-71
264. Bartkowiak J, Kulczyck-Wojdala D, Blonski JZ, Robak T. 2002. Molecular diversity of gammadelta T cells in peripheral blood from patients with B-cell chronic lymphocytic leukaemia. *Neoplasma* 49:86-90
265. Mangan BA, Dunne MR, O'Reilly VP, Dunne PJ, Exley MA, et al. 2013. Cutting edge: CD1d restriction and Th1/Th2/Th17 cytokine secretion by human Vδ3 T cells. *J Immunol* 191:30-4
266. Petrasca A, Melo AM, Breen EP, Doherty DG. 2018. Human Vδ3(+) γδ T cells induce maturation and IgM secretion by B cells. *Immunol Lett* 196:126-34
267. Riganti C, Massaia M, Davey MS, Eberl M. 2012. Human γδ T-cell responses in infection and immunotherapy: common mechanisms, common mediators? *Eur J Immunol* 42:1668-76
268. Harly C, Guillaume Y, Nedellec S, Peigné CM, Mönkkönen H, et al. 2012. Key implication of CD277/butyrophilin-3 (BTN3A) in cellular stress sensing by a major human γδ T-cell subset. *Blood* 120:2269-79
269. Rhodes DA, Chen HC, Price AJ, Keeble AH, Davey MS, et al. 2015. Activation of human γδ T cells by cytosolic interactions of BTN3A1 with soluble phosphoantigens and the cytoskeletal adaptor periplakin. *J Immunol* 194:2390-8
270. Rigau M, Ostrouska S, Fulford TS, Johnson DN, Woods K, et al. 2020. Butyrophilin 2A1 is essential for phosphoantigen reactivity by γδ T cells. *Science* 367
271. Das H, Groh V, Kuijl C, Sugita M, Morita CT, et al. 2001. MICA engagement by human Vgamma2Vdelta2 T cells enhances their antigen-dependent effector function. *Immunity* 15:83-93
272. Groh V, Rhinehart R, Randolph-Habecker J, Topp MS, Riddell SR, Spies T. 2001. Costimulation of CD8alphabeta T cells by NKG2D via engagement by MIC induced on virus-infected cells. *Nat Immunol* 2:255-60
273. Rincon-Orozco B, Kunzmann V, Wrobel P, Kabelitz D, Steinle A, Herrmann T. 2005. Activation of V gamma 9V delta 2 T cells by NKG2D. *J Immunol* 175:2144-51
274. Guerra N, Tan YX, Joncker NT, Choy A, Gallardo F, et al. 2008. NKG2D-deficient mice are defective in tumor surveillance in models of spontaneous malignancy. *Immunity* 28:571-80

275. Raulet DH. 2003. Roles of the NKG2D immunoreceptor and its ligands. *Nat Rev Immunol* 3:781-90
276. Salih HR, Antropius H, Gieseke F, Lutz SZ, Kanz L, et al. 2003. Functional expression and release of ligands for the activating immunoreceptor NKG2D in leukemia. *Blood* 102:1389-96
277. Cimini E, Sacchi A, De Minicis S, Bordoni V, Casetti R, et al. 2019. Vδ2 T-Cells Kill ZIKV-Infected Cells by NKG2D-Mediated Cytotoxicity. *Microorganisms* 7
278. Lawand M, Déchanet-Merville J, Dieu-Nosjean MC. 2017. Key Features of Gamma-Delta T-Cell Subsets in Human Diseases and Their Immunotherapeutic Implications. *Front Immunol* 8:761
279. Couzi L, Pitard V, Sicard X, Garrigue I, Hawchar O, et al. 2012. Antibody-dependent anti-cytomegalovirus activity of human γδ T cells expressing CD16 (FcγRIIIa). *Blood* 119:1418-27
280. Lafont V, Liautard J, Liautard JP, Favero J. 2001. Production of TNF-alpha by human V gamma 9V delta 2 T cells via engagement of Fc gamma RIIIA, the low affinity type 3 receptor for the Fc portion of IgG, expressed upon TCR activation by nonpeptidic antigen. *J Immunol* 166:7190-9
281. Braza MS, Klein B, Fiol G, Rossi JF. 2011. γδ T-cell killing of primary follicular lymphoma cells is dramatically potentiated by GA101, a type II glycoengineered anti-CD20 monoclonal antibody. *Haematologica* 96:400-7
282. Tokuyama H, Hagi T, Mattarollo SR, Morley J, Wang Q, et al. 2008. V gamma 9 V delta 2 T cell cytotoxicity against tumor cells is enhanced by monoclonal antibody drugs--rituximab and trastuzumab. *Int J Cancer* 122:2526-34
283. Capietto AH, Martinet L, Fournié JJ. 2011. Stimulated γδ T cells increase the in vivo efficacy of trastuzumab in HER-2+ breast cancer. *J Immunol* 187:1031-8
284. Dokouhaki P, Schuh NW, Joe B, Allen CA, Der SD, et al. 2013. NKG2D regulates production of soluble TRAIL by ex vivo expanded human γδ T cells. *Eur J Immunol* 43:3175-82
285. Li Z, Xu Q, Peng H, Cheng R, Sun Z, Ye Z. 2011. IFN-γ enhances HOS and U2OS cell lines susceptibility to γδ T cell-mediated killing through the Fas/Fas ligand pathway. *Int Immunopharmacol* 11:496-503
286. Xiao L, Chen C, Li Z, Zhu S, Tay JC, et al. 2018. Large-scale expansion of Vγ9Vδ2 T cells with engineered K562 feeder cells in G-Rex vessels and their use as chimeric antigen receptor-modified effector cells. *Cytotherapy* 20:420-35
287. Sutton KS, Dasgupta A, McCarty D, Doering CB, Spencer HT. 2016. Bioengineering and serum free expansion of blood-derived γδ T cells. *Cytotherapy* 18:881-92
288. Wang RN, Wen Q, He WT, Yang JH, Zhou CY, et al. 2019. Optimized protocols for γδ T cell expansion and lentiviral transduction. *Mol Med Rep* 19:1471-80
289. Zoine JT, Knight KA, Fleischer LC, Sutton KS, Goldsmith KC, et al. 2019. Ex vivo expanded patient-derived γδ T-cell immunotherapy enhances neuroblastoma tumor regression in a murine model. *Oncoimmunology* 8:1593804
290. Salot S, Laplace C, Saïagh S, Bercegeay S, Tenaud I, et al. 2007. Large scale expansion of gamma 9 delta 2 T lymphocytes: Innacell gamma delta cell therapy product. *J Immunol Methods* 326:63-75
291. Godder KT, Henslee-Downey PJ, Mehta J, Park BS, Chiang KY, et al. 2007. Long term disease-free survival in acute leukemia patients recovering with increased gammadelta T cells after partially mismatched related donor bone marrow transplantation. *Bone Marrow Transplant* 39:751-7
292. Saura-Esteller J, de Jong M, King LA, Ensing E, Winograd B, et al. 2022. Gamma Delta T-Cell Based Cancer Immunotherapy: Past-Present-Future. *Front Immunol* 13:915837
293. Xu Y, Xiang Z, Alnaggar M, Kouakanou L, Li J, et al. 2021. Allogeneic Vγ9Vδ2 T-cell immunotherapy exhibits promising clinical safety and prolongs the survival of patients with late-stage lung or liver cancer. *Cell Mol Immunol* 18:427-39
294. Lin M, Zhang X, Liang S, Luo H, Alnaggar M, et al. 2020. Irreversible electroporation plus allogenic Vγ9Vδ2 T cells enhances antitumor effect for locally advanced pancreatic cancer patients. *Signal Transduct Target Ther* 5:215

295. Kaufmann KB, Büning H, Galy A, Schambach A, Grez M. 2013. Gene therapy on the move. *EMBO Mol Med* 5:1642-61
296. Yu CY, Yuan Z, Cao Z, Wang B, Qiao C, et al. 2009. A muscle-targeting peptide displayed on AAV2 improves muscle tropism on systemic delivery. *Gene Ther* 16:953-62
297. Griffey MA, Wozniak D, Wong M, Bible E, Johnson K, et al. 2006. CNS-directed AAV2-mediated gene therapy ameliorates functional deficits in a murine model of infantile neuronal ceroid lipofuscinosis. *Mol Ther* 13:538-47
298. Naidoo J, Stanek LM, Ohno K, Trewman S, Samaranch L, et al. 2018. Extensive Transduction and Enhanced Spread of a Modified AAV2 Capsid in the Non-human Primate CNS. *Mol Ther* 26:2418-30
299. Wassmer SJ, Carvalho LS, György B, Vandenberghe LH, Maguire CA. 2017. Exosome-associated AAV2 vector mediates robust gene delivery into the murine retina upon intravitreal injection. *Sci Rep* 7:45329
300. Sullivan JA, Stanek LM, Lukason MJ, Bu J, Osmond SR, et al. 2018. Rationally designed AAV2 and AAVrh8R capsids provide improved transduction in the retina and brain. *Gene Ther* 25:205-19
301. Cabanes-Creus M, Hallwirth CV, Westhaus A, Ng BH, Liao SHY, et al. 2020. Restoring the natural tropism of AAV2 vectors for human liver. *Sci Transl Med* 12
302. Niemeyer GP, Herzog RW, Mount J, Arruda VR, Tillson DM, et al. 2009. Long-term correction of inhibitor-prone hemophilia B dogs treated with liver-directed AAV2-mediated factor IX gene therapy. *Blood* 113:797-806
303. Batty P, Mo AM, Hurlbut D, Ishida J, Yates B, et al. 2022. Long-term follow-up of liver-directed, adeno-associated vector-mediated gene therapy in the canine model of hemophilia A. *Blood* 140:2672-83
304. Russell S, Bennett J, Wellman JA, Chung DC, Yu ZF, et al. 2017. Efficacy and safety of voretigene neparvovec (AAV2-hRPE65v2) in patients with RPE65-mediated inherited retinal dystrophy: a randomised, controlled, open-label, phase 3 trial. *Lancet* 390:849-60
305. Maguire AM, Simonelli F, Pierce EA, Pugh EN, Jr., Mingozzi F, et al. 2008. Safety and efficacy of gene transfer for Leber's congenital amaurosis. *N Engl J Med* 358:2240-8
306. Maguire AM, Bennett J, Aleman EM, Leroy BP, Aleman TS. 2021. Clinical Perspective: Treating RPE65-Associated Retinal Dystrophy. *Mol Ther* 29:442-63
307. Ellis BL, Hirsch ML, Barker JC, Connelly JP, Steininger RJ, 3rd, Porteus MH. 2013. A survey of ex vivo/in vitro transduction efficiency of mammalian primary cells and cell lines with Nine natural adeno-associated virus (AAV1-9) and one engineered adeno-associated virus serotype. *Virol J* 10:74
308. Ng R, Govindasamy L, Gurda BL, McKenna R, Kozyreva OG, et al. 2010. Structural characterization of the dual glycan binding adeno-associated virus serotype 6. *J Virol* 84:12945-57
309. Xie Q, Lerch TF, Meyer NL, Chapman MS. 2011. Structure-function analysis of receptor-binding in adeno-associated virus serotype 6 (AAV-6). *Virology* 420:10-9
310. Rutledge EA, Halbert CL, Russell DW. 1998. Infectious clones and vectors derived from adeno-associated virus (AAV) serotypes other than AAV type 2. *J Virol* 72:309-19
311. Rogers GL, Huang C, Clark RDE, Seclén E, Chen HY, Cannon PM. 2021. Optimization of AAV6 transduction enhances site-specific genome editing of primary human lymphocytes. *Mol Ther Methods Clin Dev* 23:198-209
312. Song L, Kauss MA, Kopin E, Chandra M, Ul-Hasan T, et al. 2013. Optimizing the transduction efficiency of capsid-modified AAV6 serotype vectors in primary human hematopoietic stem cells in vitro and in a xenograft mouse model in vivo. *Cytotherapy* 15:986-98
313. Wang J, DeClercq JJ, Hayward SB, Li PW, Shivak DA, et al. 2016. Highly efficient homology-driven genome editing in human T cells by combining zinc-finger nuclease mRNA and AAV6 donor delivery. *Nucleic Acids Res* 44:e30

314. Tran R, Lam WA. 2020. Microfluidic Approach for Highly Efficient Viral Transduction. *Methods Mol Biol* 2097:55-65
315. Tran R, Myers DR, Denning G, Shields JE, Lytle AM, et al. 2017. Microfluidic Transduction Harnesses Mass Transport Principles to Enhance Gene Transfer Efficiency. *Mol Ther* 25:2372-82
316. Tran R, Ahn B, Myers DR, Qiu Y, Sakurai Y, et al. 2014. Simplified prototyping of perfusable polystyrene microfluidics. *Biomicrofluidics* 8:046501
317. Pandya J, Ortiz L, Ling C, Rivers AE, Aslanidi G. 2014. Rationally designed capsid and transgene cassette of AAV6 vectors for dendritic cell-based cancer immunotherapy. *Immunol Cell Biol* 92:116-23
318. Pandya M, Britt K, Hoffman B, Ling C, Aslanidi GV. 2015. Reprogramming Immune Response With Capsid-Optimized AAV6 Vectors for Immunotherapy of Cancer. *J Immunother* 38:292-8
319. Krotova K, Day A, Aslanidi G. 2019. An Engineered AAV6-Based Vaccine Induces High Cytolytic Anti-Tumor Activity by Directly Targeting DCs and Improves Ag Presentation. *Mol Ther Oncolytics* 15:166-77
320. Baskar JF, Smith PP, Nilaver G, Jupp RA, Hoffmann S, et al. 1996. The enhancer domain of the human cytomegalovirus major immediate-early promoter determines cell type-specific expression in transgenic mice. *J Virol* 70:3207-14
321. An DS, Wersto RP, Agricola BA, Metzger ME, Lu S, et al. 2000. Marking and gene expression by a lentivirus vector in transplanted human and nonhuman primate CD34(+) cells. *J Virol* 74:1286-95
322. Astrakhan A, Sather BD, Ryu BY, Khim S, Singh S, et al. 2012. Ubiquitous high-level gene expression in hematopoietic lineages provides effective lentiviral gene therapy of murine Wiskott-Aldrich syndrome. *Blood* 119:4395-407
323. Raikar SS, Fleischer LC, Moot R, Fedanov A, Paik NY, et al. 2018. Development of chimeric antigen receptors targeting T-cell malignancies using two structurally different anti-CD5 antigen binding domains in NK and CRISPR-edited T cell lines. *Oncoimmunology* 7:e1407898
324. Fleischer LC, Becker SA, Ryan RE, Fedanov A, Doering CB, Spencer HT. 2020. Non-signaling Chimeric Antigen Receptors Enhance Antigen-Directed Killing by γδ T Cells in Contrast to αβ T Cells. *Mol Ther Oncolytics* 18:149-60
325. Mendell JR, Al-Zaidy SA, Rodino-Klapac LR, Goodspeed K, Gray SJ, et al. 2021. Current Clinical Applications of In Vivo Gene Therapy with AAVs. *Mol Ther* 29:464-88
326. Lu Y, He W, Huang X, He Y, Gou X, et al. 2021. Strategies to package recombinant Adeno-Associated Virus expressing the N-terminal gasdermin domain for tumor treatment. *Nat Commun* 12:7155
327. Reul J, Frisch J, Engeland CE, Thalheimer FB, Hartmann J, et al. 2019. Tumor-Specific Delivery of Immune Checkpoint Inhibitors by Engineered AAV Vectors. *Front Oncol* 9:52
328. Santiago-Ortiz JL, Schaffer DV. 2016. Adeno-associated virus (AAV) vectors in cancer gene therapy. *J Control Release* 240:287-301
329. Nawaz W, Huang B, Xu S, Li Y, Zhu L, et al. 2021. AAV-mediated in vivo CAR gene therapy for targeting human T-cell leukemia. *Blood Cancer J* 11:119
330. Huang KC, Lai CY, Hung WZ, Chang HY, Lin PC, et al. 2023. A Novel Engineered AAV-Based Neoantigen Vaccine in Combination with Radiotherapy Eradicates Tumors. *Cancer Immunol Res* 11:123-36
331. Hensel JA, Khattar V, Ashton R, Ponnazhagan S. 2019. Recombinant AAV-CEA Tumor Vaccine in Combination with an Immune Adjuvant Breaks Tolerance and Provides Protective Immunity. *Mol Ther Oncolytics* 12:41-8
332. Cappell KM, Kochenderfer JN. 2023. Long-term outcomes following CAR T cell therapy: what we know so far. *Nat Rev Clin Oncol*:1-13
333. Hoeres T, Smetak M, Pretscher D, Wilhelm M. 2018. Improving the Efficiency of Vγ9Vδ2 T-Cell Immunotherapy in Cancer. *Front Immunol* 9:800

334. Wu Z, Miller E, Agbandje-McKenna M, Samulski RJ. 2006. Alpha2,3 and alpha2,6 N-linked sialic acids facilitate efficient binding and transduction by adeno-associated virus types 1 and 6. *J Virol* 80:9093-103
335. Huang LY, Patel A, Ng R, Miller EB, Halder S, et al. 2016. Characterization of the Adeno-Associated Virus 1 and 6 Sialic Acid Binding Site. *J Virol* 90:5219-30
336. Bi S, Baum LG. 2009. Sialic acids in T cell development and function. *Biochim Biophys Acta* 1790:1599-610
337. Scherer LD, Brenner MK, Mamonkin M. 2019. Chimeric Antigen Receptors for T-Cell Malignancies. *Front Oncol* 9:126
338. Fleischer LC, Spencer HT, Raikar SS. 2019. Targeting T cell malignancies using CAR-based immunotherapy: challenges and potential solutions. *J Hematol Oncol* 12:141
339. Kalos M, Levine BL, Porter DL, Katz S, Grupp SA, et al. 2011. T cells with chimeric antigen receptors have potent antitumor effects and can establish memory in patients with advanced leukemia. *Sci Transl Med* 3:95ra73
340. Wada M, Zhang H, Fang L, Feng J, Tse CO, et al. 2020. Characterization of an Anti-CD5 Directed CAR T-Cell against T-Cell Malignancies. *Stem Cell Rev Rep* 16:369-84
341. Alcantara M, Tesio M, June CH, Houot R. 2018. CAR T-cells for T-cell malignancies: challenges in distinguishing between therapeutic, normal, and neoplastic T-cells. *Leukemia* 32:2307-15
342. Mamonkin M, Rouce RH, Tashiro H, Brenner MK. 2015. A T-cell-directed chimeric antigen receptor for the selective treatment of T-cell malignancies. *Blood* 126:983-92
343. Burnham RE, Zoine JT, Story JY, Garimalla SN, Gibson G, et al. 2020. Characterization of Donor Variability for γδ T Cell ex vivo Expansion and Development of an Allogeneic γδ T Cell Immunotherapy. *Front Med (Lausanne)* 7:588453
344. Rosenberg SA, Restifo NP, Yang JC, Morgan RA, Dudley ME. 2008. Adoptive cell transfer: a clinical path to effective cancer immunotherapy. *Nat Rev Cancer* 8:299-308
345. McCreedy BJ, Senyukov VV, Nguyen KT. 2018. Off the shelf T cell therapies for hematologic malignancies. *Best Pract Res Clin Haematol* 31:166-75
346. Mirzaei HR, Mirzaei H, Lee SY, Hadjati J, Till BG. 2016. Prospects for chimeric antigen receptor (CAR) γδ T cells: A potential game changer for adoptive T cell cancer immunotherapy. *Cancer Lett* 380:413-23
347. Brandes M, Willimann K, Bioley G, Lévy N, Eberl M, et al. 2009. Cross-presenting human gammadelta T cells induce robust CD8+ alphabeta T cell responses. *Proc Natl Acad Sci U S A* 106:2307-12
348. Jarry U, Chauvin C, Joalland N, Léger A, Minault S, et al. 2016. Stereotaxic administrations of allogeneic human Vγ9Vδ2 T cells efficiently control the development of human glioblastoma brain tumors. *Oncoimmunology* 5:e1168554
349. Lamb LS, Jr., Musk P, Ye Z, van Rhee F, Geier SS, et al. 2001. Human gammadelta(+) T lymphocytes have in vitro graft vs leukemia activity in the absence of an allogeneic response. *Bone Marrow Transplant* 27:601-6
350. Gu S, Sachleben JR, Boughter CT, Nawrocka WI, Borowska MT, et al. 2017. Phosphoantigen-induced conformational change of butyrophilin 3A1 (BTN3A1) and its implication on Vγ9Vδ2 T cell activation. *Proc Natl Acad Sci U S A* 114:E7311-e20
351. Thomas ML, Samant UC, Deshpande RK, Chiplunkar SV. 2000. gammadelta T cells lyse autologous and allogenic oesophageal tumours: involvement of heat-shock proteins in the tumour cell lysis. *Cancer Immunol Immunother* 48:653-9
352. Zhang H, Hu H, Jiang X, He H, Cui L, He W. 2005. Membrane HSP70: the molecule triggering gammadelta T cells in the early stage of tumorigenesis. *Immunol Invest* 34:453-68
353. Wu J, Groh V, Spies T. 2002. T cell antigen receptor engagement and specificity in the recognition of stress-inducible MHC class I-related chains by human epithelial gamma delta T cells. *J Immunol* 169:1236-40

354. Hoeres T, Pretscher D, Holzmann E, Smetak M, Birkmann J, et al. 2019. Improving Immunotherapy Against B-Cell Malignancies Using γδ T-Cell-specific Stimulation and Therapeutic Monoclonal Antibodies. *J Immunother* 42:331-44
355. Shah NN, Lee DW, Yates B, Yuan CM, Shalabi H, et al. 2021. Long-Term Follow-Up of CD19-CAR T-Cell Therapy in Children and Young Adults With B-ALL. *J Clin Oncol* 39:1650-9
356. Zhang X, Zhu L, Zhang H, Chen S, Xiao Y. 2022. CAR-T Cell Therapy in Hematological Malignancies: Current Opportunities and Challenges. *Front Immunol* 13:927153
357. Mehta PH, Fiorenza S, Koldej RM, Jaworowski A, Ritchie DS, Quinn KM. 2021. T Cell Fitness and Autologous CAR T Cell Therapy in Haematologic Malignancy. *Front Immunol* 12:780442
358. Fraietta JA, Lacey SF, Orlando EJ, Pruteanu-Malinici I, Gohil M, et al. 2018. Determinants of response and resistance to CD19 chimeric antigen receptor (CAR) T cell therapy of chronic lymphocytic leukemia. *Nat Med* 24:563-71
359. Topp MS, Gökbuget N, Stein AS, Zugmaier G, O'Brien S, et al. 2015. Safety and activity of blinatumomab for adult patients with relapsed or refractory B-precursor acute lymphoblastic leukaemia: a multicentre, single-arm, phase 2 study. *Lancet Oncol* 16:57-66
360. Linke R, Klein A, Seimetz D. 2010. Catumaxomab: clinical development and future directions. *MAbs* 2:129-36
361. Thakur A, Huang M, Lum LG. 2018. Bispecific antibody based therapeutics: Strengths and challenges. *Blood Rev* 32:339-47
362. Ma J, Mo Y, Tang M, Shen J, Qi Y, et al. 2021. Bispecific Antibodies: From Research to Clinical Application. *Front Immunol* 12:626616
363. Li H, Er Saw P, Song E. 2020. Challenges and strategies for next-generation bispecific antibody-based antitumor therapeutics. *Cell Mol Immunol* 17:451-61
364. Hoffmann P, Hofmeister R, Brischwein K, Brandl C, Crommer S, et al. 2005. Serial killing of tumor cells by cytotoxic T cells redirected with a CD19-/CD3-bispecific single-chain antibody construct. *Int J Cancer* 115:98-104
365. Jonus HC, Burnham RE, Ho A, Pilgrim AA, Shim J, et al. 2022. Dissecting the cellular components of ex vivo γδ T cell expansions to optimize selection of potent cell therapy donors for neuroblastoma immunotherapy trials. *Oncoimmunology* 11:2057012
366. Burnham RE, Tope D, Branella G, Williams E, Doering CB, Spencer HT. 2021. Human serum albumin and chromatin condensation rescue ex vivo expanded γδ T cells from the effects of cryopreservation. *Cryobiology* 99:78-87
367. Lamb LS, Pereboeva L, Youngblood S, Gillespie GY, Nabors LB, et al. 2021. A combined treatment regimen of MGMT-modified γδ T cells and temozolomide chemotherapy is effective against primary high grade gliomas. *Sci Rep* 11:21133
368. Lamb LS, Jr., Bowersock J, Dasgupta A, Gillespie GY, Su Y, et al. 2013. Engineered drug resistant γδ T cells kill glioblastoma cell lines during a chemotherapy challenge: a strategy for combining chemo- and immunotherapy. *PLoS One* 8:e51805
369. Story JY, Zoine JT, Burnham RE, Hamilton JAG, Spencer HT, et al. 2021. Bortezomib enhances cytotoxicity of ex vivo-expanded gamma delta T cells against acute myeloid leukemia and T-cell acute lymphoblastic leukemia. *Cytotherapy* 23:12-24
370. Fisher J, Anderson J. 2018. Engineering Approaches in Human Gamma Delta T Cells for Cancer Immunotherapy. *Front Immunol* 9:1409
371. Haso W, Lee DW, Shah NN, Stetler-Stevenson M, Yuan CM, et al. 2013. Anti-CD22-chimeric antigen receptors targeting B-cell precursor acute lymphoblastic leukemia. *Blood* 121:1165-74
372. Park JA, Santich BH, Xu H, Lum LG, Cheung NV. 2021. Potent ex vivo armed T cells using recombinant bispecific antibodies for adoptive immunotherapy with reduced cytokine release. *J Immunother Cancer* 9
373. de Weerdt I, Lameris R, Ruben JM, de Boer R, Kloosterman J, et al. 2021. A Bispecific Single-Domain Antibody Boosts Autologous Vγ9Vδ2-T Cell Responses Toward CD1d in Chronic Lymphocytic Leukemia. *Clin Cancer Res* 27:1744-55

374. Ganesan R, Chennupati V, Ramachandran B, Hansen MR, Singh S, Grewal IS. 2021. Selective recruitment of γδ T cells by a bispecific antibody for the treatment of acute myeloid leukemia. *Leukemia* 35:2274-84
375. Huang Y, Kim BYS, Chan CK, Hahn SM, Weissman IL, Jiang W. 2018. Improving immune-vascular crosstalk for cancer immunotherapy. *Nat Rev Immunol* 18:195-203
376. Kankeu Fonkoua LA, Sirpilla O, Sakemura R, Siegler EL, Kenderian SS. 2022. CAR T cell therapy and the tumor microenvironment: Current challenges and opportunities. *Mol Ther Oncolytics* 25:69-77
377. Gentles AJ, Newman AM, Liu CL, Bratman SV, Feng W, et al. 2015. The prognostic landscape of genes and infiltrating immune cells across human cancers. *Nat Med* 21:938-45
378. Simões AE, Di Lorenzo B, Silva-Santos B. 2018. Molecular Determinants of Target Cell Recognition by Human γδ T Cells. *Front Immunol* 9:929
379. Sahin U, Karikó K, Türeci Ö. 2014. mRNA-based therapeutics--developing a new class of drugs. *Nat Rev Drug Discov* 13:759-80
380. Ladak RJ, He AJ, Huang YH, Ding Y. 2022. The Current Landscape of mRNA Vaccines Against Viruses and Cancer-A Mini Review. *Front Immunol* 13:885371
381. Foster JB, Choudhari N, Perazzelli J, Storm J, Hofmann TJ, et al. 2019. Purification of mRNA Encoding Chimeric Antigen Receptor Is Critical for Generation of a Robust T-Cell Response. *Hum Gene Ther* 30:168-78
382. Zhou S, Liu M, Ren F, Meng X, Yu J. 2021. The landscape of bispecific T cell engager in cancer treatment. *Biomark Res* 9:38
383. Chen YH, Wang Y, Liao CH, Hsu SC. 2021. The potential of adoptive transfer of γ9δ2 T cells to enhance blinatumomab's antitumor activity against B-cell malignancy. *Sci Rep* 11:12398
384. Oberg HH, Peipp M, Kellner C, Sebens S, Krause S, et al. 2014. Novel bispecific antibodies increase γδ T-cell cytotoxicity against pancreatic cancer cells. *Cancer Res* 74:1349-60
385. Foeng J, Comerford I, McColl SR. 2022. Harnessing the chemokine system to home CAR-T cells into solid tumors. *Cell Rep Med* 3:100543
386. Di Stasi A, De Angelis B, Rooney CM, Zhang L, Mahendravada A, et al. 2009. T lymphocytes coexpressing CCR4 and a chimeric antigen receptor targeting CD30 have improved homing and antitumor activity in a Hodgkin tumor model. *Blood* 113:6392-402
387. Idorn M, Skadborg SK, Kellermann L, Halldórsdóttir HR, Holmen Olofsson G, et al. 2018. Chemokine receptor engineering of T cells with CXCR2 improves homing towards subcutaneous human melanomas in xenograft mouse model. *Oncoimmunology* 7:e1450715
388. Brown HC, Zakas PM, George SN, Parker ET, Spencer HT, Doering CB. 2018. Target-Cell-Directed Bioengineering Approaches for Gene Therapy of Hemophilia A. *Mol Ther Methods Clin Dev* 9:57-69
389. Ujiie H, Shevach EM. 2016. γδ T Cells Protect the Liver and Lungs of Mice from Autoimmunity Induced by Scurfy Lymphocytes. *J Immunol* 196:1517-28
390. Penaud-Budloo M, François A, Clément N, Ayuso E. 2018. Pharmacology of Recombinant Adeno-associated Virus Production. *Mol Ther Methods Clin Dev* 8:166-80
391. Denard J, Jenny C, Blouin V, Moullier P, Svinartchouk F. 2014. Different protein composition and functional properties of adeno-associated virus-6 vector manufactured from the culture medium and cell lysates. *Mol Ther Methods Clin Dev* 1:14031
392. Wright JF. 2014. AAV empty capsids: for better or for worse? *Mol Ther* 22:1-2
393. Gao K, Li M, Zhong L, Su Q, Li J, et al. 2014. Empty Virions In AAV8 Vector Preparations Reduce Transduction Efficiency And May Cause Total Viral Particle Dose-Limiting Side-Effects. *Mol Ther Methods Clin Dev* 1:20139
394. Zeltner N, Kohlbrenner E, Clément N, Weber T, Linden RM. 2010. Near-perfect infectivity of wild-type AAV as benchmark for infectivity of recombinant AAV vectors. *Gene Ther* 17:872-9
395. van den Boogaard WMC, Komninos DSJ, Vermeij WP. 2022. Chemotherapy Side-Effects: Not All DNA Damage Is Equal. *Cancers (Basel)* 14

396. Sermer D, Brentjens R. 2019. CAR T-cell therapy: Full speed ahead. *Hematol Oncol* 37 Suppl 1:95-100
397. Sun S, Hao H, Yang G, Zhang Y, Fu Y. 2018. Immunotherapy with CAR-Modified T Cells: Toxicities and Overcoming Strategies. *J Immunol Res* 2018:2386187
398. Jhita N, Raikar SS. 2022. Allogeneic gamma delta T cells as adoptive cellular therapy for hematologic malignancies. *Explor Immunol* 2:334-50
399. Siegel RL, Miller KD, Fuchs HE, Jemal A. 2021. Cancer Statistics, 2021. *CA Cancer J Clin* 71:7-33
400. Vadillo E, Dorantes-Acosta E, Pelayo R, Schnoor M. 2018. T cell acute lymphoblastic leukemia (T-ALL): New insights into the cellular origins and infiltration mechanisms common and unique among hematologic malignancies. *Blood Rev* 32:36-51
401. Hefazi M, Litzow MR. 2018. Recent Advances in the Biology and Treatment of T Cell Acute Lymphoblastic Leukemia. *Curr Hematol Malig Rep* 13:265-74
402. Pui CH, Evans WE. 2006. Treatment of acute lymphoblastic leukemia. *N Engl J Med* 354:166-78
403. Bostrom BC, Sensel MR, Sather HN, Gaynon PS, La MK, et al. 2003. Dexamethasone versus prednisone and daily oral versus weekly intravenous mercaptopurine for patients with standard-risk acute lymphoblastic leukemia: a report from the Children's Cancer Group. *Blood* 101:3809-17
404. Karrman K, Johansson B. 2017. Pediatric T-cell acute lymphoblastic leukemia. *Genes Chromosomes Cancer* 56:89-116
405. Raetz EA, Teachey DT. 2016. T-cell acute lymphoblastic leukemia. *Hematology Am Soc Hematol Educ Program* 2016:580-8
406. Chuprin J, Buettner H, Seedhom MO, Greiner DL, Keck JG, et al. 2023. Humanized mouse models for immuno-oncology research. *Nat Rev Clin Oncol* 20:192-206
407. Rooney C, Sauer T. 2018. Modeling cytokine release syndrome. *Nat Med* 24:705-6
408. Brehm MA, Cuthbert A, Yang C, Miller DM, DiIorio P, et al. 2010. Parameters for establishing humanized mouse models to study human immunity: analysis of human hematopoietic stem cell engraftment in three immunodeficient strains of mice bearing the IL2rgamma(null) mutation. *Clin Immunol* 135:84-98
409. Covassin L, Jangalwe S, Jouvet N, Laning J, Burzenski L, et al. 2013. Human immune system development and survival of non-obese diabetic (NOD)-scid IL2rγ(null) (NSG) mice engrafted with human thymus and autologous haematopoietic stem cells. *Clin Exp Immunol* 174:372-88