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Developing novel cellular and gene therapies for pediatric malignancies

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Developing novel cellular and gene therapies for pediatric malignancies

By

Jaquelyn Taylor Zoine
B.S. Loyola University Maryland, 2015

Advisor: H. Trent Spencer, PhD

An abstract of a dissertation submitted to the Faculty of the
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Abstract (350 words)

Developing novel cellular and gene therapies for pediatric malignancies

By Jaquelyn Taylor Zoine

Alternative strategies for cancer therapy are necessary because existing therapeutics have not been universally effective and current treatments introduce major toxicities. In some cancers, immunotherapy, a therapy responsible for activating, suppressing, boosting, or modifying the immune system or its components, has shown tremendous promise in targeting tumor cells. Current strategies of immunotherapy include the administration of cancer vaccines, cytokines, monoclonal antibodies, checkpoint inhibitors, and adoptive cell therapy (ACT). ACT has emerged as a successful strategy for immunotherapy and focuses on the infusion of immune competent cells into a patient. The primary objectives of the studies presented in this dissertation were to explore unique strategies of ACT, including γδ T cells and the expansion of chimeric antigen receptor (CAR) T cells. γδ T cells have intrinsic anti-tumorgenicity through multiple mechanisms by their cell surface receptors NKG2D, FasL, and CD16. Peripheral blood contains between 1-5% of γδ T cells, so their expansion for therapy is essential to achieve robust responses. Our studies demonstrate an efficient method for expanding and storing γδ T cells from neuroblastoma patient-derived apheresis products. The expanded patient-derived γδ T cells were cytotoxic against neuroblastoma cell lines. Furthermore, low-dose temozolomide in combination with expanded γδ T cells and dinutuximab caused targeted killing of neuroblastoma xenografts in vivo, reducing tumor burden and prolonging survival. Additionally, we developed a novel CAR T cell, which are T cells engineered with a recombinant receptor consisting of an antigen binding domain, a costimulatory domain, and T cell activation domain. Major limitations of CAR T cells are the limited number of available tumor associated antigens and methods to target existing antigens. Therefore, we designed a ligand-based CAR targeting the thrombopoietin (TPO) receptor, MPL, is a critical survival signal for hematopoietic stem cells (HSCs) and leukemia stem cells. We designed a CAR against MPL using a fragment of TPO. The TPO-CAR targeted MPL+ leukemia cell lines *in vitro* and *in vivo* and selectively targeted MPL+ HSCs. This dissertation discusses the novel development and incorporation of γδ T cells and ligand-based CARs into immunotherapeutic strategies for pediatric malignancies.

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"She's gotta learn young, life's tough."

"Knowing information in excess is never a bad thing."

Dr. Andrea Erdas, Loyola University Maryland

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List of Abbreviations

7AAD	7-aminoactinomycin
ACT	Adoptive Cell Therapy
ACT	Adenocortical Carcinoma
ADCC	Antibody Dependent Cellular Cytotoxicity
AICD	Activation Induced Cell Death
AIDS	Acquired Immunodefficiency Syndrome
ALK	Anaplastic Lymphoma Kinase
ALL	Acute Lymphoblastic Leukemia
AML	Acute Myeloid Leukemia
AMLM7	Acute Megakaryoblastic Leukemia
ANOVA	Analysis of Variance
APC	Antigen Presenting Cell
ARG1	Arginase 1
B-ALL	B Cell Acute Lymphoblastic Leukemia
BCG	Bacillus Calmette-Guerin
B-CLL	B-cell Chronic Lymphocytic Leukemia
BCMA	B Cell Maturation Antigen
BCR	B Cell Receptor
BFU-E	Primitive Erythroid Progenitor Cells
BiTEs	Bispecific T Cell Engagers
BMT	Bone Marrow Transplant
CAR	Chimeric Antigen Receptor
CBF	Core Binding Factor
CCL	CC Chemokine Family Ligand
CCLE	Cancer Cell Line Encyclopedia
CCR	CC Chemokine Family Ligand
CD	Cluster of Differentiation
cDC	Conventional Dendritic Cell
CDC	Complement Dependent Cellular Cytotoxicity
CFSE	Carboxyfluorescein Succinimidyl Ester
CFU-GEMM	Granulocyte, Erythroid, Macrophage, Megakaryocyte Progenitor Cells
CFU-GM	Granulocyte-Macrophage Progenitor Cells
cGAS	Cytosolic Enzyme Cyclic GMP-AMP Synthase
CIN	Chromosomal Instability
CLL	Chronic Lymphocytic Leukemia
CML	Chronic Myelogenous Leukemia
COX2	Cyclooxygenase-II Enzyme
CPC	Choroid Plexus Carcinoma
CRP	C Reactive Protein
CRS	Cytokine Release Syndrome
CSF-1	Colony Stimulating Factor 1

CTLA-4	Cytotoxic T Lymphocyte Associated Protein-4
DAMP	Danger-Associated Molecular Patterns
DC	Dendritic Cell
DMSO	Dimeythl Sulfoxide
DN	Double Negative
DNA	Deoxyribonucleic Acid
DNAM-1	DNAX Acessory Molecule-1
ds	Double Strand
DTX	Dinutuximab
eGFP	Expression Green Fluorescence Protein
EGFR	Epidermal Growth Factor
EpCAM	Epithelial Cell Adhesion Molecule
EPD	Ependymoma
EphA2	Erythropoetin producing hepatocellular carcinoma A2
Fab	Fragment Antibody
FAS	Apoptosis Antigen 1
FASL	Fas Antigen Ligand
Fc	Constant Region
FCγRIII	CD16
FDC	Follicular Dendritic Cell
Fig	Figure
FRα	Folate Receptor alpha
G-CSF	Granulcyte Colony Stimulating Factor
GD2	Diasylganglioside 2
GM-CSF	Granulocyte Colony Stimulating Factor
GM-CSFPAP	GM-CSF Prostatic Acid Phosphatase
GMP	Good Manufacturing Practice
GPC3	Glypican-3
GUCY2C	Guanylyl Cyclase C
GVHD	Graft Versus Host Disease
HBS	Hepes Bufferd Saline
HCL	Hairy Cell Leukemia
HD	Healthy Donor
HEL	Human Erythropoietic Leukemia
HER1	Human Epidermal Growth Factor 1
HER2	Human Epidermal Growth Factor 2
HGG	High Grade Glioma
HIF-1α	Hypoxia Inducible Factor 1 alpha
HPV	Human Papilloma Virus
HRP	Horse Radish Peroxidase
hrs	Hours
HSC	Hematopoietic Stem Cell
hUBC	Human Ubiquitin C Promoter
IACUC	Institutional Animal Care and Use Committee
ICAM-1	Intercellular Adhesion Molecule 1

ICOS	Inducible T Cell Costimulator
IDO	Indoleamine 2,3-Deoxygenase
IFN	Interferon
IFN-γ	Interferon Gamma
Ig	Immunoglobulin
IGF1-R	Insulin Like Growth Factor 1 Receptor
IL	Interleukin
IL11Rα	Interleukin 11 Receptor alpha
IL13Rα2	Interleukin 13 Receptor alpha 2
iNOS	Inducible Nitric Oxide Synthase
ITIM	Immunoreceptor Tyrosine-Based Inhibitory Motif
IVIG	Intravenous Immunoglobulin
kg	kilo grams
KRAS	Kirsten Rat Sarcoma Viral Oncogene Homolog
L1CAM	L1-cell adhesion molecule
Lag-3	Lymphocyte Activation Gene 3
CO	Lovely Codon Optimized
LDH	Lactate Dehydrogenase
LGG	Low Grade Glioma
LK	Lineage- c-Kit+
LSK	Lineage- c-Kit+ Sca-1+
LT-HSC	Long Term-Hematopoietic Stem Cells
LTR	Long Terminal Repeat
M	Macrophage
mAB	Monoclonal Antibodies
MAC	Membrane Attack Complex
MART-1	Protein Melan-A
MB	Medulloblastoma
MDSC	Myeloid Derived Suppressor Cell
MEL	Melanoma
MFI	Mean Fluorescence Intensity
mg	milli grams
MHC	Major Histocompatability Complex
MICA/B	MHC Class I Related Chain A/B
MIN	Microsatellie Instability
mL	micro grams
MLL	Mixed Lineage Leukemia
mm	millimeters
MMR	Mismatch Repair
MOI	Multiplicity of Infection
MPL	Myeloproliferative Leukemia Protein
MUC1	Mucin 1
MUC16 ecto	Mucin 16
MYC	Proto-Oncogene
NB	Neuroblastoma
NBL	Neuroblastoma

NCO	Non Codon Optimizied
NF-κB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
NIN	Nucleotie-Excision Repair Instability
NK	Natural Killer
NKG2D	Natural Killer Group 2 Member D
NKT	Natural Killer T cells
Non-txd	Non Transduced
NSCLC	Non small cell lung cancer
NSG	NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ
NY-ESO-1	Cancer/Testic Antigen 1
OS	Osteoarcoma
PAP	Prostatic Acid Phosphatase
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Solution
pDC	Plasmacytoid Dendritic Cell
PDGF-Rα	Platelete Derived Growth Factor Receptor alpha
PD-L1	Programmed Cell Death Ligand 1
PEG	Pegylated
PI	Propidium Iodie
PRR	Pattern Recognition Receptors
PSCA	Prostate Stem Cell Antigen
RAG	Recombination-Activating Gene
RANKL	Receptor Activator of Nuclear Factor kappa-B Ligand
RB	Retinoblastoma
RHB	Rhabdosarcoma
RIG1	Retinoic Acid-Inducible Gene I Protein
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcription Polymerase Chain Reaction
scFv	Single Chain Variable Fragment
SD	Standard Deviation
SLAM	Signaling Lymphocytic Activation Molecule
SLAMF7	SLAM Family member 7
ss	Single Strand
ss	Signal Sequence
SSM	Subcapsular Sinus Macrophages
STAT	Signal Tranducer and Activator of Transcription
STING	Stimulator of IFN Genes Protein
TAA	Tumor Associated Antigen
T-ALL	T Cell Acute Lymphocytic Leukemia
TAM	Tumor Associated Macrophage
TCGA	The Cancer Genome Atlas
TCM	Central Memory T Cell
TCR	T Cell Receptor
TE	Effector T Cell
TEM	Effector Memory T cell
TGF-β	Transforming Groth Factor beta

Th	Helper T cell
TIGIT	T Cell Immunoreceptor with Ig and ITIM Domains
TIL	Tumor Infiltrating Leukocyte
TLR	Toll-like Receptor
TME	Tumor Microenvironment
TMZ	Temozolomide
TPO	Thrombopoietin
TRAIL	Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand
Treg	Regulatory T cell
TriKEs	Trispecific Killer Cell Engagers
TSCM	Self-Renewing Central Memory T Cell
TSLP	Thymic Stromal LymphoPoietin
ULBP	UL16 Binding Proteins
VCN	Vector Copy Number
VDJ	Variable, Diversity, Joining
VEGF	Vascular Endothelial Growth Factor
VPD456	Violet Proliferation Dye 450
WLM	Wilm's Tumor
WT-1	Wilm's Tumor 1
αβ	alpha beta
γδ	gamma delta
μg	micro grams
μM	micro Molar

Chapter 1

Introduction

1.1 History of Cancer Immunology

A. Overview

In the 1890's, Dr. William B. Coley was among the first to apply a strategy of cancer immunotherapy to patients presenting with soft tissue sarcomas. Dr. Coley discovered that patients presenting with erysipelas, a streptococcal infection, had remission in their sarcoma. Coley, wanting to induce a similar response in erysipelas negative patients injected heat-killed streptococcus pyogenes (Gram positive) and serratia marcescens (Gram negative), which produced an immune response[1, 2]. This treatment had impressive results published in 1893, but the mechanism as to how this treatment worked was not obvious[3]. Dr. Coley worked to understand his findings, and his research contributed to the discovery of pattern recognition receptors, signaling factors that elicit an immune response, and immune checkpoint inhibitors[4, 5]. The culmination of Dr. Coley's work developed the groundwork for basic principles for cancer immunotherapy.

The discovery of the interferons was the next major discovery in the boom of immunotherapy. Drs. Alick Isaacs and Jean Lindenmann conducted experiments by placing heat-inactivated viruses on living cells, which then became resistant to subsequent living viral infections[6, 7]. The secreted interferons inhibited growth of live influenza virus. The conferred viral resistance demonstrated interferons' role in the immune system response to unhealthy or foreign cells. Interferons, and other cytokines, have impacted cancer treatment today, and are being used as a systemic adjuvant therapy for high-risk melanoma[8] and bladder cancers[9]. While the infusion of cytokines as a cancer treatment was being developed, the first cancer vaccine study was also being conducted by Ruth and John Graham[10]. They injected tumor lysates into gynecological cancer patients, and achieved 22% incidence of remission or stable disease; however, the reason for this remained unknown.

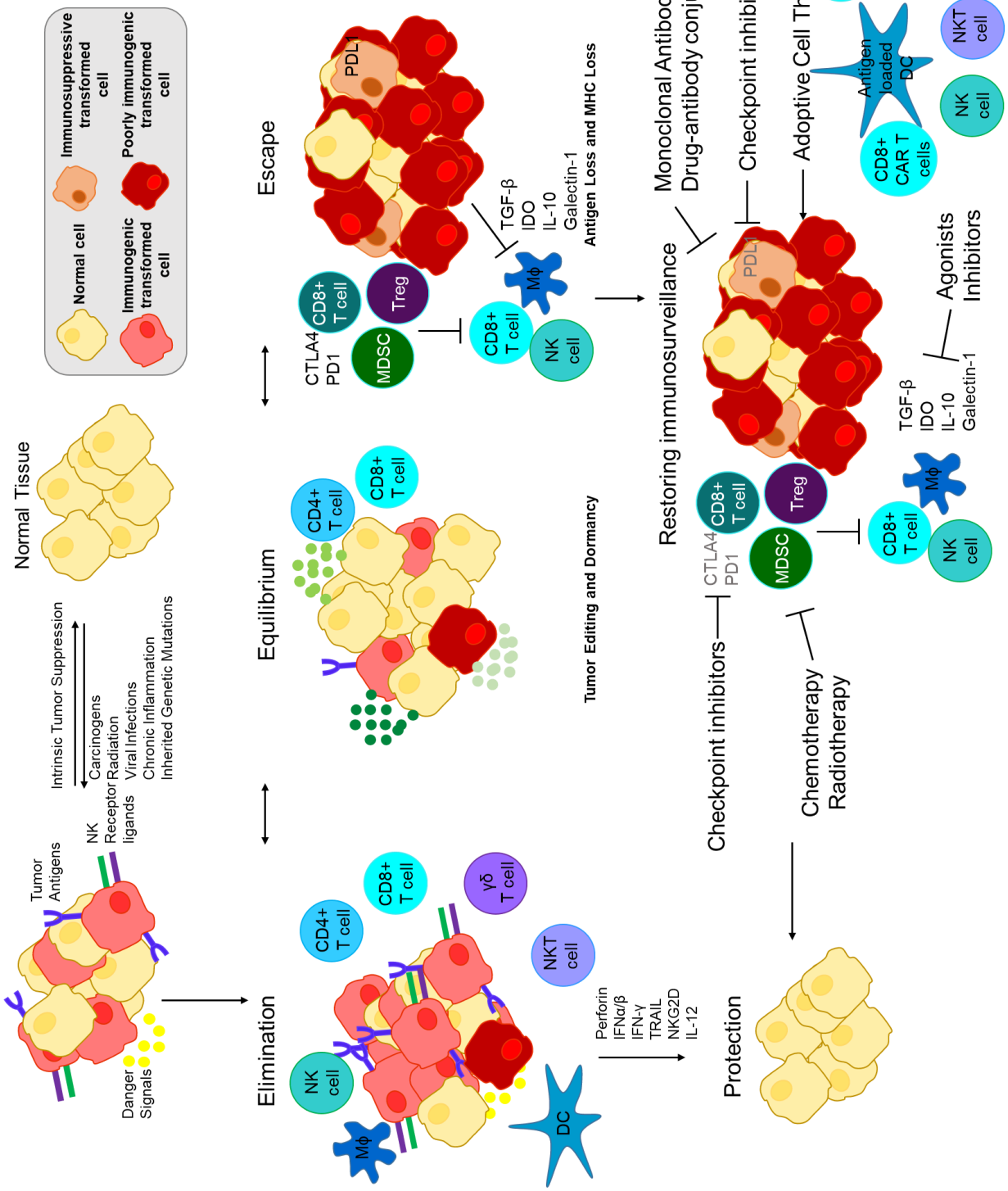
Following the Graham experiments, cancer immunotherapy progress slowed. However, major developments in the field of immunology were occurring during this time. Pertinent research that would have implications on cancer immunotherapy included the discovery of lymphocytes between 1965-1967 by

Max Cooper and Jacques Miller[11, 12], dendritic cells in 1973 by Ralph Steinman[13], Major Histocompatibility Complex (MHC) restriction by Rolf Zinkernagel and Peter Doherty in 1974[14], and Natural Killer (NK) cells by Eva Klein in 1975[15]. The next major discovery to enhance cancer therapy was the use of bone marrow transplantation (BMT) to replace leukemia cells and cancerous stem cells in patients[16]. Following these major discoveries, Robert Schreiber began his work, which became the foundation for the immunotherapy revolution.

Robert Schreiber established an immunoediting hypothesis that suggested the immune system went through phases fighting cancer[17, 18]. The three phases included elimination, equilibrium, and escape (Figure 1.1)[19]. Elimination is when the immune system is fighting and killing the cancer cells; however, sometimes not all of the cancer is cleared. This leads to the equilibrium phase, which is characterized by no outgrowth of the cancer. While this phase does not involve expansion of the cancerous phenotype, it allows for selection of certain advantageous mutations within the cancer cells. This phenomenon leads to tumor escape, the act of a tumor developing immune evasion characteristics. During this phase, cancer cells can shut off immune responses and signal for immune cells to promote the cancer cells' survival and proliferation. This reduces the immunogenicity of cancers, leading to continued growth of the tumor. Currently, one of the key challenges of cancer immunotherapy currently is how to reverse this tumor cell anti-immunogenic phenotype demonstrated by tumor cells.

The elimination phase of immunoediting would be completed if the cancer was completely eradicated by cytotoxic immune cells. A robust immune response likely involving the innate and adaptive systems would be necessary to prevent the progression to the second phase of cancer immunoediting[18-23]. Presumably, in the elimination phase the tumor has yet to develop key hallmarks of cancer that are essential for cancer progression[24, 25]. Innate cells are recruited to the site of the tumor and engage with antigens on the cancer cells. The infiltration of the innate cells into the tumor stroma and microenvironment is proinflammatory and begins the active immune response. The chemokines and cytokines in the microenvironment contribute

Figure 1.1: Cancer immunoediting



Modified from Smyth MJ Cancer Immunosurveillance and Immunoediting: The Roles of Immunity in Suppressing Tumor Development and Shaping Tumor Immunogenicity. Advanced in Immunology. 2006; 90:1-50. License number: 4678870285157

Figure Legend 1.1: The three phases of cancer immunoediting. Cancer immunoediting is the result of three processes that function either independently or in sequence to control and shape cancer. Once normal cells are transformed, the immune system may function as an extrinsic tumor suppressor by eliminating tumor cells or preventing their outgrowth. In the first phase, elimination, innate and adaptive immune cells and molecules recognize transformed cells and destroy them, resulting in a return to normal physiological tissue. However, if antitumor immunity is unable to completely eliminate transformed cells, surviving tumor variants may enter into the equilibrium phase, where cells and molecules of adaptive immunity prevent tumor outgrowth. These variants may eventually acquire further mutations that result in the evasion of tumor cell recognition, killing, or control by immune cells and progress to clinically detectable malignancies in the escape phase. The goal of cancer immunotherapy is to restore immunosurveillance and clear the tumor. This can be done by establishing an environment that is conducive to T-cell priming, trafficking, and activation. T-cell antitumor immunity can be reengaged by delivering tumor antigen in the form of a vaccine or chimeric antigen receptor (CAR) T cells. This can also be accomplished through adoptive cell transfer of immune competent cells. In addition, to creating a more favorable environment and *ex vivo* transfer of immune cells, checkpoint inhibitors can be used to restimulate existing and specific T cells. Successful immunosurveillance restoration is influenced by tumor antigenicity, tumor immunogenicity, and the immune profile of the tumor microenvironment. (Abbreviations: CTLA-4, cytotoxic T lymphocyte associated protein-4; IDO, indoleamine 2,3-deoxygenase; IFN, interferon; IL, interleukin; M, macrophage; MDSC, myeloid-derived suppressor cells; NK, natural killer; NKG2D, NK group 2, member D; PD-L1, programmed cell death 1 ligand 1; TGF-β, transforming growth factor-β; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; Treg, regulatory T cell; VEGF, vascular endothelial growth factor.)

to Natural Killer T cells (NKT) cells, γδ T cells, NK cells, and macrophages homing and cytotoxicity[26-28]. The innate response depends on the production of interferon-γ (IFN-γ) for tumor clearance[17, 29]. IFN-γ promotes the immune response and engagement of NK cells[30]. NK cells will infiltrate the tumor and produce more IFN-γ, with downstream effects causing macrophage secretion of interleukin (IL)-12[31]. As the extracellular matrix and stromal microenvironment are being broken down, the NK cells cause antiproliferative, proapoptotic and angiostatic tumor responses[26]. As tumor cells die, they release tumor associated antigens (TAAs), which are then processed by antigen presenting cells (APCs) for an adaptive immune response. Dendritic cells (DCs) [32] process these antigens and when activated, can migrate to the lymph nodes to present to helper T cells (Th), Th1 CD4+ T cells. Th1 cells cross-present antigens to cytotoxic CD8+ T cells. This enables tumor specific elimination by T cells[28]. Helper T cells produce IL-2 and IL-15 to expand and enhance the cytotoxic T cell response. The T cells continually produce IFN-γ, perpetuating the response from other immune infiltrating cells[27]. This process continues until the cancer progresses into the equilibrium phase or it is completely eliminated.

Equilibrium is a dynamic phase. Tumor cells have survived elimination and are under constant selective pressure from T cells and IFN-γ release[33, 34]. During this phase, tumors are genetically mutating and evolving to survive the immune pressure. While some tumor cells are being eliminated in equilibrium, new cancer cells are forming with advantageous mutations that causes the cancer to develop superior characteristics to avoid immune detection. This results in a heterogeneous and clonally mature tumor. The three types of genetic instability causing the immunologically undetectable tumor variants are nucleotide-excision repair instability (NIN), microsatellite instability (MIN), and chromosomal instability (CIN)[35]. Genomic instability is one of the hallmarks of cancer and an increase in this instability allow for tumor initiation and progression[35]. As the disease progresses and becomes immunologically undetectable, the tumor can escape clearance by the immune system.

The escape phase is the result of the tumor cells resisting the immune systems surveillance and functions. Tumors can develop multiple immunosuppressive phenotypes to elicit their escape, including production

of cytokines and hijacking immune cells[36-40]. Tumor cells can downregulate MHC complexes and lose their tumor associated antigens by antigen shedding or downregulation[41-43]. An essential component for creating a successful immune-based therapy is to stimulate the immune response against escaped tumor cells.

The numerous discoveries in cancer immunotherapy led to many therapeutic advances, which can be classified as therapies that either target the tumor directly or activate immune cells. One type of tumor directed immunotherapy includes the use of monoclonal antibodies[44]. Monoclonal antibodies target antigens and receptors on the tumor cell surface to elicit an immune mediated response through the FCγRIII receptor (CD16) by antibody dependent cellular cytotoxicity (ADCC) [45, 46]. Additionally, monoclonal antibodies have been conjugated to immunotoxins or drugs to facilitate a faster immune response[29, 47-51]. Cancer vaccines are used for both cancer directed as well as immune stimulating immunotherapeutic responses. For example, the vaccine Sipuleucel-T was approved for prostate cancer[52], demonstrating the effectiveness of DC vaccination targeted to the antigen prostatic acid phosphatase (PAP).

Researchers worked to develop ways to restimulate the immune response after the escape phase of immunoediting occurred. One example of successfully overcoming the escape phase was with checkpoint inhibitors. The discovery of checkpoint inhibitors facilitated a reverse in exhaustion and long term activation in T cells that were no longer responding to cancer[53]. Checkpoint inhibitors being used clinically setting today target cytotoxic T lymphocyte antigen 4 (CTLA-4/CD152) and programmed cell death 1 (PD-1) pathways[54]. By blocking the signaling of these pathways, T cell activation and proliferation can be enhanced. Checkpoint inhibitors allow for specific and primed T cells to continue working, but the technology to genetically engineer T cells was being developed to create T cells that specifically target cancer cells. T cells were being engineered for specific T cell receptors[55], for high affinity and specificity to a cancer neoantigen, or engineered with a chimeric antigen receptor (CAR) [56], which specifically targets tumor associated antigens, but also bypasses MHC processing essential for T cell engagement and activation.

Currently, research is being done to improve all aspects of cancer immunotherapy. Some exciting work is being done to develop cytokine therapy, monoclonal antibodies, immune checkpoint inhibitors, cancer vaccines, and adoptive cell transfer. Research groups are now working to combine immunotherapies or combine an immunotherapy with radiation therapy, surgery, and/or chemotherapy. These avenues of combined therapies have demonstrated promising *in vitro* and *in vivo* effects[57], but are also a major opportunity for research development. However, it has become apparent that more research needs to be done to better understand the contributions made by the immune system to cancer development and destruction by looking into mechanisms of how these immune-based therapies are affecting the body.

B. Innate immune system's response to cancer

The first line of defense in the innate immune response is an ancient system called complement. The complement cascade is one of the earliest defense mechanisms to any foreign substance. This system is comprised of three different pathways of activation including the classical, alternative, and lectin[58]. The classical complement pathway is activated by antigen-antibody complexes. Permissive surfaces activate the alternative pathway, and the lectin pathway is triggered by mannose binding lectins. The convergence point for all three pathways is after the C3 convertase is formed and ready to carry out effector functions. The C3 convertase cleaves the C3 protein into C3a and C3b. C3a is a major anaphylatoxin and C3b is an opsonin, which binds to the surface of cells and marks them for phagocytosis by macrophages. In addition to facilitating phagocytosis and an inflammatory response, the C3 convertase results in the formation of the C5 convertase which is an enzyme responsible for the formation of the membrane attack complex (MAC). The MAC disrupts the phospholipid bilayer of the cell membrane, leading to massive calcium influx, loss of mitochondrial membrane potential, and cell lysis. C3a, C3b, and MAC are the major mechanisms as to how complement induces cell death. Some of the first discovered chemokines and chemoattractants were discovered within the complement system. In cancer patients, complement proteins have been detected on the surface of cancer cells, as well as in biological fluid. These data suggests that complement proteins have a role in combatting cancer throughout its development and progression[59]. It is unclear which complement

pathway responds to cancer but, components of each pathway have been found across a variety of malignant tumors. While complement can induce a potent immune response, it is one of the easiest systems to evade, as evidenced by the frequency of bacterial infections that evade detection in the general human population[60]. Antigens on cancer cells or foreign cells simply upregulate complement regulators to bypass this detection. In addition, evidence suggests that immune-surveillance is predominantly a cell-mediated response suggesting complement is not playing an antitumorigenic response. Further analysis of tumors that have activated complement, release C5a and C3a components and subsequently promotes tumor growth, through recruitment of myeloid derived suppressor cells (MDSCs, myeloid cell with immunosuppressive functions) and reduction of IL-10[61]. It could be advantageous to target complement components and discover inhibitors to complement proteins to control tumor microenvironment and stromal cells, which can lead to reversal of the immunosuppressive phenotypes. Despite its limitations, the complement system in healthy subjects plays a role in tumor antigen processing and presentation, B cell activation, and T helper and effector T cell survival and differentiation[62].

The key cellular players in the innate immune response are NK cells, DCs, innate lymphoid cells (ILCs), macrophages, neutrophils eosinophils, basophils, and mast cells. Invariant NK cells (iNKT), NK cells and γδ T cells function in the innate immune system and have adaptive mechanisms of activation, but mainly function through innate mechanisms to target cancer (described in more detail below). Harnessing the innate immune system to target cancer is an attractive strategy for immunotherapy because there is a faster response to harmful signals, and the activation and stimulation of the innate response leads to robust management of non-self-antigens.

NK cells are one of the most effective cells in the innate response to cancer. The NK cell is commonly defined by its activating and inhibitory receptors and is capable of secreting potent inflammatory cytokines, including type I interferons, IL-12, IL-18, and IL-15[63, 64]. NK cells are unique in that they do not require the priming necessary to activate T cells to elicit a strong immune response[65]. They are not only capable of

homing and infiltrating tumors, but they can respond to stress antigens expressed on tumor cells through their NKG2D receptor[66]. Additionally, NK cells can recognize DNA (deoxyribonucleic acid) damage and replication stress through the DNAM-1 (DNAX accessory molecule-1) ligands on cells through their DNAM-1 receptor[67]. NK cells naturally combat cancer, once activated, by many pathways: secreting perforin and granzymes, Fas (Apoptosis antigen 1) antigen ligand (FASL) and Fas receptor interaction[68], TNR-related, apoptosis-inducing ligand (TRAIL) and TRAIL receptor interaction[69], or by their CD16 receptor through ADCC[45]. Tumors can evade NK cell recognition by downregulating these ligands and receptors. The NKG2D receptor ligands are commonly downregulated across all cancers, but they are inducible through chemotherapy treatments[70].

There are a variety of cells that function in the innate system with adaptive properties. Interestingly, some cells such as iNKT possess machinery for both NK cells and T cells. iNKT cells are activated through antigen recognition. iNKT type I recognize glycolipid antigens by CD1d[71]. The T cell receptor (TCR) on these cells recognizes the lipid antigens by directly binding CD1d leading to secretion of IL-2, IL-4, and IFN-γ[72-75]. iNKT cells can upregulate costimulatory molecules on DCs, having an indirect result on antigen presentation for activation of an adaptive immune response. The type II NKT cells that secrete IL-13 are immunosuppressive and support cancer growth. IL4R-STAT6 (signal tranducer and activator of transcription) signaling, and TGF-β secretion are alternative mechanisms where type II NKTs have supported a pro-tumor environment[76]. Harnessing the type I invariant cells and depleting type II would eb therapeutically beneficial as a cancer immunotherapeutic approach.

Similar to the NK and NKT cells, the γδ T cells bridge the adaptive and innate immune response. There are two types of T cells: αβ (functionality is explained further below) and γδ T cells. Both sets of T cells undergo Variable Diversity Joining (VDJ) Recombination, a recombination genetic process in early T cells and B cells resulting in diverse T cell receptors (TCR) and B cell receptors (BCR). All T cells arise from the double negative (DN, CD4-, and CD8-) precursors in the thymus. During DN2 (CD44+ CD25+)

rearrangements begin in the δ, γ, and β TCR[77]. The αβ and γδ T cells diverge in DN3 (CD44- CD25+)[78]. γδ T cells are considered committed to the lineage after expression of CD24[79] and CD73[80]. γδ T cells have multiple γ and δ chains that comprise of the repertoire of the T cell population. In humans, there are only 3 Vδ genes on chromosome 14 with 4 J segments; whereas, the Vγ chains have 12 genes of which only 7 are functional with 5 J segments, all located on chromosome 7[81]. The limited number of rearrangements possible, accounts for the low diversity of γδ T cells. When γδ T cells exit the thymus they already have their effector function and there is limited plasticity of these cells. There are very few reports of Vδ3 cells due to a lack of abundance, but the majority of them have been found in the epithelium of the liver and gut and functionally behave similarly to NKT cells[82]. Vδ1 γδ T cells and Vγ9 Vδ2 T cells are the two γδ T cell types that respond to cancer. Vδ1 γδ T cells do not have a preferred γ chain. Unlike the Vγ9 Vδ2 T cells, Vδ1 cells when cytotoxic against cancer persist and exhibit a memory like phenotype. The Vγ9Vδ2 T cells are of particular interest to combating cancer because they are innately anti-tumorigenic, function independent of MHC processing, can act as APCs, secrete pro-inflammatory cytokines (including perforin and granzymes), and kill through all the same mechanisms as a NK cell[83-86]. An advantage of the Vγ9Vδ2 T cells is they do not have any of the inhibitory components NK cells have. These γδ T cells do not have a memory phenotype like other innate cells and only consist of 1-5% of circulating peripheral blood. Therefore, they are only a small piece of the innate anti-cancer response, but can be powerful if expanded to elicit a large anti-tumorigenic response[87]. Despite the anti-tumor response, there are γδ T cells that are protumorigenic, including γδ Tregs, IL-17 producing γδ T cells, and some Vδ1 T cells. IL-17 secreting γδ T cells support angiogenesis and metastasis resulting in cancer progression[88, 89]. Notably, the role of γδ Tregs is similar to αβ Tregs (described below) and Vδ1 T cells are equally immunosuppressive when secreting IL-17A and TGFβ[90, 91].

ILCs are a growing family of immune cells that mirror the phenotypes and functions of T cells. NK cells can be considered the innate counterparts of cytotoxic CD8+ T cells, whereas ILC1s, ILC2s, and ILC3s may represent the innate counterparts of CD4+ T helper 1 (TH1), TH2, and TH17 cells[92]. Group 1 ILC can

produce IFNγ granulocyte macrophage- colony stimulating factor (GM-CSF), granzyme and perforin in response to IL-12 and IL-18 and activate macrophages. Group 2 ILCs can produce IL-4, IL-5, IL-13, IL-9, and amphiregulin in response to IL-25, IL-33, and TSLP (Thymic Stromal LymphoPoietin). Group 2 ILCs are essential in the immune response against parasites and allergens and their production of amphiregulin promotes tissue damage repair. Group 3 ILCs can be activated by IL-23 or IL-1β and they are involved in the immune response against extracellular microbes such as fungi or bacteria. However, in contrast to T cells, ILCs do not express antigen receptors or undergo clonal selection and expansion when stimulated. Instead, ILCs react promptly to signals from infected or injured tissues and produce an array of secreted cytokines. Thus, the power of ILCs may be controlled or unleashed to regulate or enhance immune responses in disease prevention and therapy[93].

Another key part of the innate immune system is the cells responsible for antigen presentation. Antigen presentation is essential for T cell activation and cytotoxicity and is primarily done by dendritic cells. There are two types of dendritic cells: plasmacytoid DCs (pDCs) [94] and conventional DCs (cDCs). pDCs are found in lymphoid tissues and blood. They secrete high levels of type I interferons upon interaction with Toll like receptors (TLRs), which is a mechanism for cancer destruction[95, 96]. Release of Type I IFNs by tumor and immune cells stimulates an adaptive immune response against dead tumor cell-associated antigens via autocrine and paracrine activation of the IFN signaling pathway[97]. pDCs, though they present less antigen than cDCs, provide critical antitumor effect via IFN[98]. In contrast, cDCs are the superior population of cells responsible for antigen presentation to CD8+ cytotoxic T cells. cDCs are primarily in nonlymphoid tissues collecting antigens and subsequently migrate to the draining lymph nodes to prime T cells for an anticancer immune response. The two types of cDCs, cDC1 and cDC2, are both capable of antigen presentation, but present antigens from different dangers and pathogens[99]. cDC1 cells present viruses, tumors, and intracellular pathogens whereas the cDC2 cells present intracellular pathogens, parasites, allergens, extracellular bacteria, and fungi[100]. Typically, cancer antigens are presented to T cells by cDC1s, which are dependent on MHCI complexes for recognition. The dependence of the antigen presentation and triggering

of the immune response heavily relies on MHCI; however, this protein is often downregulated or lost by cancer cells so they can escape immune surveillance[101].

Tumor associated macrophages (TAMs) are among the most influential immune cells in the tumor microenvironment. There are two types of macrophages: the anti-tumorigenic M1, which secretes IFNγ and tumor necrosis factor α (TNFα), and the pro-tumorigenic M2 phenotype, which secretes IL-10 which turns off anti-tumor cytokine IL-12 [102]. It is possible that a macrophage can switch between the M1 and M2 state depending on what cytokines are present in its environment. For example, cancers will secrete CCL2 (CC chemokine ligand 2), CSF-1(colony stimulating factor-1) and IL-10 and Th cells can secrete IL-4 and IL-13 to turn M1 into M2 macrophages[102]. IL-10 antibodies or IFN-γ are typically able to reverse an M2 macrophage to an M1[103]. The polarization of macrophages between pro- and anti-tumorigenic is constantly changing the tumor microenvironment (TME), so combining therapies to make a more favorable TME has potential to be an effective way of halting tumor progression.

Key mechanisms of action for immune cells, including neutrophils eosinophils, basophils, and mast cells, rely on essential innate recognition machinery and proteins. These cells facilitate immediate host protection by phagocytosis of cells that express non-self-antigens or altered self-antigens by killing them with lysosomal enzymes. These innate processes rely heavily on type I IFNs which lead to antigen presentation[104]. Recognizing tumor cells can occur through the release of nucleic acids or RNA (ribonucleic acid) that are then detected by TLRs. The TLRs are a family of pattern recognition receptors (PRR) which play an important role in immune responses by recognizing pathogen-associated molecular patterns and danger-associated molecular patterns (DAMPs). Essential TLRs for cancer detection include TLR 3, which recognizes dsRNA (double-stranded RNA), TLR7, which recognizes ssRNA (single-stranded RNA), and TLR9, which recognizes CpG DNA. Sensing of cytosolic RNA can also be achieved through the RIG1 protein (retinoic acid-inducible gene I protein), which signals type 1 IFN production[105]. Furthermore, studies show TLR1 and TLR2 promotes cell death by NF-κB (nuclear factor kappa-light-chain-enhancer of

activated B cells) and the upregulation of TLR3 inhibits proliferation of cancer cells[106, 107]. Additionally, the cGAS (cytosolic enzyme cyclic GMP-AMP synthase)/STING (stimulator of IFN genes protein) pathway can be activated by DNA causing type I IFN production. It is apparent that tumor recognition and presentation by the innate immune system relies on successful recognition of danger signals and subsequent secretion of type I IFNs for a powerful immune response to tumor cells. However, studies have shown certain cancers overexpress TLRs which, contributes to an immunosuppressive microenvironment (discussed further below). Aberrant TLR signaling in cancer cells activated by TLR signals can release cytokines and chemokines. This can result in the recruitment of immune cells and cytokine profiles associated with immune tolerance, cancer progression and propagation of the tumor microenvironment[108].

Effectively, an adaptive immune response relies on innate immune activation that leads to productive T cell priming. Therefore, a deeper understanding of the mechanisms as to how the innate immune system engages with cancer is essential to develop new therapeutics. Expanding our knowledge of how the innate response can be used therapeutically to create a robust adaptive response will benefit patient outcomes and treatment strategies.

C. Adaptive immune system's response to cancer

The adaptive immune system differs from the innate system in that it is capable of forming immunological memory is capable of being formed. Immunological memory is the ability for the immune system to readily respond to a specific antigen after the primary immune response. While the adaptive immune response takes longer to engage with foreign antigen, it is a more robust and long-lasting response that is capable of specificity. The adaptive immune response is primarily composed of lymphocytes, including B and T cells.

B cells are being capable of secreting antibodies, which are immunoglobulins used to neutralize pathogens. Antibodies are potent in that they have a binding domain, which confers specificity to an antigen, and a constant region, which engages with receptors to facilitate ADCC or CDC (complement dependent

cytotoxicity) [45, 46]. Immature B cells will mature into plasma cells and will secrete immunoglobulins (Ig) A, G, and E. B cells rely on MHCII molecules to present antigens to activate[109, 110]. MHCII are found on helper T cells; however, a B cell can activate in the absence of a T cell if there is sufficient antigen load[111]. B cell activation begins with the BCR engaging soluble or membrane bound antigen. BCRs develop from VDJ recombination events, similar to TCRs. Before leaving the bone marrow, immature B cells are tested for auto-reactivity against self-antigens. They are then capable of migrating to the B-cell follicle in the spleen for the final stages of maturation and antigen presentation. These antigens are presented by a follicular dendritic cell (FDC), which are primarily found in periphery or residing in lymph nodes near a B cell follicle, or subcapsular sinus macrophages (SSMs) on the subcapsular sinus. SSMs are not required for a humoral response. B cells will either secrete low-affinity antibodies or travel to a germinal center after antigen engagement. The B cell will undergo somatic hypermutation but need to complete affinity maturation in the follicle. Somatic hypermutation alters the binding specificity and binding affinity of antibodies produced by the plasma cells whereas affinity maturation occurs as a result of repeated antigen exposure to produce the highest affinity immune response[112]. If B cells are unable to activate, they cannot secrete tumor specific antibodies, effectively rendering them useless in eliminating cancer.

The αβ T cell is the T cell in the adaptive immune response. Similar to the γδ T cell and B cells, αβ T cells undergo VDJ recombination. Thymocytes mature and develop in the thymus where they will begin with a CD4- and CD8- phenotype DN1. As they move through the cortex of the thymus they mature to DN2 and proceed to DN3 and DN4, an immature double negative thymocyte in the subcapsular region. As thymocytes exit the thymus they are double positive for CD4+ and CD8+ receptors. Double positive thymocytes undergo positive and negative selection before being considered mature. Positive selection is the process of separating T cells that are reactive with MHCI into CD4- CD8+ T cells and cells reactive with MHCII become CD4+ CD8- T cells. Remaining T cells undergo negative selection. Negative selection occurs as a result of a T cell receptor affinity reactivity. If the TCR engages strongly with self-antigen the T cell is negatively selective through clonal deletion. If the TCR affinity for self-peptide is too low the T

cell dies of neglect[112]. CD4+ T cells are classified into the helper T cell subsets Th1, Th2, and T regulatory Tregs. Each cell secretes cytokines to perform a function to the immune response whether priming cells, polarizing macrophages, or to reduce inflammation. The CD8+ T cell population is categorized by its cytotoxic properties. CD8+ T cells rely on MHC class I proteins, found on all nucleated cells, to distinguish between self and non-self. The CD8+ T cells will target non-self by recognizing the cell displaying no MHC class I. Alternatively, APCs can present to CD8+ T cells by MHC class II proteins so the CD8+ T cells can target a specific antigen. The action of a T cell activating and causing cytotoxicity heavily relies on MHC processing, which remains problematic in aggressive cancers due to the ability for cancer cells to downregulate antigens and MHC complexes[37, 113-115]. This is a major reason as to why T cells are unable to detect cancer, thereby allowing the tumor to escape immune detection.

D. Cancer evasion of the immune response

Despite a functioning immune system, thousands of people die from cancer every year. This is in part due to the fact that the cancer is constantly evolving to escape detection. The tumor is also capable of exploiting immune mechanisms to become immunologically undetectable or tolerant. There have been numerous reports of the cancer hijacking immune cells to make a suppressive microenvironment for favorable cancer outgrowth[116, 117]. Cancer cells have been reported to use Tregs, MDSCs, and M2 macrophages to hijack the immune response. Data suggests that Tregs cytokine profiles secreted by the tumor contribute to how aggressive the Treg function in the tumor microenvironment[118, 119]. Additionally, MDSCs and M2 macrophages promote an immunosuppressive phenotypes for cancer to gain metastatic capabilities and achieve angiogenesis. MDSCs target T cells through immune suppression by arginase (ARG1)[120], inducible nitric oxide synthase (iNOS)[121], TGFβ[122], IL-10[120], (cyclooxygenase-II enzyme) COX2[123], indoleamine 2,3-dioxygenase (IDO) sequestration of cysteine and tryptophan[124-126], and decrease of L-selectin[127] expression by T-cells. TAMs have been shown to directly suppress T cell function through surface presentation of PD-L1 and B7-homologs[128]. Hypoxia inducible Factor 1α (HIF-1α) also induces TAMs to suppress T cell

function[129]. Tregs, MDSCS, and TAMS are responsible for maintaining an immunosuppressive microenvironment, making immune escape probable in cancer progression.

It is noteworthy that there are mechanisms that allow cancer to escape detection by downregulating MHC molecules and their machinery. Further, there are immune suppressive cytokines and growth factors being secreted by contributing immune cells, the cancer, and the microenvironment which include TGFβ, TNFα, IL-1, IL-6, CSF-1, IL-8, IL-10, VEGF (vascular endothelial growth factor) and EGFR (epidermal growth factor)[117]. These cytokines can lead to tumor outgrowth, but can also contribute to polarization of macrophages, T helper cells, and increases in highly suppressive regulatory cells. Further, the immune cells that are working to fight the cancer such as the CD8+ T cells can upregulate exhaustion and activation markers so they are no longer effective. These markers include PD1 and CTLA-4[130]. Additionally, all T cells need costimulation to effectively kill and tumors have ways of turning off the costimulatory partners. While cancer successfully escapes immune recognition and manipulates the immune response to further growth, many immunotherapies are being discovered and employed to reverse these effects and eliminate cancer cells, which are described below.

1.2 Cancer Immunotherapy overview

Immunotherapy is activating, suppressing, boosting, or modifying the immune system or its components for therapeutic benefit and has shown promising clinical results. Strategies using tumor directed or immune stimulatory therapies have shown efficacy in both solid and hematological malignancies. Currently utilized approaches to cancer immunotherapy include cytokines, monoclonal antibodies, cancer vaccines, immune checkpoint inhibitors, and adoptive cell therapy.

A. Cytokines

Cytokines are the key molecules for communication within the immune system. They are capable of both positive and negative immune responses and ultimately have major control of immunological outcomes. It

is unsurprising that the use of cytokines for cancer therapy has been studied and employed clinically because of the downstream signaling pathways they can activate and their ability to signal to different parts of the body. However, cytokines are not a targeted therapy and simply stimulate or suppress an immune response. The immune response caused by the influx of cytokines can enhance the immune cell reaction to cancer cells, but there are also immune inhibitory consequences that can come from the infusion of certain cytokines. Some side effects of cytokine therapy include inducing immune checkpoints and systemic toxicities. However, there are clinical benefits of cytokines in many cancers.

Type I, II, and III IFNs are being used clinically to treat cancer[131]. IFNα and PEG (pegylated)-IFNα 2b are used in the treatment for melanoma, metastatic renal cell carcinoma, Acquired Immunodeficiency Syndrome (AIDS)-related Kaposi's sarcoma, follicular lymphoma, hairy cell leukemia (HCL), chronic myelogenous leukemia (CML), condyloma acuminate, and cervical intraperitoneal neoplasms[132]. Depending on the dose, IFNα can be extremely toxic with symptoms including fever, headache, gastrointestinal symptoms, myalgias, increase in hepatic enzymes, thrombocytopenia, leukopenia, neutropenia, and neuropsychiatric issues[133]. Since IFNγ never showed efficacy in cancer patients, it was never approved for therapy.

GM-CSF stimulates neutrophils, eosinophils, macrophages, megakaryocytes, erythrocytes, and antigen presentation. GM-CSF has been systemically introduced with positive outcomes in melanoma and prostate cancer patients[134, 135]. GM-CSF is being used to promote dendritic cell activity in a variety of anti-cancer trials. It was also used to decrease the recovery time of patients with neutropenia, but caused too much stimulation of the macrophage compartment[136]. To reduce this toxicity, granulocyte-colony stimulating factor (G-CSF) was substituted to decrease macrophage stimulation[137]. In addition, G-CSF can enhance ADCC and neutrophil activity[138]. However, both GM-CSF and G-CSF have had protumorigenic side effects

by influencing the immune microenvironment; therefore, more work is being done to expand and regulate the use of these cytokines *in vivo*.

Interleukins (IL) are naturally occurring glycoproteins that are produced by leukocytes to regulate the immune response. Many interleukins have been tested in oncology settings, including IL-12, IL-2, IL-21, IL-7, and IL-15. IL-12 is involved in helper T cell differentiation into Th1 cells and contributes to cytotoxic T cell and B cell survival. IL-12 had some efficacy in cutaneous T cell lymphoma, Hodgkin's and non-Hodgkin's lymphoma, melanoma, and Kaposi's sarcoma, but preclinical work is still being done to develop the use of this cytokine as part of the standard of care[139]. IL-2 is a powerful cytokine that induces NK and T cell proliferation, but can also induce T cell death[140-143]. IL-2 has been an incredibly problematic cytokine due to extreme toxicities with high dosing and minimal effects with low dosing. IL-2 is rarely used as a stand-alone treatment, but has clinical applications in cellular immunotherapy and adoptive cell therapy[144]. IL-21 contributes to B cell differentiation, development of T follicular helper cells and Th17, and promotes CD8+ antitumor activity[145]. IL-7 was tested preclinically for its benefits to T cell development[145, 146]. Infusion of IL-21 and IL-7 into patients has ceased due to side effects, such as induction of colon cancer, but it is still being used in the *ex vivo* expansion of effector cells. IL-15 is similar to IL-2 in that it contributes to the development of cytotoxic T cells, B cells' production of immunoglobulins, and NK cell proliferation[147-150]. IL-15 is similar to IL-2 but typically needs cell-cell contact and also does not induce activation induced cell death (AICD) capillary syndrome, or stimulate Tregs. IL-15 is still being tested preclinically with combinations of monoclonal antibodies to produce robust responses. The interleukin family, while powerful regulators of the immune response, cause major toxicities in patients and have the greatest benefit when used as an adjunct to existing treatments.

B. Monoclonal antibodies

Monoclonal antibody therapies have successfully provided therapeutic benefit. Monoclonal antibodies provide a tumor antigen targeted therapy to elicit engagement of the immune system to perform ADCC or

CDC. Antibodies can also block a targeted molecule from functioning and induce apoptosis. In addition, monoclonal antibodies have been conjugated to radionucleotides, immunotoxins, and cytokines to elicit cytotoxicity. Part of the difficulty in finding these targets/antigens is ensuring they have minimal on-target off-tumor side effects. Once the epitope is determined, a monoclonal antibody is made comprising of the antigen binding domain (Fab) and a constant region (Fc). The binding domain is similar to the structure of consists of a variable heavy and light chain, which are a result of B cell VDJ recombination. Monoclonal antibodies that are mouse derived need to be humanized to reduce the immunogenicity of these products prior to infusion. Monoclonal antibodies will always have problems with immunogenicity because of the nature of antigen-specific combining sites. However, humanization, the replacement of mouse constant regions and V framework regions for human sequences, results in a significantly less immunogenic product[151]. Human and humanized antibodies have a lower risk of inducing immune responses in humans than mouse or chimeric antibodies.

Currently, there are many monoclonal antibodies that have been approved by the FDA and are being used for cancer treatment. Table 1.1 summarizes some of the targeted monoclonal antibodies being used to treat cancer. These monoclonal antibodies, as well as those still under preclinical development, have shown great efficacy in combination with standard of care treatments. This targeted immunotherapy approach to engage activation of the immune system highlights the importance of finding tumor associated antigens with limited expression on healthy tissue.

Monoclonal antibody therapy has advanced to directly engage the cancer cell with an immune cell. Bispecific T cell engagers (BiTEs)[152] and trispecific killer cell engagers (TriKEs)[153] have developed as a result of the successes of monoclonal antibodies. A BiTE aims to target a tumor antigen as well as a receptor found on an immune cells; for example, CD16 on NK cells and CD33 on AML cells. TriKEs are slightly more complex in that they add a third component to enhance proliferation of the immune cells. Typically, IL-2 and IL-15 have been added to stimulate NK cell proliferation. The idea of using these next generation monoclonal antibodies elevates the immune engagement and response to the tumor. Currently, there is only

Table 1.1: Clinically available monoclonal antibodies for cancer treatment

Monoclonal Antibody	**Cancer Type**	**Antigen**
Alemtuzumab[154]	B-cell chronic lymphocytic leukemia (B-CLL)	CD52
Bevacizumab[155]	Metastatic cancer	VEGF-A
Brentuximab[156]	Hodgkin lymphoma, anaplastic large-cell lymphoma	CD30
Capromab[157]	Prostatic carcinoma cells	For detection
Catumaxomab[158]	Ovarian cancer, malignant ascites, and gastric cancers	Epithelial cell adhesion molecule (EpCAM)/CD3
Cetuximab[159]	Metastatic colorectal cancer, metastatic non-small cell lung cancer (NSCLC) and head and neck cancer	EGFR
Cixutumumab[160]	Solid tumors	Insulin like growth factor-1 receptor (IGF1-R)
Daratumumab[161]	Multiple myeloma	CD38
Denosumab [162]	Metastatic cancer that affects the bones	Receptor activator of nuclear factor kappa-B ligand (RANKL)
Dinutuximab[163, 164]	High-risk neuroblastoma	Diasylganglioside 2 (GD-2)
Elotuzumab [165]	Multiple myeloma	Signaling lymphocytic activation molecule family member 7 (SLAMF7)
Etaracizumab[166]	Melanoma, prostate cancer, and ovarian cancer	Integrin $\alpha v\beta 3$
Gemtuzumab [167]	Acute myelogenous leukemia (AML)	CD33
Girentuximab[168]	Clear cell renal cell carcinoma	Carbonic anhydrase 9
Ibritumomab[169]	Non-Hodgkin's lymphoma	CD20
Inotuzumab[170]	Acute lymphoblastic leukemia (ALL)	CD22
Mogamulizumab[171]	Adult T-cell leukemia/lymphoma	C-C chemokine receptor type 4 (CCR4)
Moxetumomab[172, 173]	Hairy cell leukemia	CD22
Necitumumab[174]	Non-small cell lung carcinoma	EGFR
Nimotuzumab[175]	Squamous cell carcinoma, head and neck cancer, nasopharyngeal cancer, and glioma	EGFR
Obinutuzumab[176]	Chronic lymphatic leukemia	CD20
Ofatumumab[177]	Chronic lymphocytic leukemia (CLL)	CD20
Olaratumab[178]*	Solid tumors	Platelete derived growth factor receptor α (PDGF-Rα)
Panitumumab[179]	Colon and rectum cancers	EGFR
Pertuzumab[180]	Human epidermal growth factor 2(HER2) positive breast cancer	HER2/neu
Polatuzumab[181]	Diffuse large B-cell lymphoma	CD79B
Racotumomab[182]	NSCLC	NGNA ganglioside

Ramucirumab[183]	Solid tumors	VEGFR2
Rituximab[184]	B cell Non-Hodgkin's lymphoma	CD20
Siltuximab[185]	Pan-cancers	IL-6
Tositumomab[186]	Follicular lymphoma	CD20
Trastuzumab[187]	Breast cancer	HER2

*recently withdrawn for the treatment of soft tissue sarcoma because it did not demonstrate a clinical benefit

one BiTE that is FDA approved targeting CD19 and CD3 called Blinatumomab for pre B-ALL[188]. This particular BiTE aims to engage T cells through CD3 and cancerous B cells through CD19. More preclinical work is being done to enhance the efficacy of BiTEs and TriKEs and they are expected to elicit promising results similar to the monoclonal antibody revolution.

C. Immune Checkpoint Inhibitors

The natural immune response has checkpoints to regulate how and when it is functions. Because T cells can produce strong immune responses, they are highly regulated. T cells that are activated for too long will upregulate checkpoint proteins to indicate their exhaustion and chronic activation. Cancer cells are able to hijack T cells to upregulate these checkpoints and render them inactive against the tumor cells. To combat this, monoclonal antibodies were developed to prevent cancer cells ability to turn on the off switch.

CTLA-4 is one of the proteins expressed by T cells after they become activated[189]. It acts as an inhibitory signal and typically binds CD80 and CD86 at a higher affinity than CD28, thereby outcompeting the costimulatory signal transmitted by CD28 that is necessary for T cell activation[190]. Ipilimumab is a monoclonal antibody targeting CTLA-4 to stop the inhibition of T cell activation[191]. The concept of using a CTLA-4 checkpoint inhibitor for cancer treatment was developed by Jim Allison, who was awarded the 2018 Nobel Prize for this work and work on PD-1. Ipilimumab received FDA approval for melanoma, but clinical trials are still ongoing for its use in other cancers.

Another immune checkpoint that has been a hot target for inhibitors is PD-1 and its respective ligand, PDL-1[192]. PD-1 promotes apoptosis of T cells and reduces apoptosis in Tregs. Expression of PD-1 suggests that T-cells are exhausted and have inhibited activity, expansion, and effector functions. PDL-1 is highly expressed on cancer cells so targeted therapies have been designed to block the ligand expressed on cancer cells, including atezolizumab[193], avelumab[194], and durvalumab[195]. Monoclonal antibodies have also been designed to target PD-1 on T cells, including nivolumab[196] and pembrolizumab[197]. These checkpoint

inhibitors are effective in turning the immune response back on and have been extremely successful in combination with chemotherapy and other immunotherapy treatments.

Lymphocyte-activation gene-3 (Lag-3), Mucin-domain containing-3 (Tim-3), CD160, and T cell immunoreceptor with Ig and ITIM (immunoreceptor tyrosine-based inhibitory motif) domains (TIGIT) are key proteins upregulated in an exhausted T cell[198-201]. These proteins have emerged as potential candidates for new checkpoint inhibitors. Marin-Acevedo et al summarize the use of inhibitors targeting these proteins in clinical trials. The expansion and discovery of checkpoint inhibitors to reverse exhaustion phenotypes is advantageous to improve the microenvironment and stimulate the immune system.

D. Cancer Vaccines

Vaccines are typically developed for prevention of viruses, so cancers caused by oncoviruses were naturally good candidates against which to make vaccines. There have been two approaches to develop cancer vaccines: isolate cancer cells from a patient and immunize against those cancer antigens to stimulate the immune response and use oncolytic viruses that preferentially kill and infect cancer cells, which propagate more viruses to infect more cancer cells directly[202]. Both of these approaches heavily rely on immune detection and clearance of the cancer. Currently, there are four cancer vaccines approved by the FDA that are divided into preventative and therapeutic vaccines. The preventative vaccines include Cervarix/Guardasil that protects against human papilloma virus (HPV)[203] and the hepatitis B vaccine which prevents liver related cancers[204]. The two therapeutic vaccines include Bacillus Calmette-Guerin (BCG) for early-stage bladder cancer patients[205] and Sipuleucel-T, a dendritic cell vaccine for prostate cancer[206]. There are also personalized neoantigen vaccines that are not FDA approved, but are being tested across many cancer types. The success of personalized vaccines has been rare and inconsistent in types of cancer they are successful. Cancer vaccines are capable of having potent anti-tumor responses and as technology advances, personalized vaccines for patients may be more efficacious and economical than other available therapies.

Sipuleucel-T was approved by the US FDA in 2010 for the treatment of asymptomatic metastatic castrate-resistant prostate cancer[207]. This vaccine is developed from dendritic cells that have been pulsed with GM-CSFPAP (GM-CSF prostatic acid phosphatase), a prostate cancer antigen, and then reinfused into a patient. The primed DCs subsequently activate CD8+ T cells. A survival advantage was achieved, with a 4.1-month improvement in median survival[206]. Sipuleucel-T as the first therapeutic cancer vaccine opened exciting new paradigms for prostate cancer and other cancers because it was evidence that an immunologically "cold" tumor could have T cell infiltration with immunotherapy treatment.

BCG is a live attenuated vaccine to tuberculosis produced from *Mycobacterium bovis.* BCG is a major stimulant of the immune system, contributing to secretion of ligands such as TRAIL, IL-2, IL-8, IL-12, and IFN-γ and recruitment of CD4+ and CD8+ T cells, NK cells, and granulocytes[208]. The beneficial role of BCG in in situ carcinomas of the bladder show a 71% complete response[209]. Survival rates increase to 84% when maintenance therapy is included. BCG has been well-tolerated with mild side-effects in immune competent patients, but has been found fatal in immunocompromised patients[210]. BCG has not been surpassed with its ability to delay cancer progression and reduce the incidence of disease recurrent; however, similar vaccines have not been discovered and therefore BCG remains unique.

HPV can result in cervical cancer. The HPV vaccines Gardasil and Cervarix are prophylactic aimed at preventing the infection and the subsequent disease. HPV vaccines are prepared from empty protein shells called virus-like particles. The vaccines demonstrated 100% protection against HPV types 16 and 18, which accounts for 63% and 16% of cervical cancers[211, 212]. However, the vaccine is only effective for HPV negative patients and is not globally available in areas that have the highest prevalence of HPV. In addition, preventative vaccines have no therapeutic benefit so there is still work necessary to expand HPV vaccines as a therapeutic intervention.

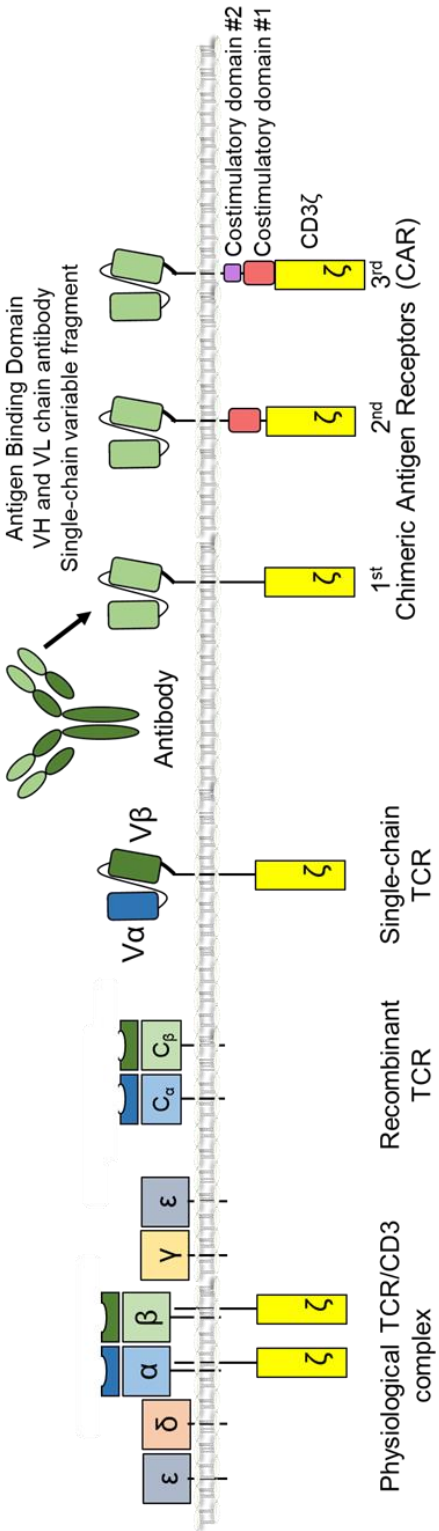
E. Adoptive Cell Therapy

In cases where the immune system is not effectively functioning in a cancer patient, adoptive cell therapy (ACT) is considered to introduce healthy and reinvigorated immune cells into the patient. ACT functions by expanding immune reactive lymphocytes from a patient *ex vivo*. The lymphocytes can be expanded from the tumor infiltrating lymphocytes (TILs) or from patient fluid then cytokines are used to induce activation and proliferation of the lymphocytes. Remaining cancer cells are depleted and cells are reinfused into the patient[213]. ACT with TILs have been shown in clinical trials to cause objective clinical responses in approximately 40% to 72% of patients with metastatic melanoma[214]. The expansion of TILs for ACT has been successfully completed in renal cell carcinoma[215], breast cancer[216], cervical cancer (resulted in 2/9 complete responses)[217], gastrointestinal cancers[218], cholangiocarcinoma[219], pancreatic cancer[220], head and neck cancer[221], ovarian cancer (100% 3 year overall survival)[222], and NSCLC[223].

Strategies that have been developed to genetically engineer lymphocytes prior to reinfusion into patients to enhance T cell function includes costimulatory molecules, homing receptors, cytokines, and anti-apoptotic genes for the benefit of making a superior T cell. The introduction of gene therapy to ACT lead to the advancement of chimeric TCRs and chimeric antigen receptors (CARs) (Figure 1.2)[224]. Chimeric TCRs aim to introduce new recognition specificities to the lymphocytes while remaining MHC-dependent. Chimeric TCRs have been successful targeting melanoma through the melanoma differentiation antigens protein melan-A (MART-1) (30% response) or gp100 (19% response) [225]. Another targeted gene for melanoma TCR gene therapy is the cancer/testis antigen 1B, NY-ESO-1, which demonstrated a 2/11 complete response in patients[226]. NY-ESO-1 is also expressed in other malignancies so there is potential to expand the therapy to other cancer types. TCR gene therapies are limited by the number of antigens available, similar to CAR T cells.

CAR T cells have emerged as one of the most effective immunotherapeutic technologies. CAR T cells bypass all MHC processing to activate against a tumor specific antigen[56]. The CAR is typically virally

Figure 1.2: T Cell and Chimeric Antigen Receptors



Modified from Field AC. Engineered T cell therapies. Expert Reviews in Molecular Medicine, Vol. 17; e19; 1 of 10. License Number: 4680810996397

Figure Legend 1.2: The αβ T cell receptor (TCR) comprises α- and β-chains associated with the γ, δ, e and ζ chains of the CD3 complex. Recombinant TCRs are engineered to be primed for and recognize specific antigens. The single chain TCR combines a recombinant TCR with a CD3ζ signaling domain but activation is still limited by costimulation and MHC processing. Chimeric antigen receptors (CARs) are recombinant molecules composed of an antigen-specific single chain variable fragment (scFv) formed from the variable heavy and variable light chains of an antibody. This is linked through a hinge region to CD3ζ alone (first-generation), or in combination with the intracellular signaling domain of a T cell co-stimulatory molecule, usually CD28 (second-generation), or more recently with an additional signaling domain from a second co-stimulatory molecule such as CD137 (4-1BB) or CD134 (third-generation).

introduced into T cells. There have been multiple iterations of CAR technology. Structurally, the CAR contains an antigen binding domain, termed the single chain variable fragment (scFv), which combines the variable heavy and variable light chain of an antibody. Connected to the antigen recognition domain is a transmembrane domain that signals to the intracellular portion of the CAR made of the T cell signaling machinery, CD3ζ. As generations of the CAR have been developed, costimulatory domains have been added to support activation and persistence of the CAR T cells. The costimulatory domains most commonly used are CD28, 4-1BB, and OX40. Second generation CARs are characterized by one costimulatory domain in the CAR construct and have had the most success in preclinical and clinical models. In the third generation CAR with two costimulatory domains there was no significant benefit acquired. Data are being collected on the different costimulatory domains and their effects on CAR function. Currently, CARs targeting CD19 have led to the most clinical success[227-230]. CD19 CAR T cells target B cells and eradicate both leukemic B cells and healthy B cells. Patients receiving CD19 CAR T cells for their leukemia experience B cell depletion and as a result require intravenous immunoglobulin (IVIG) to manage these symptoms[231]. There is ongoing research to determine the best way to turn off a CAR or clear the CAR modified T cells from the patient considering the on-target, off-tumor toxicities for other cancer associated antigens that may have greater deleterious effects than CD19. One of the major side effects of the CD19 CAR was cytokine release syndrome (CRS). CRS is an influx of inflammatory symptoms resulting from cytokine elevations associated with T cell engagement and proliferation. In patients with severe CRS, symptoms were managed by blocking IL-6 with a monoclonal antibody tocilizumab[232, 233]. In addition, multiple myeloma has been successfully targeted with the CD19 CAR but greater success has been achieved with the B cell maturation antigen (BCMA) CAR T cell (NCT02546167). Hematological malignancies including multiple myeloma, lymphoma, leukemia, follicular lymphoma, non-Hodgkin's lymphoma, diffuse large B cell lymphoma, acute lymphoblastic leukemia, acute myeloid leukemia, chronic lymphocytic leukemia, and small lymphocytic leukemias have been targeting using CAR T cells as well using multiple antigens including CD19, CD20, BCMA, CD22, CD138, CD33, and CD123. Current clinical trials have been limited to relapse and refractory hematological malignancies, but given the success of CAR

T cells in these circumstances the therapy is being expanded to include more targets and newly diagnosed patients.

Despite the success of CAR T cells in B cell malignancies, antigen loss has become a major issue rendering the CAR ineffective[234]. Therefore, strategies such as tandem CARs (2 scFvs on the same construct), coadministration of CARs (transduce 2 pools of cells one with vector A and one with vector B and infuse both cellular products), bicistronic CARs (one vector with a ribosomal skipping segment to allow for approximately equal translation of both CAR constructs), and cotransduction (transducing one pool of T cells with 2 different vectors) CAR T cells have emerged. These are all strategies to have the infused T cell product express multiple antigens for T cell activation. The most common example that has emerged is the CD20 and CD19 CAR T cell products[235]. While this addresses some of the issues associated with CAR therapy, the expansion of CAR T cells into solid tumor malignancies has not had the same success as CARs directed toward hematological malignancies.

Solid tumors have been more difficult to target due to the competing tumor microenvironment (detailed below). Despite these difficulties, CAR T cells have been designed targeting solid tumors[236]. Table 1.2 summarizes CAR T cells being used to treat solid tumors. The number of developed targets for solid tumors is impressive; however, there is still work being done to best identify tumor associated antigens that will limit on-target off-tumor side effects. Both companies and academic institutions have begun massive screens of surface antigens, proteomic screens, and mass RNA sequencing on tumor cells to find novel candidates for CAR T cell therapy. In addition, tumor infiltration and survival in the microenvironment remain major obstacles for the success of solid tumor CAR T cells.

1.3 Challenges in immunotherapy

Immunotherapy has shown great promise, yet complete remission is not always guaranteed, suggesting there is still work to achieve robust and consistent responses. The continued advancement of cancer

Table 1.2: Solid Tumor CAR T cell Targets

Antigen Target	Cancer Type	NCT (if available)
CD44v6[237, 238]	metastasized colon cancer and soft tissue sarcomas	
carboxy-anhydrase-IX[239]	metastatic renal cell carcinoma	
carcinoembryonic antigen	ovarian, gastrointestinal, colorectal, and hepatocellular carcinoma	NCT02959151, NCT02850536, NCT02349724, NCT03267173
CD133[240, 241]	ovarian, glioblastoma, and hepatocellular carcinoma	NCT02541370, NCT03423992
c-Met (hepatocyte growth factor receptor) [242]	breast, melanoma, and hepatocellular carcinoma	NCT01837602, NCT03060356, NCT03672305
EGFR[243-245]	NSCLC, glioblastoma, sarcoma, malignant pleural mesothelioma, retinoblastoma, glioma, medulloblastoma, osteosarcoma, Ewing sarcoma	NCT03152435, NCT03182816, NCT03542799, NCT03638167, NCT03618381
EGFRvIII[246]	glioblastoma, glioma, colorectal, sarcomas, and pancreatic	NCT03283631, NCT02844062, NCT01454596, NCT03267173, NCT03726515, NCT03423992
EpCAM[247]	hepatocellular carcinoma, lung, ovarian, colorectal, breast, gastric, stomach, esophogeal, pancreatic, liver, prostate, gynecological cancers, and nasopharyngeal carcinoma	NCT02915445, NCT03563326, NCT03013712, NCT02729493, NCT02725125
EphA2 (Erythropoetin producing hepatocellular carcinoma A2) [248, 249]	glioblastoma and glioma	NCT03423992
fetal acetylcholine receptor[250]	osteosarcoma and rhabdomyosarcoma	
FRα (folate receptor α) [251]	ovarian and urothelial bladder carcinoma	NCT03185468
GD2[252-255]	neuroblastoma, melanoma, osteosarcoma, rhabdomyosarcoma, Ewing's sarcoma, and cervical cancer	NCT03721068, NCT01953900, NCT03373097, NCT03635632, NCT02765243, NCT02919046, NCT02761915, NCT03356795, NCT03423992, NCT03356782
GPC3 (Glypican-3)	hepatocellular carcinoma, and squamous cell carcinoma	NCT02959151, NCT03084380, NCT02932956, NCT02905188, NCT02876978, NCT02715362, NCT03130712, NCT03198546, NCT03146234, NCT03302403
GUCY2C (Guanylyl cyclase C) [256]	metastatic colorectal cancer	
HER1 (human epidermal growth factor receptor 1) [257]	lung and prostate cancer	
HER2 (ERBB2) [244, 258-262]	breast, ovarian, osteosarcoma, glioblastoma, medulloblastoma, gastric, malignant pleural	NCT03696030, NCT02713984, NCT03740256, NCT02442297, NCT03500991, NCT03198052,

	mesothelioma, sarcoma, and pediatric CNS	NCT00902044, NCT03267173, NCT03389230, NCT03423992, NCT02792114
ICAM-1 (Intercellular adhesion molecule 1) [263]	thyroid cancer	
IL13Rα2 (interleukin 13 receptor α2) [264, 265]	glioma and glioblastoma	NCT03423992, NCT02208362
IL11Rα (interleukin 11 receptor α) [250]	osteosarcoma	
Kras (Kirsten rat sarcoma viral oncogene homolog) [266]	lung adenocarcinoma and pancreatic	
Kras G12D[267]	pancreatic ductal adenocarcinoma, colorectal and lung cancer	
L1CAM (L1-cell adhesion molecule) [268]	ovarian cancer	
MET[269]	malignant pleural mesothelioma	
Mesothelin[270-273]	pancreatic ductal adenocarcinoma, malignant pleural mesothelioma, ovarian, lung adenocarcinoma, and glioblastoma	NCT02930993, NCT02959151, NCT03545815, NCT03182803, NCT01583686, NCT03030001, NCT03747965, NCT03198052, NCT03615313, NCT03267173, NCT03356795, NCT02792114, NCT02414269, NCT03608618
MUC1 (mucin 1) [240]	hepatocellular carcinoma, NSCLC, pancreatic, breast, glioma, colorectal, and gastric cancers	NCT03179007, NCT02587689, NCT03706326, NCT03525782, NCT03198052, NCT03267173, NCT03356795, NCT03356782, NCT03633773
MUC16 ecto (mucin 16)	ovarian cancer[241]	
NKG2D[241, 274]	Ewing's sarcoma, osteosarcoma, and ovarian cancer	
NY-ESO-1[275-277]	liposarcoma, neuroblastoma, synovial sarcoma, melanoma, ovarian, breast, glioblastoma, and NSCLC	
PSCA (prostate stem cell antigen) [240, 278]	pancreatic and prostate cancers	NCT03198052, NCT03267173
WT-1 (Wilms tumor 1) [34]	ovarian cancer[241]	

immunotherapy depends on a deeper understanding of the tumor microenvironment and effective drug combinations. The benefit of immunotherapy is that it functions independent of driver mutations and not all types depend on the presentation of a novel antigen. However, it is apparent there are still many problems that must be considered when designing all types of immunotherapy. Immune suppression in the tumor microenvironment is one of the major obstacles immune based therapies must overcome. While the hosts immune system is equipped with ways of fighting the cancer, immune suppression can come from this same machinery including Tregs, cytokines/chemokines, T cell tolerance/exhaustion, antigen escape, or metabolic proteins.

A. Immunosuppressive tumor microenvironment

Many tumor associated antigens develop from self-antigens, which is partially why Tregs are more actively engaged in suppressing T cell responses to cancer. Tregs are attracted to the site of cancer by chemokines and their infiltration is associated with poor prognosis in cancer patients[18, 39, 116]. It is hypothesized that Tregs take up antigens from dying cancer cells and upregulate checkpoints for cytotoxic T cells, such as CTLA-4[116]. The active suppression of the immune response by Tregs enables tumor growth and metastasis. Other immune cells, such as MDSCs and type II iNKTs, also promote tumor progression. As expected, these cells are recruited by chemokines and growth factors. MDSCs suppress the immune system by production of arginase, NOS[129], TGFβ[122], and IL-10[121]. These factors make for a rich immunosuppressive microenvironment. For example, T cell metabolism requires L-arginine to properly function, and cells like MDSCs secrete the two major enzymes (arginase and NOS) that break down L-arginine[120]. This leads to metabolically compromised T cells that are no longer able to function at the site of the tumor. Other immunosuppressive metabolic regulators include adenosine, prostaglandin E2, and IDO. Adenosine has high concentrations within a tumor that contribute to angiogenic events to promote tumor growth, as does prostaglandin E2[123]. In addition, prostaglandin E2 promotes tumor cell migration, inhibits functioning of DCs, and downregulates proinflammatory cytokines. Some tumors and tumor draining lymph nodes have

high expression of IDO, an enzyme that degrades essential amino acids. IDO mediates immune tolerance by depleting tryptophan from tumor microenvironments and areas for T cell maturation[125]. The immunosuppressive immune cell infiltration, cytokine profiles, and metabolic restrictions in the tumor microenvironment contribute to incomplete responses in cancer patients on immunotherapy.

B. T cell tolerance and exhaustion

The immune escape described in Schreiber's hypothesis relies on an immunosuppressive microenvironment and T-cell tolerance to tumor[279]. In the absence of costimulation, T cells are unable to activate and become anergic[280]. T cell tolerance is broken into positive and negative costimulation, where CD4+ T cells require both and CD8+ T cells only require positive costimulation. Positive costimulation depends on CD28 that binds to B7.1 and B7.2 or inducible T cell costimulator (ICOS) that binds B7-H3. Without this interaction, there is defective T cell functioning. CD4+ cells require an additional negative costimulation signal to become tolerant. Exhaustion markers such as CTLA-4 and PD-1 and B7-H3, and B7-S1 are proteins that cause anergic T cells[279]. Part of the issue with targeting the positive and negative costimulation proteins is they mediate T cell positive and negative selection, mechanisms that prevent autoreactive T cells. Anergic and tolerant T cells contribute to the immune escape and allow for an immunosuppressive tumor microenvironment.

T cell exhaustion contributes to impair functioning of cytotoxic cells. It is important to recognize that an exhausted T cell started in an activated state, but was chronically exposed to antigen. PD-1, Lag-3, Tim-3, CD160, and T cell immunoreceptor with Ig and ITIM, and TIGIT are upregulated when a T cell is exhausted, though typically one protein is not enough to define exhaustion[198-201]. These proteins are also markers of an activated T cell depending on the set of circumstances. Exhausted T cells lose functioning in a succession of steps: loss of IL-2 production, loss of TNFα, IFNγ, beta chemokines and degranulation. T cells reaching this stage of exhaustion are likely to complete apoptosis[281, 282]. However, depending on the degree of exhaustion, exhausted T cells still have minor effector functions and tumor infiltration of

exhausted T cells can still correspond with some clinical benefit[283, 284]. Major advances have been made to target exhaustion markers and these checkpoint inhibitors have been largely successful at increasing T cell activation at tumor sites.

C. Biomarkers

A remaining problem in cancer immunotherapy, especially in checkpoint inhibitor therapy, is that there are a lack of biomarkers to determine clinical success. This translates into unpredictable responses from patients receiving immunotherapy. The lack of identifiable soluble proteins from serum, receptor expression and patterns, the microenvironment landscape, or naïve genomic markers to monitor patient response to checkpoint inhibitors remains an obstacle in the field making it difficult for clinicians to decide appropriate treatments regimens. Most of the biomarker analysis has been completed retrospectively because of the lack of understanding of immunotherapy mechanisms. Finding biomarkers is especially difficult due to the T cells and cancer cells being dynamic and having a constantly changing landscape of protein expression of potential biomarkers. The two most predictive biomarkers are cytotoxic lymphocyte infiltration and PD-L1 expression in the microenvironment[285].

Biomarkers can be found at the soluble, cellular, or genomic level in serum, peripheral blood, and the tumor. Serum biomarkers include IL-6, C-reactive protein (CRP), VEGF, lactate dehydrogenase (LDH), sCD25, NY-ESO-1 antibody. IL-6 and CRP has been prognostic for high dose IL-2 treatments in metastatic renal cell carcinoma for decreased overall survival[286]. In advanced melanoma, serum levels of VEGF showed a clinical response with high-dose IL-2 treatment[287]. VEGF also showed positive outcomes in serum for patients on ipilimumab in melanoma patients[288]. In ipilimumab treatment, high LDH and low soluble CD25 have been a biomarker for a negative clinical outcome[289].

Serum biomarkers and peripheral blood biomarkers are of greater clinical utility due to the minimal invasiveness to collect these data from a patients. Some of the peripheral blood biomarkers from

immunotherapy include neutrophils/leukocytes, lymphocytes, CD8+ T cells, eosinophils, CD4+ ICOS+ T cells, and MDSCs. Within the peripheral blood of metastatic melanoma patients, high concentrations of neutrophils was associated with poor prognoses[290]. As MDSCs are an immunosuppressive cell, it is unsurprising that high concentrations in the peripheral blood correlates with an overall worse survival in patients receiving checkpoint inhibitors[291]. High-dose IL2 results in lymphopenia, but a rebound in lymphocyte count correlates with a favorable clinical outcome[292].

High levels of tumor infiltrating lymphocytes within patients receiving immunotherapy leads to favorable outcomes. High levels of CD8+ T cells within the tumor of patients receiving checkpoint inhibitor therapy has caused an overall survival benefit and predictive of a positive outcome[293-295]. In addition, within the tumor biomarkers include PD-L1, tumor mutational load, and DNA mismatch repair (MMR) [296]. PD-L1 status within a tumor has been highly debated if indicative of a response to PD-1/PD-L1 checkpoint blockades[196, 297-299]. This controversy is partially due to the variability of the expression within patients, thus, it is one of the most studied biomarkers. The mutational load of a tumor[300] and MMR proteins have been thought of as predictive biomarkers in other cancer therapies and studies are still ongoing to validate that these are also predictive of an immunotherapeutic response. While these biomarkers are promising, they each have specific clinical significance that has been tested in few cancer models and with limited therapeutics. The available data on biomarkers while promising, is limited to retrospective analyses that are often incomplete and variable.

D. Neoantigens and antigen escape

One of the greatest challenges in cancer therapy is finding tumor neoantigens to target. ACT and monoclonal antibody therapy depend on targeting a specific antigen restricted to the tumor cells. These tumor associated antigens must be homogenously expressed on the cancer as well. While many targets have been found to be highly expressed on the surface of tumors, they are also expressed on healthy tissue. Finding the balance between on-target off-tumor toxicity is essential when creating CAR T cells and

monoclonal antibodies. Through VDJ recombination, there are a limited number of BCRs that can be arranged. This limited number contributes to the difficulty in targeting tumor neoantigens[301]. An additional, a problem in CAR-based therapies is the lack of efficacious methods to turn the CAR off once targeted to an antigen, meaning if the antigen is expressed on any healthy tissues, the CAR will have deleterious effects on the healthy cells.

Despite finding an antigen with low on-target off-tumor toxicity, there is still potential for antigen loss and escape. Antigen loss is one of the major problems emerging from CAR T cell therapy caused by selective pressure from CAR T cells on the cancer to evolve and become undetectable[302]. The most compelling evidence of antigen loss has been seen with CAR T cells is with the CD19 CAR. In patients with B-ALL treated with the CD19 CAR, antigen loss is seen after patients relapse with a similar disease with loss of CD19 surface expression. It is hypothesized that the CAR selects for the CD19 negative tumor cells, but phenotypically the B-ALL functions similarly to pre-CAR treatment[303]. Another mechanism of antigen loss is that the lineage of the leukemia switches from a B-ALL to AML phenotypically[304]. This has been seen in two pediatric patients with a mixed lineage leukemia (MLL) rearrangement and one adult patient in clinical trials. Related to antigen loss is low antigen density that is typically detectable by natural TCRs, but CARs have been reported to only be functional with high antigen loads[305, 306]. Due to the CARs simplicity and need for high antigen loads, it is hypothesized that one target for CAR activation and engagement will be insufficient.

1.4 Overcoming problems in immunotherapy

Important work is being conducted to test hypotheses and solutions to all of the challenges presented by immunotherapy. The expansion of ACT research has led to the prioritization of the problems associated with this technology. Research is ongoing and two areas that have recently seen great development are the utilization of alternative cellular sources and development of alternative antigen binding domains for CAR

therapy. This dissertation addresses these two issues within the context of high risk and relapsed pediatric malignancies.

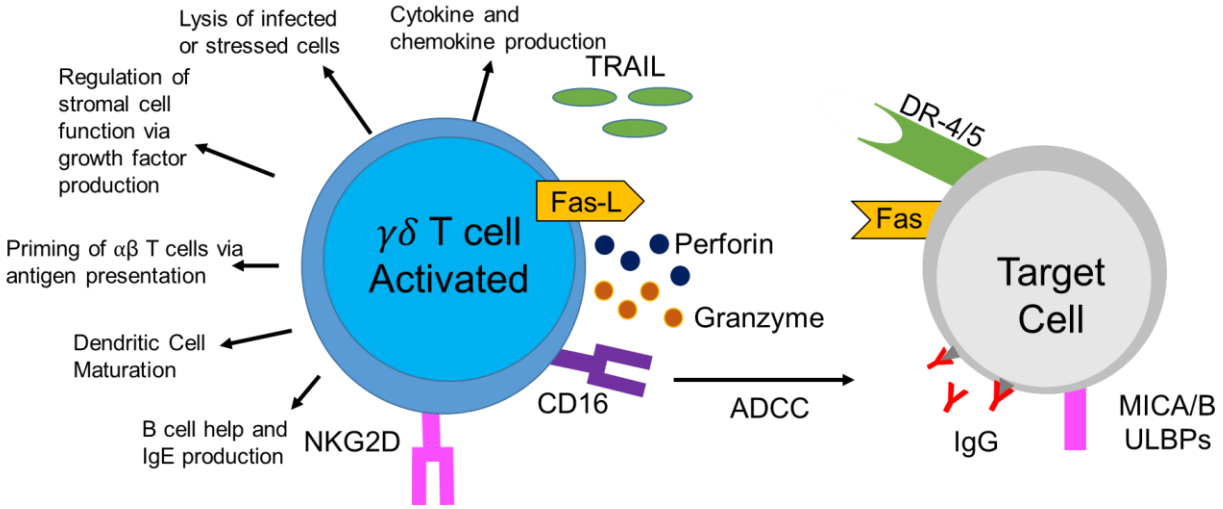
A. Utilization of alternative cell sources

Primarily, cell-mediated immunotherapies rely upon the αβ T cell, due to its cytotoxic function in the adaptive immune system. But, in general, αβ T cells do not have inherent cytotoxicity to tumor cells and require MHC processing to mediate cell killing. In contrast, γδ T cells are specific innate cells that remains an attractive candidate for immunotherapy because, unlike the αβ T cell, it is not restricted by MHC complexes. Additionally, γδ T cells require no priming, have intrinsic anti-tumorigenicity, and respond to tumors via recognition of stress antigens and antibody-mediated mechanisms (Figure 1.3)[70, 85, 307]. Target cells are recognized by the innate immune system through pattern recognition receptors, or through a damaged or stressed target cell sending a signal to initiate an immune response. The response associated with processing of an antigen through MHC recognition is associated with the adaptive immune response, but γδ T cells are capable of bridging this adaptive and innate immune response[70, 85, 307]. γδ T cells are able to reduce tumor burden using the major mechanisms of ADCC and stress antigen recognition without additional priming. CD16 positive γδ T cells can efficiently mediate ADCC, even though CD16 is a low affinity receptor, the γδ T cell will activate when the receptor interacts with antibody coated cells[45, 46]. Combination therapy involving *ex vivo*-expanded γδ T cells could thus improve patient outcomes in both safety and efficacy.

B. Expanding the repertoire of antigen recognition

Most CAR T cells rely on an antibody-based scFv for antigen recognition. scFvs have instability, immunogenicity, and aggregation that can render a CAR ineffective. Affinity and specificity are key criteria for a functional CAR and this is not guaranteed when using a scFv. An alternative approach to using a scFv is using a natural receptor ligand interaction to engage a CAR. Utilization of a ligand can reduce

Figure 1.3: γδ T cells anti-tumor and pro-immunogenic activity



Modified Vantourout P and Hayday A. Nature Reviews Immunology 2013

Modified Lafont, V et al. Frontiers in Immunology 2014

Figure Legend: γδ T cells can defend against a broad range of infectious and sterile stresses by directly eliminating infected or stressed cells; by producing a diversified set of cytokines and chemokines to regulate other immune and non-immune cells; by directly promoting immune cell maturation and activation by triggering B cell help, DC maturation and αβ T cell priming via antigen presentation; and by regulating stromal cell function. γδ T cells can recognize tumor cells through interaction with (i) TCR ligands, such as phosphoantigens (ii) innate receptor ligands. Following sensing of tumor antigens or stress signals, γδ T cells are activated and can kill tumor cells through cytotoxic mechanisms that rely on the perforin/granzyme pathway, the death receptor pathway in response to TRAIL or Fas-L expression, and ADCC in the presence of tumor-specific antibodies and expanding targets for CARs can benefit more cancer patients, but gives potential for the advancement of CAR therapy for other non-cancer diseases.

immunogenicity, better predict on-target off-tumor interactions, and increase binding affinity for natural receptors[308, 309]. There are few ligand-based CARs in preclinical and clinical development including the IL-13Rα, IL-11, adectin, follicle stimulating hormone receptor, and granulocyte- macrophage colony stimulating factor[310-314]. There has been success and clinical advancement of the IL-13 Rα CAR in glioblastoma and the T1E in head and neck cancers, while the remaining ligand-based CARs are still being developed preclinically [265, 315]. Considering there have been successes using ligand-based CARs, creating more ligand-based CARs can positively impact cancer therapy through targeting more epitopes. Additionally, actively pursuing natural receptors

Chapter 2

Ex vivo expanded patient-derived γδ T-cell immunotherapy enhances neuroblastoma tumor regression in a murine model.

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Jaquelyn T. Zoine performed and designed all experiments, wrote and edited the paper

Kristopher A. Knight assisted with mouse work

Lauren C. Fleischer contributed to supplementary figure 3

Kathryn S. Sutton and Kelly C. Goldsmith contributed with neuroblastoma expertise and editing the paper

Christopher B. Doering and H. Trent Spencer conceived the experiments and edited the paper

2.1 Abstract

An effective therapy regimen for relapsed/refractory high-risk neuroblastoma (NB) includes the anti–GD2 monoclonal antibody, dinutuximab, in combination with temozolomide and irinotecan, supporting a role for chemo-immunotherapy in NB. γδ T cells are an attractive anti-tumor immunotherapy because of their direct cytotoxic activity mediated through cell surface receptors NKG2D and CD16. NKG2D facilitates the innate recognition of stress-induced ligands whereas CD16 recognizes antibody bound to tumors and activates mechanisms of antibody-dependent cellular cytotoxicity (ADCC). This study demonstrates an efficient method for expanding and storing γδ T cells from NB patient-derived apheresis products at clinically relevant amounts. The expanded patient-derived γδ T cells were cytotoxic against the K562 cell line and multiple NB cell lines. Combining γδ T cells with dinutuximab led to a 30% increase in tumor cell lysis compared to γδ T cells alone. Furthermore, low-dose temozolomide in combination with expanded γδ T cells and dinutuximab resulted in increased IFNγ secretion and increased γδ T-cell surface expression of FasL and CD107a. IMR5 NB cell line xenografts established subcutaneously in NSG mice were treated with a regimen of dinutuximab, temozolomide, and γδ T cells. This combination caused targeted killing of NB xenografts *in vivo*, reducing tumor burden and prolonging survival. These data support the continued preclinical testing of dinutuximab and temozolomide in conjunction with γδ T-cell immunotherapy for patients with recurrent/refractory NB.

2.2 Introduction

Neuroblastoma (NB), the most common extracranial pediatric solid tumor, is treated with multimodal therapy, including cytotoxic chemotherapy, autologous stem cell transplantation, local control with surgery and radiation, and maintenance immunotherapy. Despite increases in the intensity of therapy, high-risk NB has a 5-year event-free survival rate of <50% [316, 317]. Further dose escalation to improve survival is limited by the acute and chronic toxicities already encountered with current chemotherapy regimens [318-320]. Cytotoxic chemotherapy has rarely been curative in high-risk NB as an individual treatment, leading to the evaluation of more targeted agents and novel modalities, most recently immunotherapy (for example see:

NCT03294954, NCT01460901, NCT01576692, NCT03242603, NCT03373097, and NCT02311621). Chemotherapy can positively impact the efficacy of immunotherapy by limiting the interference of protumorigenic immune regulatory cells systemically and within the tumor microenvironment [321-326]. It can also be detrimental, as chemotherapy can be toxic to the therapeutic immunocompetent cells, whether innate or adaptive [327, 328]. While chemotherapy lacks specificity and generally targets proliferating cells, it can still be used as an effective method to sensitize tumors to anti-tumor cytotoxic T-cell lymphocytes[329-331].

The use of monoclonal antibodies (mAb) as a cancer therapeutic has been clinically evaluated in an array of neoplastic disorders [332]. Specifically for NB, the development of antibodies to GD2 (3-F8, ch14.18 now dinutuximab, hu14.18K322A, etc. [333]), a diasylganglioside found on a subset of NB cells, proved useful in the setting of minimal residual disease, yet showed little effect on the growth of bulky tumors in the relapsed and refractory setting [334-341]. However, the combination of dinutuximab with chemotherapy resulted in a 47% response rate, with complete and partial responses seen in relapsed/refractory patients with bulky metastatic disease [342]. Furthermore, the combination of chemotherapy and a similar monoclonal anti-GD2 antibody in newly diagnosed high risk NB patients has shown thus far response rates as high as 80% [338, 343, 344]. Although increased response rates have been achieved, the effect of anti-GD2 antibody and chemotherapy combinations on cure rates in newly diagnosed high-risk NB patients is currently unknown and, therefore, actively under investigation in pilot clinical trials. Overall, the success of dinutuximab and chemotherapy combinations has established a paradigm of trial concepts and preclinical investigations to identify additional agents that will augment this effective chemo-immunotherapy backbone (NCT01576692 and NCT0379349).

The biological effectiveness of anti-GD2 antibodies, including dinutuximab, is dependent on antibody-dependent cellular cytotoxicity (ADCC) through the CD16, FcγRIII, receptor [45, 46]. We hypothesized the efficiency of monoclonal antibodies (mAbs), such as dinutuximab, could be improved by infusion of cytotoxic cells capable of recognizing the Fc portion of mAbs. γδ T cells are innate immunocompetent cells

that are an attractive candidate for immunotherapy because, unlike αβ T cells, they are not restricted by major histocompatibility complexes (MHC). Importantly, subsets of expanded γδ T cells have been shown to express high levels of CD16 [345], which can enhance ADCC through Fc recognition. In addition, γδ T cells have intrinsic anti-tumor activity as they also express FasL and recognize stress antigens [87, 346]. γδ T cells have the inherent ability to recognize stress antigens including MHC class I related chain A/B (MICA/B) and UL16 binding protein (ULBPs) via the NKG2D receptor (natural killer group 2, member D) [70, 347]. The interaction of NKG2D with stress-inducible ligands produces rapid cell lysis and secretion of pro-inflammatory cytokines, including IFNγ [87, 348]. Studies have also shown that host γδ T- cell tumor infiltration results in an overall better prognosis [349]. Taken together, we hypothesize that administration of an *ex vivo* expanded γδ T-cell product could be an effective and novel treatment for high-risk NB.

Unfortunately, efforts aimed at expanding γδ T cells *in vivo* have not shown clinical benefits. For example, stimulating the production of γδ T cells *in vivo* with IL-2 can concurrently stimulate production of regulatory T cells, potentially inhibiting immune surveillance of cancer cells [350, 351]. We therefore devised a novel method to successfully expand γδ T cells from peripheral blood. Our previous studies demonstrated γδ T cells from healthy donor frozen peripheral blood mononuclear cells (PBMCs) can be expanded using a serum-free expansion protocol [352]. Notably, newly diagnosed high risk NB patients undergo hematopoeitic stem cell collection and storage in anticipation of autologous stem cell transplant as standard of care, yet many of these apheresis products go unused. One goal of these investigations was to assess whether γδ T cells from NB patient apheresis products could be used as a potential source for a viable and active expansion.

NKG2D is highly expressed on healthy donor expanded γδ T cells [307, 345, 346]. Prior studies have shown that chemotherapy induces the expression of stress antigens such as, MHC class I chain-related protein A or protein B (MICA/B) or UL16-binding proteins (ULBPs), on the tumor cell surface, increasing tumor cell vulnerability [353]. By increasing susceptibility of cancer cells to recognition via the NKG2D receptor on γδ

T cells, chemo-immunotherapy combinations can provide a therapeutic benefit not seen by either modality alone [70, 329, 330, 354-356]. The alkylating agent, temozolomide (TMZ), is used in heavily pre-treated relapsed patients to induce tumor cell killing [357]. TMZ is known to induce transient expression of NKG2D ligands [70, 329, 330]. We therefore hypothesized that dinutuximab and TMZ in combination with *ex vivo* expanded γδ T cells may provide a benefit to NB treatment outcomes. Herein, our data supports the ability to expand γδ T cells in serum-free conditions from apheresis hematopoietic stem cell (HSC) products collected from patients with NB, and illustrates a survival benefit when combining these cells with chemotherapy and mAb therapy.

2.3 Results

Robust NB patient-derived γδ T cell expansion in serum free media

Recently, we published a good manufacturing practice (GMP)-compliant process using serum-free media to expand γδ T cells with aminobisphosphonates (e.g. zoledronic acid) combined with IL-2 [352]. To determine whether these methods could be translated to frozen primary NB patient mobilized and apheresed PBMCs, the serum-free protocol with zoledronic acid and IL-2 supplementation was employed using cells harvested from 5 NB patients and compared to healthy controls, which were included to replicate our previous findings. Overall, the percentage of γδ T cells from NB patient donors during 2 week cultures increased from 1.15 ± 0.90% to greater than 75% of the population (**Fig 2.1A**). Mean-fold expansion of NB patient-derived γδ T cells ranged from 25- to 310-fold. Reproducibility was tested by expanding cells from one donor in triplicate, which showed no significant variability (paired *t* test) in the percentage of γδ T cells during the expansion process (**Fig 2.1B**). Flow cytometry analysis confirmed the resulting cell populations to be of similar composition among the NB patient and healthy PBMCs, including populations of γδ T cells ($CD3^+$, pan-$\gamma\delta^+$), αβ T cells ($CD3^+$, pan-$\gamma\delta^-$), and a low percentage of $CD3^-$ cells. Specifically, by day 14 of expansion, the myeloid/lymphoid non-T-cell population ($CD3^-$, $CD56^-$) comprised 2-10% of the population, NK cells ($CD3^-$, $CD56^+$) accounted for 4-12%, the non- γδ lymphocytes range from 4-26%, with the γδ

Figure 2.1: Expansion of γδ T cells from NB patient-derived PBMCs.

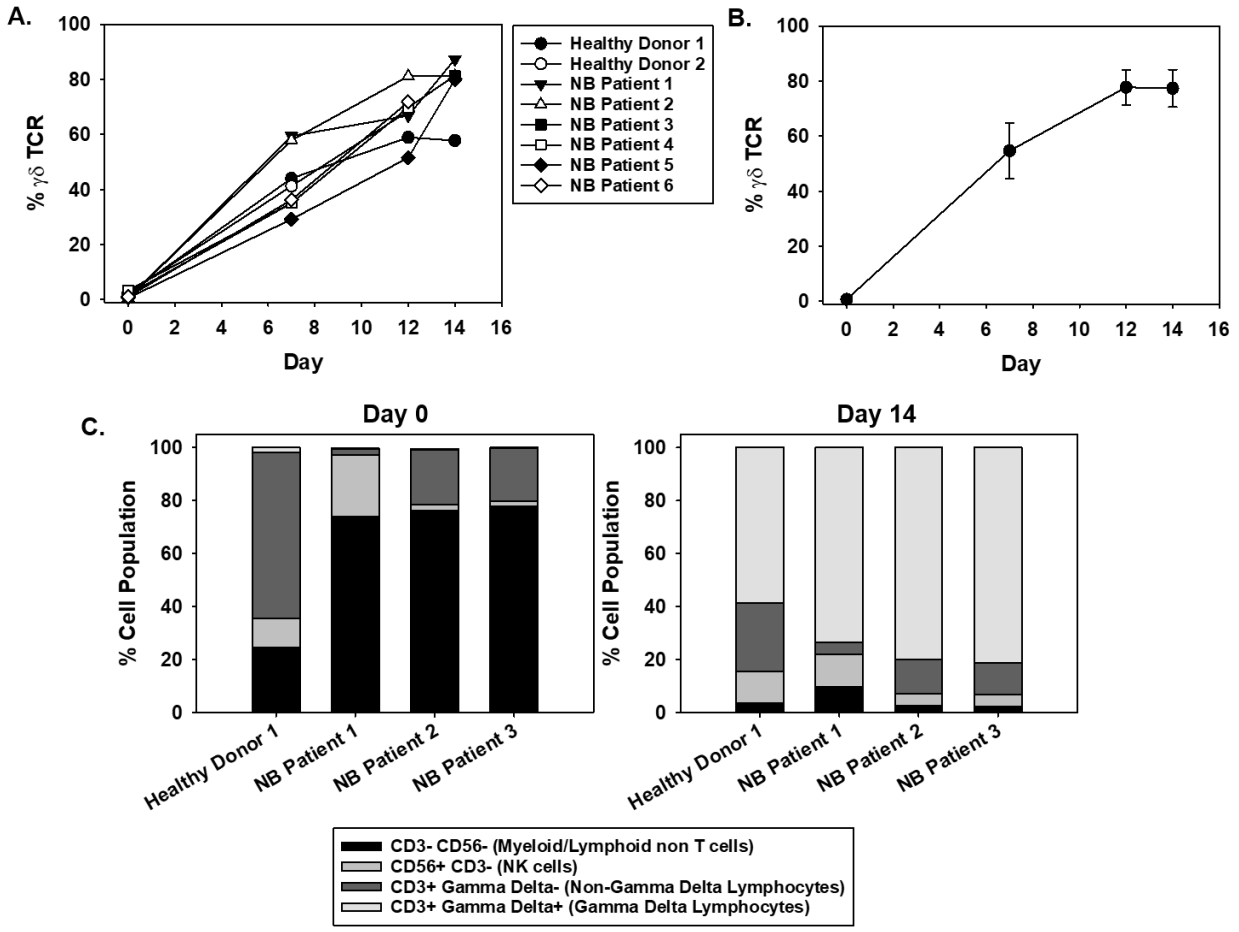


Figure Legend 2.1: A. γδ T cells were expanded using serum-free conditions from commercially available healthy donor PBMCs (n=2, with one repeated expansion using the same donor) or NB patients (n=6, where some patient samples were expanded multiple times). All cultures were supplemented with IL-2 on days 0 (500 IU/mL), 3 (500 IU/mL), 6 (1,000 IU/mL), and 9 (1,000 IU/mL) and zoledronic acid (5 μM) on days 3 and 6. Live cells were gated for CD3+, CD56-, and pan γδ TCR+ to determine γδ T-cell percentage. Data comparing a healthy donor versus a NB patient starting product were not significantly different by non-paired, two tail t-test on day 7 ($p=0.97$) and 12 ($p=0.55$). B. An apheresis product from a single NB patient was divided into two samples and each was expanded three times. The average γδ T-cell percentage and standard deviation are shown. C. The cellular distribution of a starting PBMC product on day 0 differs from the cellular components on day 14 of expansion, and the change is not dependent on the donor being healthy

or having NB. γδ T cells comprise less than 5% of the starting cellular product and increases to 60-90% of the expanded cells.

lymphocytes constituting the majority of the population, at 60-82% (**Fig 2.1C, Supplemental Table 2.1**). Additional flow cytometry characterization was performed to further classify the different γδ T-cell populations, predominantly the Vδ2 subtype (**Supplemental Figure 2.1, Supplemental Table 2.2**). The majority of cells are CD3+ (95.57±0.00) and of these, the majority are γδ T cells (85.63±0.85 Vδ2 and 7.00±0.07 Vδ1). Of the Vδ2 γδ T cells, we further subdivided into CD28+ CD27+ (65.90±0.71), CD28- CD27+ (19.73±1.56), and CD28- CD27- (12.37±0.99) (Table 2). These results therefore show that the bulk population of the γδ T cells are defined as Vδ2 γδ effector memory T cells, denoted by CD28+ CD27+ CD16+ CD45RA-CD45RO+ and CD62L-phenotype[86]. Based on preliminary RNAseq data (data not shown) the transcripts for perforin and granzyme are high (in the top 10% of RNA reads). Therefore, supporting that effector memory cells have high granzyme/perforin expression. Additionally, the NB patient-derived γδ T cells lack PD1/PDL1 expression, suggesting the cells can function despite a PD1/PDL1 rich tumor environment (**Supplemental Table 2.2**). As expected there is a mixed expression of CD57, as this senescence marker indicates some cells are further down the senescence pathway than others (**Supplemental Table 2.2**). Together, these data demonstrate the ability to consistently achieve similar expansion of active NB patient-derived γδ T-cell populations and the subtypes associated with these expansions.

NB patient-derived γδ T cells remain cytotoxic after freezing

To evaluate cytotoxicity of patient-derived γδ T cells against a standard K562, chronic myelogenous leukemia cell line, expanded γδ T cells were co-incubated at a 5:1 effector to target ratio. The γδ T cells derived from NB patients (N=5) and healthy donors (N=3) were used against the target cell line to determine the percentage of cells killed by effectors, which was evaluated by flow cytometry (**Fig 2.2A and 2.2C**). To ensure the γδ T cells would be uniform and useful as a cellular product, we evaluated γδ T-cell cytotoxicity following a serum-free freezing process. The viability of the γδ T cells post-thaw was assessed using trypan blue exclusion and was consistently greater than 70%. Eight hours after the cells were thawed,

Figure 2.2: Cytotoxic activity of NB patient-derived γδ T cells

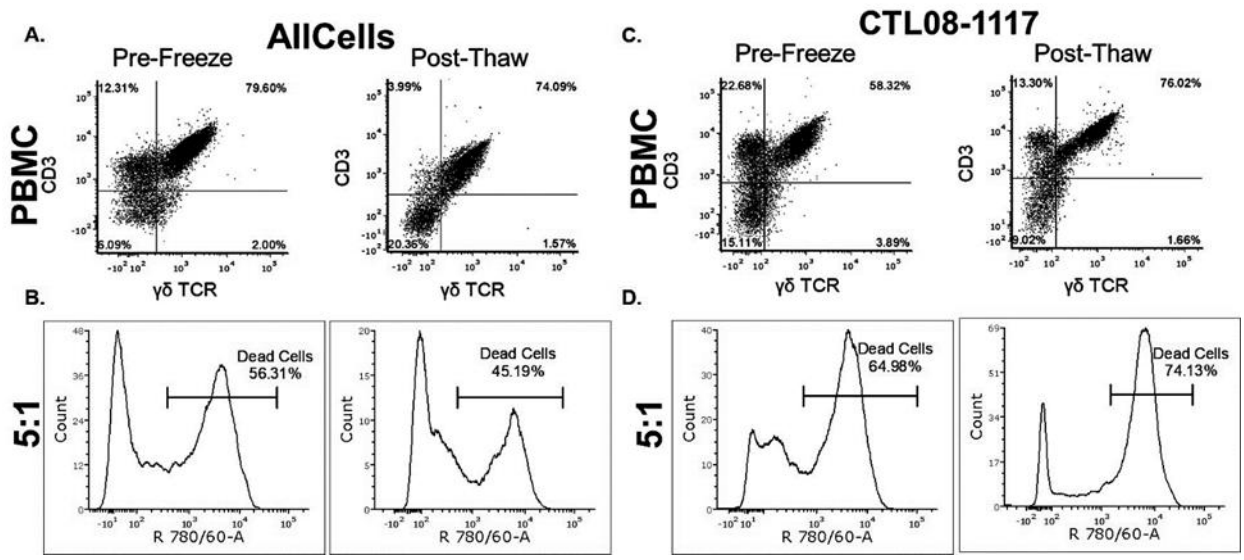


Figure Legend 2.2: Healthy donor (A, B) and NB patient (C, D) γδ T cells were expanded to day 14 and frozen at $1x10^7$ cells/mL. Prior to freezing, cells were tested for cytotoxic potential against K562 cells. The left side panels show data from cells prior to freezing and the right side panels show data from cells after freezing. For the post thaw samples, cells were thawed at 37°C and incubated in growth media for 8 hours prior to use in the 4 hr cytotoxicity assay. (B, D) γδ T cells were used at a 5:1 effector to target ratio and incubated for 4 hr with K562 cells. Cytotoxicity was measured using flow cytometry by gating on the target cells stained with VPD450 and then analyzing the target cells for the dead stain dye, eFluor Alexa 780. There was no significant difference between pre-freeze and post-thaw γδ T-cell percentage as well as cytotoxicity (one-way ANOVA p=0.618, N=4). Raw representative flow cytometry data are shown.

cell killing was normalized to background cell death and maintained at 51.9±5.5% (N=5), which was not significantly different from the 58.7±10.3% prior to freezing (N=5) (**Fig 2.2B, 2.2D**).

Killing by NB patient-derived γδ T cells is enhanced when combined with Dinutuximab

In the presence of mAb, the FcγRIII receptor, CD16, facilitates ADCC and this mechanism has been evaluated pre-clinically in NB models [163, 164, 358, 359]. The serum-free expansion and activation of γδ T cells demonstrated that patient-derived and healthy donor cells have robust expression of CD16 (**Fig 2.3A-B**). CD16 expression at the start of expansion was <20% and increased to >80% by day 6 on the total and γδ T cell populations.

To first determine the binding potential of dinutuximab to a number of NB cell lines, dinutuximab was biotinylated and flow cytometry was used to measure its binding to NGP, NLF, SMS-SAN, NB1691, LAN5, NB1643, SMS-KCNR, IMR5, Kelly, SKNBE2C (*MYCN* amplified) and SKNAS, CHLA15, and CHLA20 (*MYCN* single copy) NB cell lines. These cell lines were derived from a variety of human NB tumors with variable GD2 expression, aggressiveness of disease, and genomic profiles, including those with and without *MYCN* amplification and/or *ALK* mutations. High GD2 expression correlated with greater dinutuximab binding (non-linear regression second order polynomial R^2=0.94) compared with dinutuximab binding to non-NB cell lines that lack GD2 expression such as K562, Jurkat, and 697, which were used as references for non-specific binding (**Fig 2.3C**). Interestingly there is less correlation between the amount of GD2 expressed on the cell line surface and the increase in cytotoxicity with DTX. This suggests that the γδ T cells are able to recognize antibody targets on the cell surface regardless the concentration of antibody bound. To assess the cytotoxicity potential of patient-derived γδ T cells against human derived NB cell lines, 3 separate patient-derived γδ T-cell expansions were used in cytotoxicity assays with high GD2-expressing NB cell lines at 5:1 effector to target ratios, with and without dinutuximab (**Fig 2.4A**). The average cell killing of IMR5, Kelly, CHLA15, CHLA20, and SMS-SAN NB cell lines increased 97.9%, 62.9%, 32.7%, 33.0%, and 54.5%, respectively, when target cells were incubated with γδ T cells and

Figure 2.3: CD16 expression of NB patient-derived γδ T cells and dinutuximab binding to NB cell lines

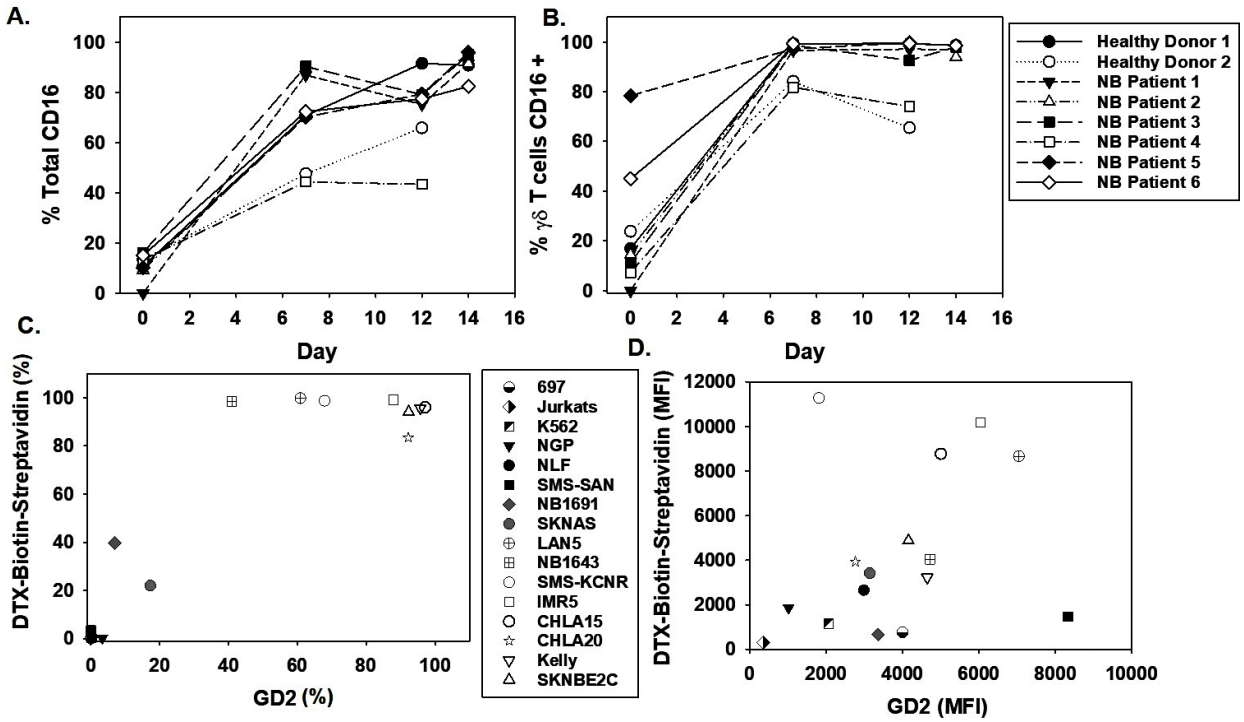


Figure Legend 2.3: A. γδ T cells were expanded using serum-free conditions and examined for surface expression of CD16 by flow cytometry. CD16 expression on days 0, 6, 12, and 14 of expansion is shown for the bulk culture (A) and specifically on γδ T cells (B). Multiple NB cell lines of variable genomic profiles and disease aggressiveness were evaluated for the percentage of GD2 surface expression and dinutuximab (DTX) binding (C). Biotinylated DTX (0.5 μg) was used to demonstrate the specificity of antibody binding to NB cell lines, which showed a trend between GD2 expression and DTX binding with increased surface GD2 corresponding to increased DTX binding (N=3 per sample and the mean is shown).

Figure 2.4: Evaluating ADCC with NB patient-derived γδ T cells

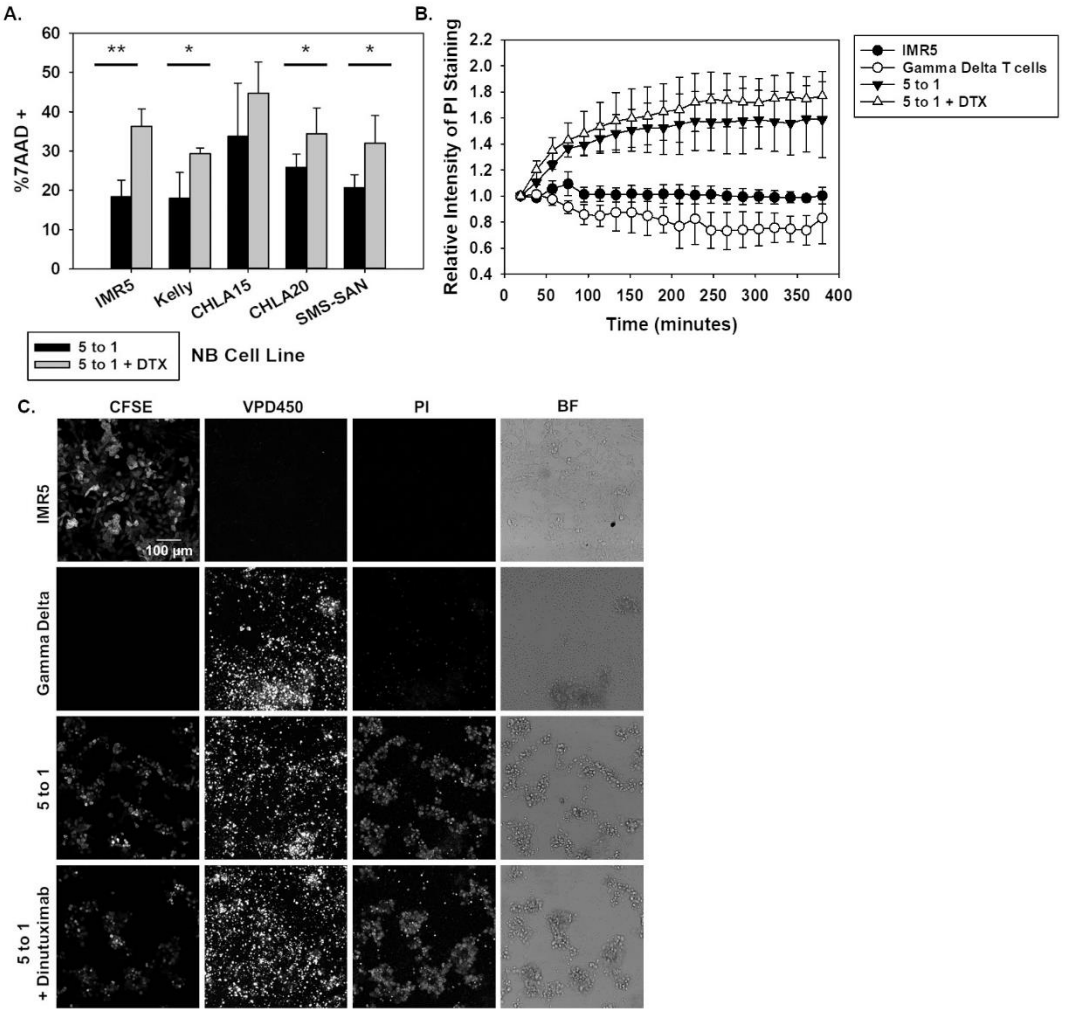


Figure Legend 2.4: A. Cytotoxicity assay using NB cell lines that showed high GD2 expression. Black bars indicate NB patient-derived γδ T cells at a 5 to 1 effector to target ratio while the grey bars represent the same assay with dinutuximab (DTX) included at 5 µg/mL. DTX alone did not show significant killing (data not shown). Expanded NB patient cells functioned as the effector cells and experiments were repeated in triplicate, mean and standard deviations (SD) are shown. (Significance determined by paired t-test * $p<0.05$; **$p<0.01$). B. Live cell images at 6 hrs of IMR5 cells (stained with CFSE) co-incubated with or without γδ T cells (stained with VPD450 proliferation dye) and with or without DTX (5 µg/mL). Cell death was quantified by PI intensity (100 µM). The mean and SD (N=4) were quantified by the relative intensity of PI staining over 6 hrs during live cell imaging. C. Representative still images at 6 hrs: CFSE stain shows

the IMR5 target cells, VPD450 shows the effector γδ T cells, PI staining identifies dead cells and bright field are captured.

dinutuximab relative to baseline cell death of untreated cells. We performed live cell imaging over 6 hr, which confirmed increased cell killing of IMR5 cells when treated with γδ T cells and dinutuximab compared to background cell killing in target or effector cells alone (**Fig 2.4B-C**). Representative still images from the live cell imaging reveals the high intensity of PI staining at 6 hrs when γδ T cells were incubated with IMR5 cells, clearly demonstrating cytotoxic potential of patient-derived γδ T cells against NB (**Fig 2.4C**).

Patient-derived γδ T cells in combination with dinutuximab do not affect NB growth in a xenograft murine model

To test the effectiveness of patient derived γδ T cells *in vivo*, NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG) mice were used to establish subcutaneous NB tumors with IMR5 cells. Once the tumor was palpable (125 mm^3), the animals were randomized to receive γδ T cells alone or γδ T cells with 200 or 400 μg of dinutuximab, all delivered intravenously. Injections of γδ T cells were administered every 3 days over 15 days and dinutuximab was administered on days 1 and 10 (**Fig 2.5A**). Mice were monitored for tumor growth (**Fig 2.5B**), weight, and survival. There were no significant differences in the average tumor volume, weight, or survival between these treatment groups by one-way ANOVA on days 10 and 20.

To determine the efficiency of tumor targeting by dinutuximab and γδ T cells, biotinylated dinutuximab was administered and on days 1, 4, and 7 after injection, tumors were harvested and antibody-opsonized tumor cells were measured by flow cytometry. The greatest binding to tumor cells was observed on day 4 with a geometric mean fluorescence intensity (MFI) of 1848. Day 1 and 7 MFI were 1459 and 1056, respectively (**Fig 2.5C**). As expected, γδ T-cell homing to IMR5 cells was low, with less than 1% of the tumor being γδ T cells, which was approximately 10-fold less than the percentage of the γδ T cells found in peripheral blood (**Supplemental Figure 2.2**) [360]. In addition, the persistence of γδ T cells in peripheral blood demonstrated a steady decline with values decreasing to near baseline by 1 week after administration (**Supplemental Figure 2.3**).

Figure 2.5: *In vivo* effectiveness of NB patient-derived γδ T cells and dinutuximab

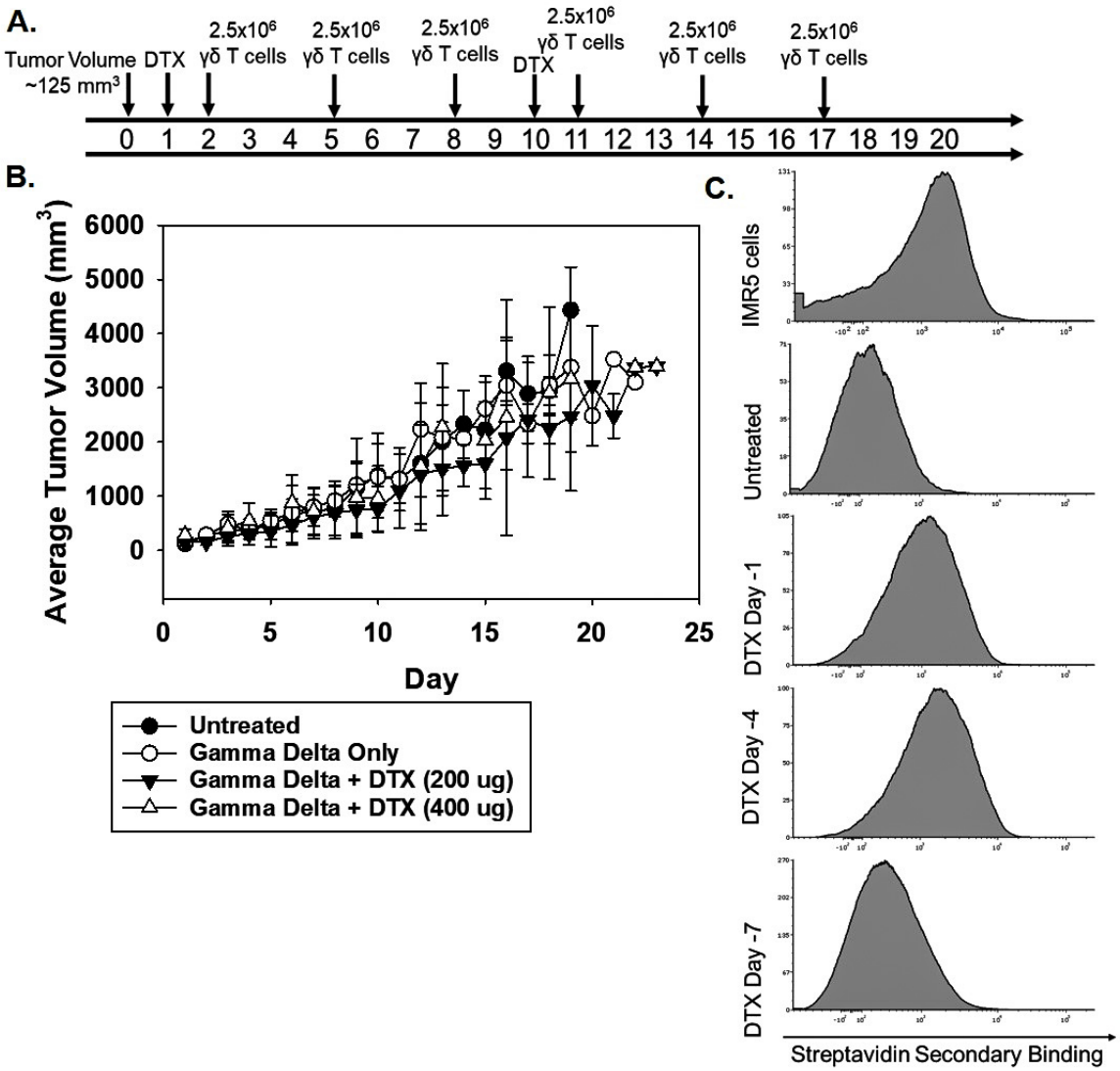


Figure Legend 2.5: A. Schematic of the treatment plan for NSG mice injected subcutaneously with IMR5 cells and treated with γδ T cells, dinutuximab (DTX), or a combination of the two. B. The mean tumor volume is shown for each group: untreated (N=8), γδ T cells only (N=8), γδ T cells + DTX (200 μg) (N=6), γδ T cells + DTX (400 μg) (N=4). The mean and standard deviation is shown for each cohort. C. NSG mice with established IMR5 subcutaneous tumors were injected with 200 μg biotinylated DTX. Mice were sacrificed and tumors processed prior to injection (untreated) and after injection on days 1, 4, or 7. Representative flow cytometry for N=3 is shown.

Temozolomide enhances dinutuximab and γδ T cell in vitro and in vivo killing of NB cells

Temozolomide (TMZ) has been reported to induce stress ligands in models of glioblastoma, leading to increased γδ T cell-based killing of glioblastoma cells [70]. When incubating IMR5 NB cells with TMZ, there was no detected inducible increase in NKG2DL (MICA/B, ULBP1, ULBP2/5/6) with doses ranging from 100 μM to 2 mM (**Supplemental Figure 2.4**). However, when IMR5 cells were incubated at a dose of 400 μM TMZ for 1 hr prior to performing a cytotoxicity assay with γδ T cells in combination with dinutuximab, there was a 10% increase in cell death compared to IMR5 treated with only γδ T cells and dinutuximab (non-paired t test $p<0.05$) (**Fig 2.6A**). To analyze the secretion of cytokines that could potentially be influencing cell death, the media from a cytotoxicity assay was collected and probed for cytokine expression (**Fig 2.6B**). An increase in the secretion of IFNγ and TNFα was observed when γδ T cells/dinutuximab were cultured with IMR5 cells, but there was no significant difference when TMZ was added to this combination. When IMR5 cells were incubated with γδ T cells, dinutuximab, and TMZ there was an increase in MIF expression. In addition to measuring cytokine expression, the γδ T cells from these assays were examined for cytotoxicity markers, including FASL and CD107a, to determine if TMZ pretreatment of NB cells induces γδ T cells FAS-mediated killing. FASL and CD107a were indeed increased on γδ T cells when cultured with NB cells pretreated with dinutuximab and TMZ (**Fig 2.6C**). CD112, CD15, TRAIL-R1, TRAIL-R2, and FAS were all expressed in IMR5 cells, and 400 μM TMZ did not alter the expression of these ligands on IMR5 (**Supplemental Figure 2.5**).

To determine if the enhanced killing observed with TMZ *in vitro* occurs *in vivo*, TMZ sensitivity was tested on established IMR5 subcutaneous xenografts growing in NSG mice. Once tumors were 125mm^3, TMZ was administered intraperitoneally at 125 mg/kg, 85 mg/kg, 40 mg/kg, or 20 mg/kg once a day every 3 days (for a total of 6 doses) (**Fig 2.7A, 2.7C**). The 125 mg/kg dose was lethal and the 85 mg/kg dose resulted in nearly complete tumor eradication. Doses below 85 mg/kg resulted in tumor growth, but only after completion of the 6 doses. Mice that received 20 mg/kg had diminished tumor growth, however tumors progressed in every mouse in this cohort. A clear dose response was achieved between 0 and 85 mg/kg

Figure 2.6: Increasing γδ T-cell function when combined with dinutuximab and TMZ

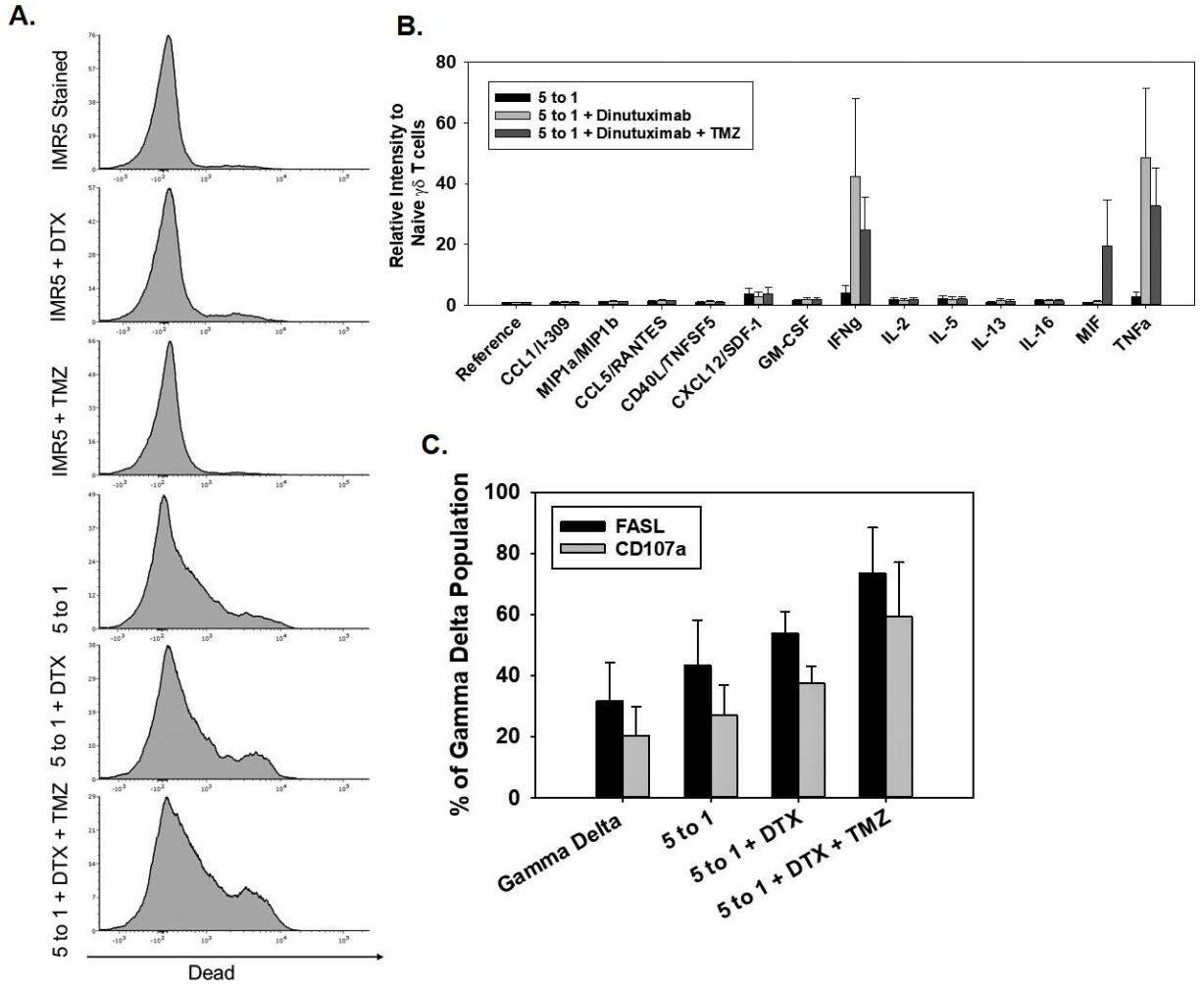


Figure Legend 2.6: A. Representative flow cytometry of IMR5 cell killing with combinations of dinutuximab (DTX), TMZ, and γδ T cells. From top to bottom , panel A shows killing for non-treated cells or cells treated with DTX, TMZ, γδ T cells (5:1 effector:target), γδ T cells (5:1 effector:target) with DTX, or γδ T cells (5:1 effector:target) with DTX and TMZ. IMR5 background dead cell staining was 6%, which was similar to treatments with DTX (5 μg/mL; 11% dead cells) and TMZ (400 μM; 11% dead cells). Average cell death increased to approximately 30% with γδ T cells, 39% with γδ T cells and DTX, and 46% with γδ T cells, DTX, and TMZ (N=3, $p<0.05$). B. Average and SD (N=3) of human cytokine array quantified using ImageJ analysis. Consistant with previous findings, γδ T cells secrete proinflammatory cytokines IFNγ and TNFα mixed with NB cells. There is an increase in cytokine production when DTX

and TMZ is included. All data is quantified as relative intensity to naïve γδ T cells. There is no significant difference between effector to target ratios 5 to 1 + DTX and 5 to 1 + DTX + TMZ. Data in these groups is significantly different compared to 5 to 1 effector to target ratio alone $p<0.05$. C. Flow cytometry was performed to measure FASL and CD107a on γδ T cells 4 hrs after intiation in cytotoxicity assays. Black and grey bars represent FASL and CD107a, respectively. Expression of these proteins is elevated when γδ T cells are incubated with IMR5 cells and further elevated when combined with DTX and TMZ.

Figure 2.7: Enhancing NB patient-derived γδ T-cell effectiveness *in vivo* by combination therapy

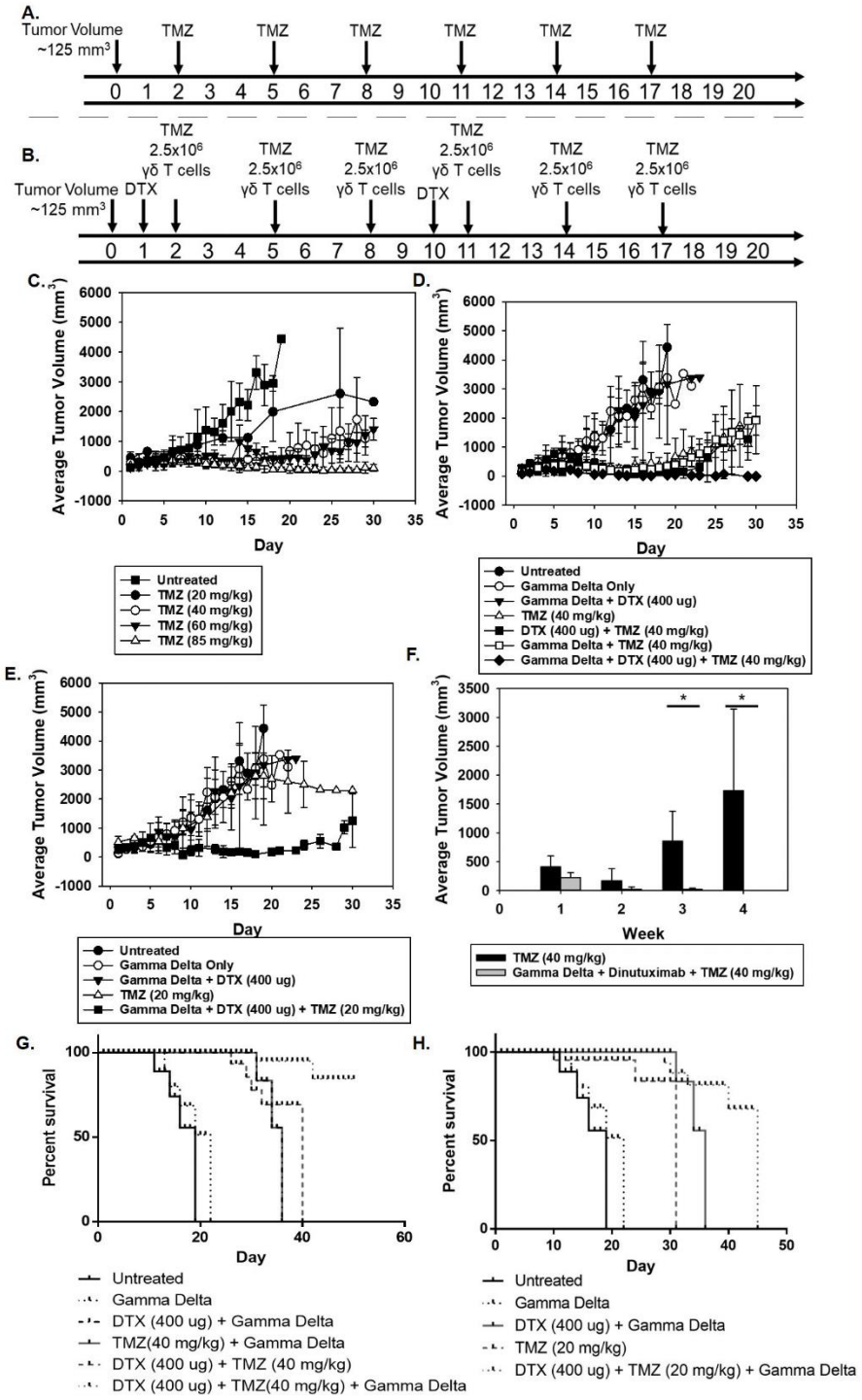


Figure Legend 2.7: A-B. Schematic representation of the treatment plan for NSG mice injected subcutaneously with IMR5 cells. Time T=0 refers to when the tumor reaches a minimum of 125 mm^3 and the start of treatment. NSG mice with established IMR5 subcutaneous tumors were treated over a 17 day

period at varied doses of TMZ, DTX, and γδ T cells . C. Tumor volume was measured and average tumor volume over time and standard deviation was calculated for TMZ doses of 20 mg/kg (N=4), 40 mg/kg (N=5), 60 mg/kg (N=2), 85 mg/kg (N=5), and compared to untreated controls. D. Untreated (N=8), $2.5x10^6$ γδ Only (N=8), γδ + dinutuximab (DTX) (400 μg) [N=4], TMZ (40 mg/kg) [N=5], DTX (400 μg) + TMZ (40 mg/kg) [N=4], γδ + TMZ (40 mg/kg) [N=5], and γδ + DTX (400 μg) + TMZ (40 mg/kg) [N=6] were evaluated through day 30. E. A lower dose of TMZ (20 mg/kg) [N=4] was used alone or with various combinations of DTX and γδ T cells [minimum of N=4 per cohort]. F. TMZ (40 mg/kg) [N=5] and γδ + DTX (400 μg) + TMZ (40 mg/kg) [N=6] are compared by paired t-test over 4 weeks (*p=0.029 at week 4). G. Survival curves to day 50 from the start of treatment, shows a significant survival advantage among animals that received 40 mg/kg TMZ with 400 μg DTX and $2.5x10^6$ γδ T cells compared to γδ T cells alone, γδ T cells + DTX, γδ T cells + TMZ, and TMZ + DTX (log-rank $p<0.001$). H. Survival curves to day 50 from the start of treatment, demonstrates significance in survival when using lower doses of TMZ (20 mg/kg) with 400 μg DTX and $2.5x10^6$ γδ T cells compared to untreated animals, γδ T cells only, γδ T cells + DTX, and TMZ (20 mg/kg) only (log-rank $p<0.001$).

TMZ. Combining 40 mg/kg TMZ with dinutuximab did not affect tumor growth, nor did the combination of γδ T cells and TMZ (**Fig 2.7B, 2.7D**). In all combination treatments using TMZ and γδ T cells, TMZ was administered 8 hrs prior to the γδ T cells. In contrast to TMZ plus γδ T cells, mice treated with the combination of patient-derived γδ T cells, dinutuximab, and 20 or 40 mg/kg TMZ dose showed a significant reduction in tumor growth compared to untreated or mice treated with any single therapy alone ($p=0.01$) (**Fig 2.7D, 2.7E, 2.7F**). In addition to tumor reduction, the combination of immunotherapies (γδ T cells and dinutuximab) with TMZ resulted in significant survival benefits for mice treated with 40 mg/kg TMZ (log-rank Mantel-Cox, $p=0.0059$) (**Fig 2.7G**). Mice treated with a combination of γδ T cells, dinutuximab and TMZ at 20 mg/kg also showed significant survival advantage (log-rank Mantel-Cox, $p<0.05$) compared to TMZ treatment alone (**Fig 2.7H**). Additionally, there is a statistically significant survival advantage of γδ T cells + dinutuximab + 40 mg/kg TMZ compared to γδ T cells + dinutuximab + 20 mg/kg TMZ (log-rank Mantel-Cox, $p=0.04$).

2.4 Discussion

The goal of these studies was to determine if a readily available cellular source material could be expanded into a cytotoxic γδ T-cell product, and if the expanded cells could be used to treat NB in a preclinical model. NB is currently treated with chemotherapy, radiation therapy, surgery, autologous stem cell transplantation, and maintenance immunotherapy containing dinutuximab [316]. For high-risk patients, survival outcomes remain poor [318, 328]. Since most children undergoing stem cell transplantation have additional unused apheresis products, there is potential to expand γδ T cells from these banked cells to be used as a therapeutic. Although substantial progress is being made in the field of cellular immunotherapy, NB specific advances have been limited. For example, the development and standardization of autologous chimeric antigen receptor (CAR) T-cell protocols allowed for the development of anti-GD2-based CARs [164, 361, 362]. However, the GD2 CAR has had some setbacks, such as severe off-tumor toxicities including fatal encephalitis in preclinical models [363]. In the current study, γδ T cells are presented as an alternative to αβ-based CAR T

cells as γδ T cells should have specific advantages since multiple killing mechanisms are inherent to these cells, including ADCC-based mechanisms, FasL expression, and targeting of stress antigens.

Recently, we developed protocols for a serum-free expansion method for γδ T cells from normal donors and showed that cells expanded using this GMP-compliant process provided a sufficient cell source to support clinical testing [352]. In this current study, the protocol was expanded to include cell products from children who underwent standard treatments for NB. This is the first study to utilize NB patient stem cell collection products for generating γδ T cells, which supports the expansion manufacturing process. The composition of the expanded product on day 14 is not significantly different compared to expansions using healthy donor PBMCs. In addition, the data shows that patient-derived cells retain their cytotoxic activity after storage in liquid nitrogen, which is important as it is predicted that multiple doses of the cellular product would be needed for each subject. The ability of patient-derived γδ T cells to recognize and kill tumor cells through mechanisms of ADCC and stress antigen recognition is important because it is anticipated the cells will be combined with standard of care therapy for relapsed and ultimately newly diagnosed NB, that includes both antibodies and chemotherapy that can enhance cytotoxicity by γδ T cells [83, 307]. Importantly, CD16 is upregulated during γδ T cells expansion and supports ADCC-based killing. Because the use of dinutuximab has demonstrated improved clinical outcomes in newly diagnosed and relapsed patients, the expansion and infusion of $CD16^+$ γδ T cells in combination with dinutuximab is predicted to be beneficial, and our *in vitro* data shows that this combination does provide some benefit when targeting highly expressing GD2 NB cell lines. However, the tumor killing by the combination of γδ T cells and dinutuximab using an immunocompromised NSG mouse, lacking in NK, B, and T cells, is insufficient for NB tumor eradication. Furthermore, the in situ microenvironment may also affect γδ T cells/dinutuximab efficacy possibly via immune cell exhaustion of the cellular product, which may benefit from cytokine supplementation which is done clinically with anti-GD2 antibody therapy. Our data strongly supports that the lack of *in vivo* efficacy of the dinutuximab/ γδ T cell combination alone is most likely due

to insufficient trafficking of γδ T cells to the tumor, which is a well-defined issue for cellular products [360, 364, 365].

To enhance the effectiveness of patient-derived γδ T cells, we tested the combination of immunotherapy and chemotherapy. Combining chemotherapy and cell-based therapeutics is typically complicated by chemotherapy-induced lymphopenia. Because TMZ is rapidly metabolized to inactive products, it provides a unique opportunity for combining treatment modalities. We hypothesized that TMZ could upregulate stress antigens and would subsequently be systemically inactivated/eliminated prior to administration of the γδ T cells, thereby providing a rational means of timing of the combination treatment. However, the *in vitro* data contradicts the use of TMZ as an inducer of stress-ligands in NB cells. Instead, the combination of TMZ and dinutuximab induced an increase in cytokine secretion, increased FasL expression on γδ T cells, and enhanced degranulation when γδ T cells were co-cultured with target cells. Therefore, based on these mechanisms, and apart from stress antigen expression, it was predicted the combination would be more effective *in vivo* than TMZ alone.

In an IMR5 *in vivo* murine model of NB, TMZ effectively reduced tumor growth in a dose-dependent manner. When using doses of TMZ that do not eradicate tumor growth alone, a significant benefit was observed when combining TMZ with γδ T cells and dinutuximab. This response was not achieved with either of the single or double treatment regimens. Interestingly, the benefit of γδ T cells was achieved without co-administration of IL-2 or zoledronic acid, which have been previously used by others to support γδ T-cell survival [358, 366]. The mechanism by which TMZ enhances the effectiveness of γδ T cells and dinutuximab is not yet fully understood. One hypothesis is that TMZ does not act directly on the tumor cells. Tumor cell growth may be prevented by affecting cells within the tumor microenvironment, such as those involved in angiogenesis [367]. It has been shown that low dose metronomic TMZ indeed inhibits tumor angiogenesis[367]. Additionally, anti-angiogenic therapies show potential benefit when combined with immunotherapies for solid tumors through normalization of abnormal tumor vasculature to allow for

increased infiltration of innate or adoptive immune effector cells[368]. Thus TMZ may directly control tumor growth and vascular architecture to allow for the immunotherapeutic component of treatment to be established in the tumor microenvironment [369], supporting this mechanism deserves further investigation in NB. Importantly, not only can the combination reduce tumor growth, but the ability to capitalize on the non-cytotoxic anti-tumor properties of chemotherapy agents allows for lower chemotherapy dosing and can benefit high-risk NB patients by reducing short- and long-term toxicities associated with chemotherapy. For example, a strategy incorporating expanded γδ T cells from a NB patients' apheresed-frozen PBMC product, collected during standard of care upfront therapy, potentially allows for a decrease in subsequent chemotherapeutic dosing without forgoing effectiveness to prolong survival. Additionally, there are alternative strategies to combine TMZ and cellular therapy including genetic engineering of immunocompetent cells using vectors encoding methylguanine methyltransferase [70], producing drug resistance. This modification is required for protecting expanded cell therapy products after administration and during the chemotherapy challenge. However, it appears this engineering may not be necessary if the administration of the cellular therapy and chemotherapy are properly timed.

Overall, the data supports that NB patient-derived γδ T cells can be efficiently expanded, and that the expanded cells enhance the effectiveness of chemoimmunotherapy *in vivo*. Although effective, it remains necessary to develop methods to increase the trafficking of γδ T cells to the tumor and increase their persistence *in vivo*. The data shows patient-derived cells can provide benefit to standard of care chemotherapy and dinutuximab-based immunotherapy treatments. While the majority of cellular based immunotherapies primarily rely on αβ T cells, there are limitations to the use of these cell products in solid tumor treatments. As such, alternative approaches such as the use of γδ T cells are necessary. These results support the potential for clinical use of *ex vivo* expanded γδ T-cell products to treat patients with highly aggressive pediatric cancers, like neuroblastoma.

2.5 Materials and Methods:

Expansion of γδ T cells in serum free media

Mobilized apheresed PBMCs were obtained from consented, deceased, neuroblastoma patients at Children's Healthcare of Atlanta (Atlanta, GA). Commercially available healthy donor frozen PBMCs were obtained from AllCells (Alameda, CA). At the time of stem cell collection, each patient had undergone two cycles of induction chemotherapy. Cells were cultured with OpTmizer (Life Technologies, Carlsbad, CA) serum free media and supplemented with 2 mM L-glutamine and 1% penicillin/streptomycin. All cultures were stimulated with 500-1000 IU/ml of IL-2 (Peprotech, Rocky Hill, NJ) and 5 μM zoledronic acid (Sigma-Aldrich). Media changes were performed every 3 days. On days 0 and 3, cells were provided with 500 IU/mL of IL-2, whereas on day 6 and 9, 1000 IU/mL is given. Zoledronic acid was used at the start of culture and added again on day 3. Total cell numbers were monitored periodically over a 2 week period via Cellometer (Nexcelom, Lawrence, MA). Dead cells were identified by trypan blue exclusion. γδ T-cell percentage and PBMC cellular composition were monitored via flow cytometry on days 0, 7, 12, and 14.

Cell lines and cell culture

Neuroblastoma cell lines (courtesy of Children's Oncology Group (COG) Cell Line Repository) and K562 cells were cultured in RPMI 1640 with L-glutamine (Corning cellgro, Manassass, VA) and 10% FBS and 1% Penicillin/Strep added.

Flow cytometry

Cells were washed with phosphate buffered saline (PBS) and centrifuged at 100x*g*. The cells were decanted and incubated with Invitrogen (San Diego, CA) eBioscience Fixable Viability Dye eFluor 780 for 30 minutes with shaking at room temperature. The cells were washed in 10 volumes of PBS. Supernatant was decanted and replaced with the appropriate antibody cocktail in PBS. The antibodies used from BD Biosciences (Franklin Lakes, NJ), include: BV421 Mouse Anti-Human CD3 (Clone UCHT1), PE Mouse Anti-Human TCR-1 (Clone 11F2), BUV395 Mouse Anti-Human CD56 (Clone NCAM16.2), BV711 Mouse Anti-Human CD178 (Clone NOK-1), APC Mouse Anti-Human CD107a (Clone H4A3), PE Mouse

Anti-Human CD95 (Clone DX2), BV480 Mouse Anti-Human CD3 (Clone UCHT1), APC-R700 Mouse Anti-Human CD56 (Clone NCAM16.2), BV711 Mouse Anti-Human CD27 (Clone M-T271), BUV496 Mouse Anti-Human CD16 (Clone 3G8), BUV661 Mouse Anti-Human CD4 (Clone SK3), PerCP-Cy5.5 Mouse Anti-Human CD8 (Clone RPA-T8), BB515 Mouse Anti-Human CD45RA (Clone HI100), BV650 Mouse Anti-Human CD45RO (Clone UCHL1), BV421 Mouse Anti-Human CD57 (Clone NK-1), BUV563 Mouse Anti-Human CD62L (Clone DREG-56), BV786 Mouse Anti-Human PD1 (Clone EH12.1), PE-CF594 Mouse Anti-Human PDL1 (Clone MIH1), BUV737 Mouse Anti-Human FAS (Clone DX2), PE Mouse Anti-Human FASL (Clone NOK-1), and BUV395 Mouse Anti-Human CD107a (Clone H4A3). Antibodies used from BioLegend (San Diego, CA) include: APC anti-human CD314 (NKG2D) (Clone 1D11), Brilliant Violet 711 Anti-Human CD16 (Clone 3G8), BV605 Mouse Anti-Human TCR Vδ2 (Clone B6), and PE-Cy5 Mouse Anti-Human CD28 (Clone CD28.2). Antibodies obtained from R&D Systems (Minneapolis, MN) include: PE Mouse Anti-Human TRAIL-R1 (Clone 69036), APC Mouse Anti-Human TRAIL-R2 (Clone 71908), and APC Mouse Anti-Human CD112 (Clone 610603), and PE Mouse Anti-Human CD155 (Clone 300907). PE-Cy7 Mouse Anti-Human TCR Vδ1 was purchased from ThermoFisher Scientific (Waltham, MA). Cells were analyzed by flow cytometry using an LSRII (BD Biosciences, Franklin Lakes, NJ) and a BD FACSymphony (BD Biosciences, Franklin Lakes, NJ).

Cytotoxicity Assays

The *in vitro* cytotoxic potential of naïve γδ T cells against multiple malignant cell lines was assessed in flow cytometry-based cytotoxicity assays. Target cell lines included the myeloid leukemia cell line, K562 (ATCC, Manassas, VA), and the neuroblastoma cell lines, IMR5, CHLA15, Kelly, CHLA20, and SMS-SAN. Target cells were labeled with the Violet Proliferation Dye 450 (BD Biosciences, Franklin Lakes, NJ) and incubated with γδ T cells at the varied effector to target (E:T) ratios: 0:1, 1:1, 5:1, 10:1 for 4 hours at 37C. Target cell death was analyzed via flow cytometry using dead cell stains (eBioscience Fixable Viability Dye eFluor 780) and incubating for 30 minutes with shaking at room temperature and/or 7-aminoactinomycin D (7AAD) was immediately added prior to data acquisition.

Freezing/Thawing γδ T cells

Cells were washed once with PBS and spun at 300x*g* for 5 minutes. Cells were resuspended in Albumin (Human) U.S.P. Albutein 5% (Grifols Therapeutics Inc.) with a 9% DMSO content at a concentration of $1x10^7$ γδ T cells per mL. All reagents were kept at 4ºC during the freezing process. Cells were then slowly frozen at a rate of -1ºC per minute until they reached -80ºC and promptly moved to liquid nitrogen storage. To thaw the cells, they were incubated in a 37ºC water bath until nearly thawed and subsequently diluted in 10 times the volume of complete OpTmizer media prior to centrifugation at 300x*g* for 5 minutes. Cells were resuspended in media containing IL-2 at 1,000 IU/mL concentration.

Biotinylation of dinutuximab

Clinical grade dinutuximab was biotinylated using EZ-Link™ Sulfo-NHS-Biotin (Thermo Fisher Scientific, Waltham, MA). Five hundred micrograms of antibody was added to an Amicon Ultra filter using DPBS/Modified (GE Life Sciences, Marlborough, MA) to adjust volume. The tube was centrifuged at 300 *xg* for 15 min. The EZ Link Sulfo NHS-LC Biotin was reconstituted in water (10 mg/ml) and added to the concentrated antibody in the Amicon Ultra filter at 1 mg of biotin reagent per mg of protein. The reaction proceeded for 30 min on ice. Hepes Buffered Saline (HBS)/0.05% azide was used to concentrate biotinylated-dinutuximab.

Cytokine Release Studies

γδ T cells were incubated for 4 hrs in a cytotoxicity assay with IMR5 neuroblastoma cells in cultures of 1 mL of media per condition. Media was removed from the cells and any floating cells were centrifuged at 500x*g for* 5 min. Supernatant was removed and immediately utilized in the ProteomeProfiler kit (R&D Systems, Minneapolis, MN). A Chemidoc BioRad Imager was used to acquire images and ImageJ/Fiji (NIH, Bethesda, MD) image analysis software was used for densitometry analysis. Data were normalized to basal γδ T-cell cytokine secretion culture in media after 4 hours.

Live Cell Imaging

IMR5 cells were transduced with a lentiviral vector green fluorescent protein (GFP) construct under the EF1α promoter (Lentigen, Gaithersburg, MD) at a multiplicity of infection of 10. Cells were cultured and sorted using a Sony SH800 to collect the top 5% of GFP+ cells by MFI. γδ T cells were labelled using Violet Proliferation Dye 450. IMR5 cells were plated on Lab-Tek II Chamber Coverglass 8 well chambers in the center 4 wells 24 hours prior to imaging to allow the cells to adhere to the glass. Immediately prior to imaging, γδ T cells and 100 μM of propidium iodide (PI) (Invitrogen, Carlsbad, CA) were added to each well. Imaging was conducted over 6 hr and images were taken every 19 min. Cells were imaged using a Leica SP8 inverted confocal microscope at 10x using a 458, 488, and 514 nm argon laser. Images were analyzed using ImageJ/Fiji (NIH, Bethesda, MD) image analysis software.

In vivo mouse experiments

NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG) mice were purchased from Jackson Laboratory (Bar Harbor, ME) and were maintained in a pathogen-free environment. Mice were cared for according to the established principles of the Institutional Animal Care and Use Committee (IACUC), and all animal protocols were approved by the IACUC. Five-week-old mice were each inoculated subcutaneously via the right flank with IMR5 cells. Mice were visually monitored and tumor growth was measured with calipers, and treated when tumors reached approximately 125 mm^3 in volume typically 30 days after inoculation. Tumor volume was determined by the following equation.

$$V = \frac{4}{3}\pi \frac{r_1}{2}\left(\frac{r_2}{2}\right)^2$$

where r_1 is the length of the tumor measuring anterior to posterior and r_2 is the length of the tumor dorsal to ventral. When tumors were established mice were administered dinutuximab, TMZ, or γδ T cells. Mice were injected with 200-400 μg of dinutuximab, IV every 10 days. TMZ and γδ T cells (2.5x10^6 cells) were

injected via tail vein every three days. This treatment plan was 17 days long. Mice were weighed and measured every other day for 4 weeks.

γδ T-cell persistence in vivo

γδ T cells were expanded from healthy donor PBMCs. On day 12 of expansion, the population was determined to be 70% pan-γδ T cells, as measured by flow cytometry. NSG mice were randomized to treatment groups, with three mice per group. Mice were injected via tail-vein with either $5x10^6$, $10x10^6$ or $15x10^6$ cells. On days 1, 3, 6 and 8 following injection, blood samples from each mouse were analyzed by flow cytometry using BD LSRII Flow Cytometer (BD Biosciences, San Jose, CA). Antibodies used included FITC anti-human CD45 (BD Biosciences, San Jose, CA), APC anti-mouse CD45.1 (BD Biosciences, San Jose, CA), and PE anti-human TCR γ/δ-1 (BD Biosciences, San Jose, CA).

Stress antigen expression on NB cells and mechanism of cytotoxicity induced by TMZ

IMR5 cells were seeded at a concentration of 250,000 cells per mL in 1mL. TMZ was added at a concentration of 100 μM – 2 mM for varying lengths of from 0-24 hrs. Cells were collected from the plate using 1 mL Versene (Gibco). Cells were counted, washed, and incubated with APC human ULBP-2/5/6 (R&D systems), Alexa Fluor® 488 human ULBP-1 (R&D Systems), and PE human MICA/MICB (Biolegend) for 30 min prior to FACS analysis. A similar process was used to analyze receptor status on the surface of IMR5 cells. IMR5 cells were plated at a concentration of 500,000 cells per mL. TMZ was added at a concentration of 400 μM for 8 hr. Cells were removed using Versene, washed, counted, and stained for flow cytometry analysis.

Statistical Analysis

All statistical analysis and graphing was performed using Sigma Plot version 13 (Systat Software Inc,) and GraphPad Software Prism. The exact method, for example ANOVA, T test, or log ranked Mantel-Cox test, are described for each experiment where they are used

2.6 **Supplemental Figures, Tables, and Legends**

Supplemental Tables

Table 2.1: Lymphocyte populations in expanded NB patient PBMCs

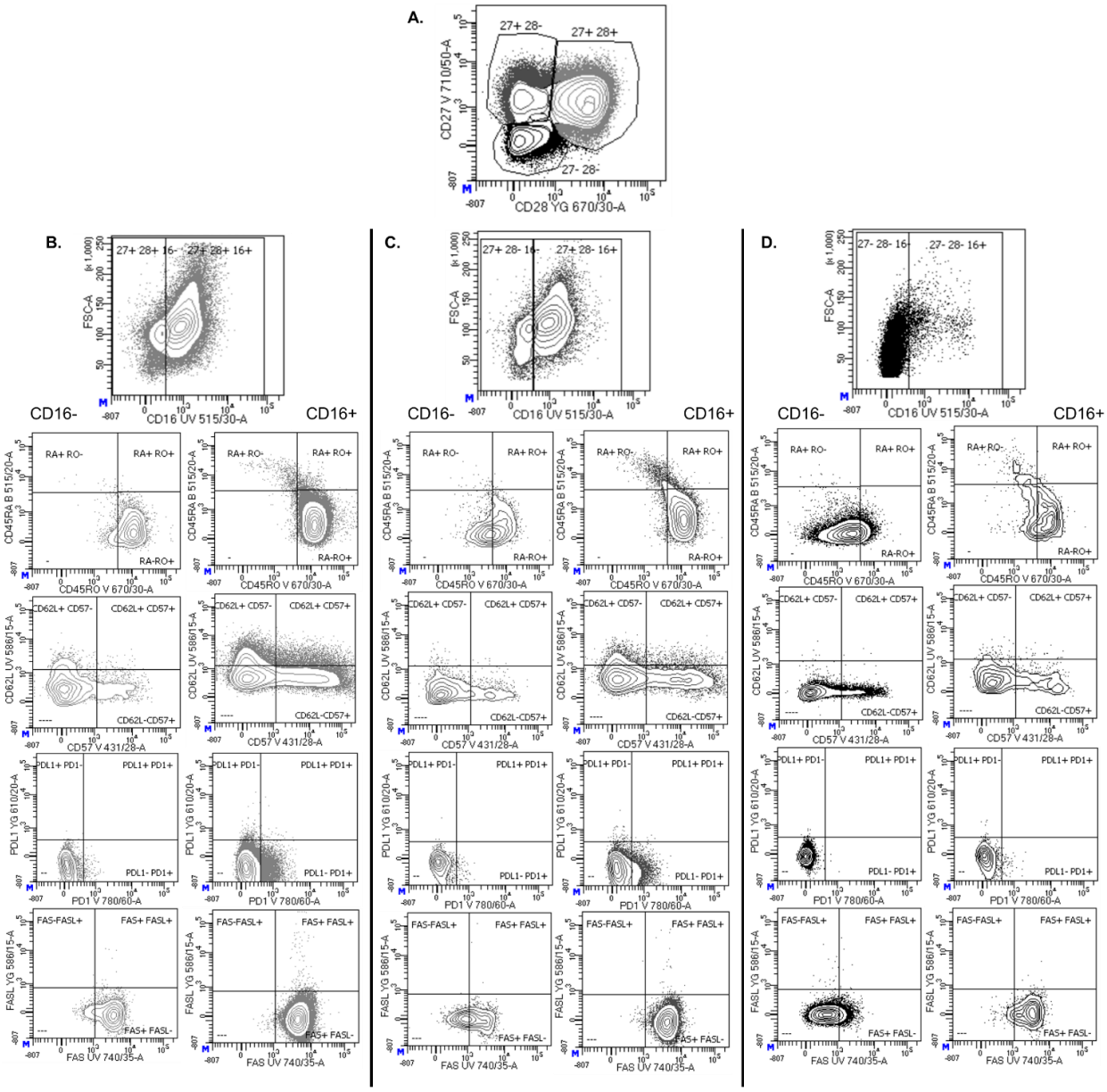
CD56				2.47±0.28
CD3				95.57±0.00
	delta -			7.23±0.85
		CD8+		12.60±1.41
			CD45RA	11.53±0.07
				CD62L+ 55.90±0.99
				CD62L- 44.10±0.99
			CD45RO	80.97±0.78
				CD62L+ 33.13±1.56
				CD62L- 66.87±1.56
		CD4		6.13±0.57
		Uncharacterized Lymphocytes		80.57±2.05
	Vδ1			7.00±0.07
	Vδ2			85.63±0.85
		CD27+, CD28+		65.90±0.71
			CD16+	90.10±5.44
			CD16-	9.90±5.44
		CD27+, CD28-		19.73±1.56

	CD16+	77.57±5.87
	CD16-	22.43±5.87
	CD27-, CD28-	12.37±0.99
	CD16+	7.33±4.10
	CD16-	93.60±3.75

Table 2.2: Characterization of Vδ2+ γδ T cell populations in expanded NB patient PBMCs

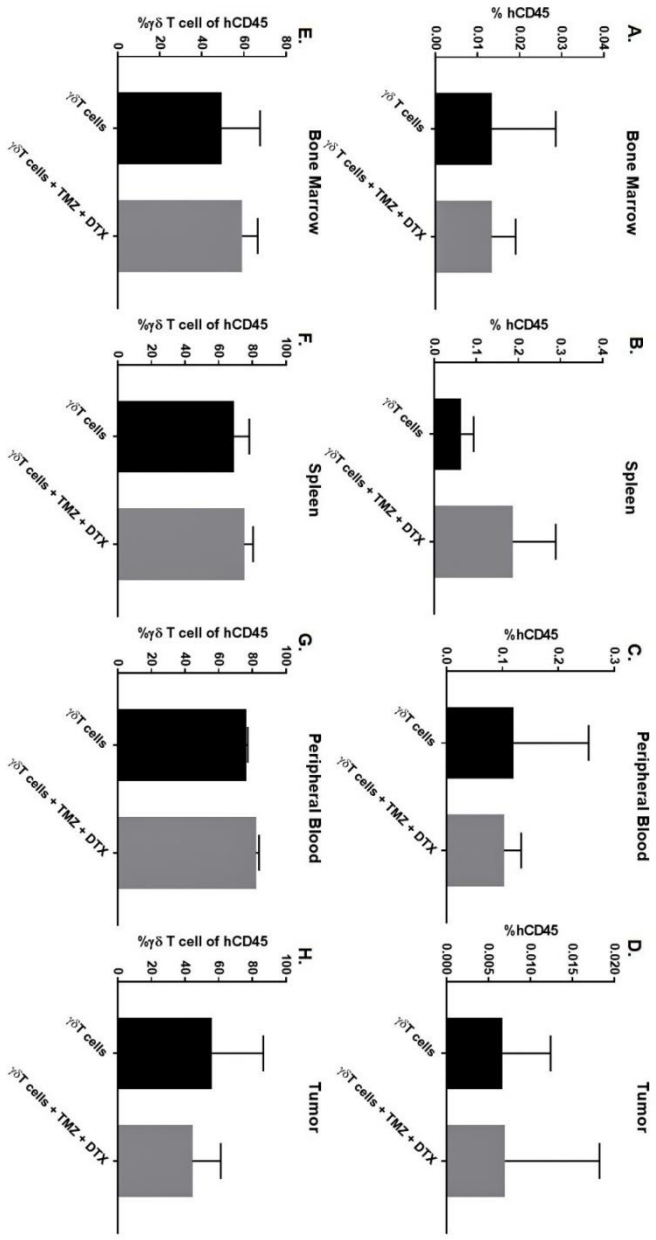
PD1	4.63±1.34	2.00±0.64	15.47±3.18	5.40±1.98	0.30±0.28	0.27±0.00
PDL1	0.13±0.14	0.10±0.14	0.20±0.21	0.07±0.07	0.10±0.21	0.97±0.07
FAS	99.37±0.42	91.73±3.25	98.57±0.85	66.60±6.86	95.95±0.07	10.23±1.70
FASL	0	1.63±3.25	0	0.83±0.21	0	0.90±0.14
CD45RO	95.70±0.35	97.37±0.57	93.70±0.35	94.30±1.06	94.57±1.34	16.20±2.62
CD45RA	1.93±0.07	0.47±0.57	3.53±0.21	0.77±0.99	1.83±1.34	0.17±0.00
CD57	25.37±2.33	16.87±0.42	30.37±3.82	24.07±0.57	23.80±3.25	20.17±0.07
CD62L	10.83±0.21	5.30±4.10	11.80±0.49	1.17±0.21	6.77±0.49	1.33±0.57
CD107a	2.97±0.92	4.73±1.70	3.77±1.27	9.13±3.68	6.67±1.98	18.17±2.05

Supplemental Figure 2.1: Characterization of NB Patient Derived γδ T cells



Supplemental Figure Legend 2.1: NB patient-derived frozen γδ T cells were thawed and flow cytometry was used to detect subpopulations of Vδ2 T cells. A. The Vδ2 T cells were first separated by CD27 and CD28. The subtypes included were CD27+ and CD28+ (B), CD27+ and CD28- (C), and CD27- and CD28- (D). Cells were then differentiated further by the absence (left) or absence (right) of CD16. Analysis for each of the 8 subtypes included surface staining for CD45RA, CD45RO, CD62L, CD57, PDL1, PD1, FAS, and FASL.

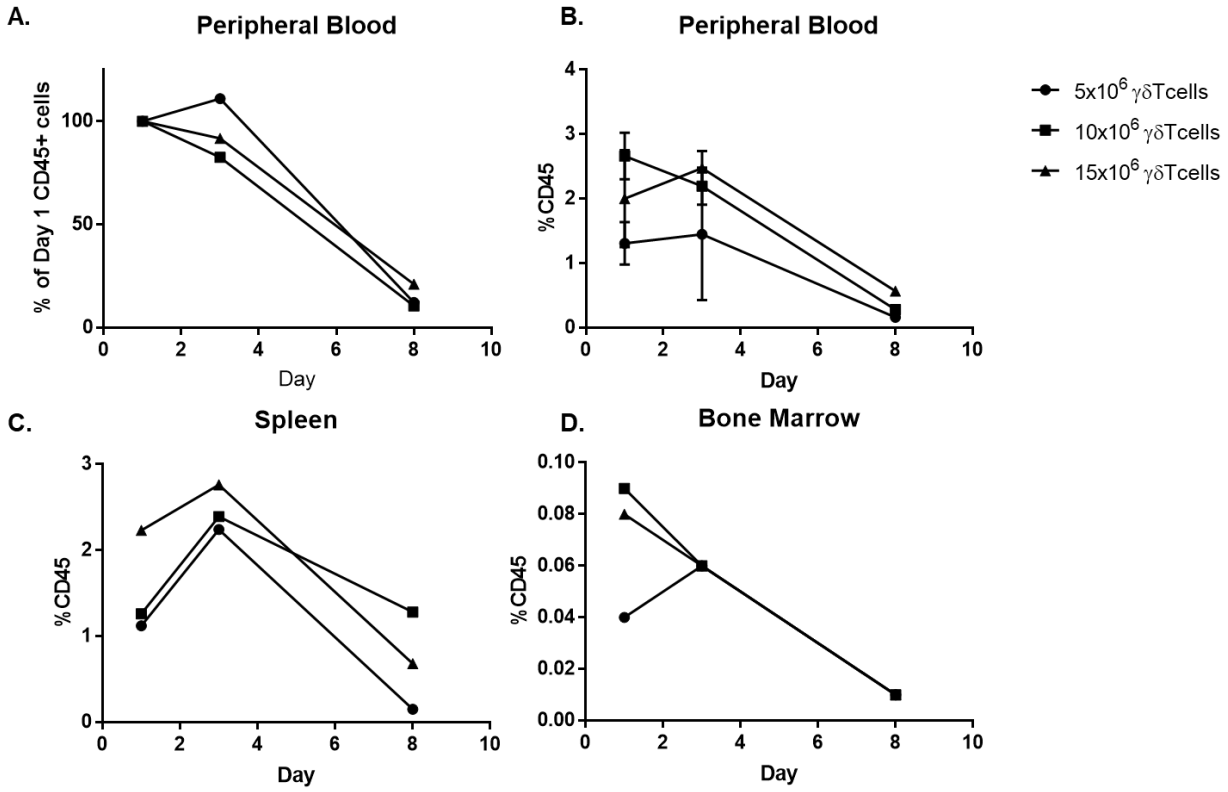
Supplemental Figure 2.2: **γδ T cells *in vivo* with combination therapy**



Supplemental Figure Legend 2.2: NSG mice with an approximate tumor volume of 500 mm^3 were left untreated or treated with $2.5x10^6$ γδ T cells ± 40 mg/kg TMZ and 400 μg of dinutuximab (DTX). Twenty four hours after treatment bone marrow, spleen, peripheral blood, and tumors were harvested. All biological samples were analyzed via flow cytometry for presence of live, human CD45 (hCD45) positive cells (A-D). The portion of hCD45 cells that were positive for the γδ TCR was also determined (E-H). There was

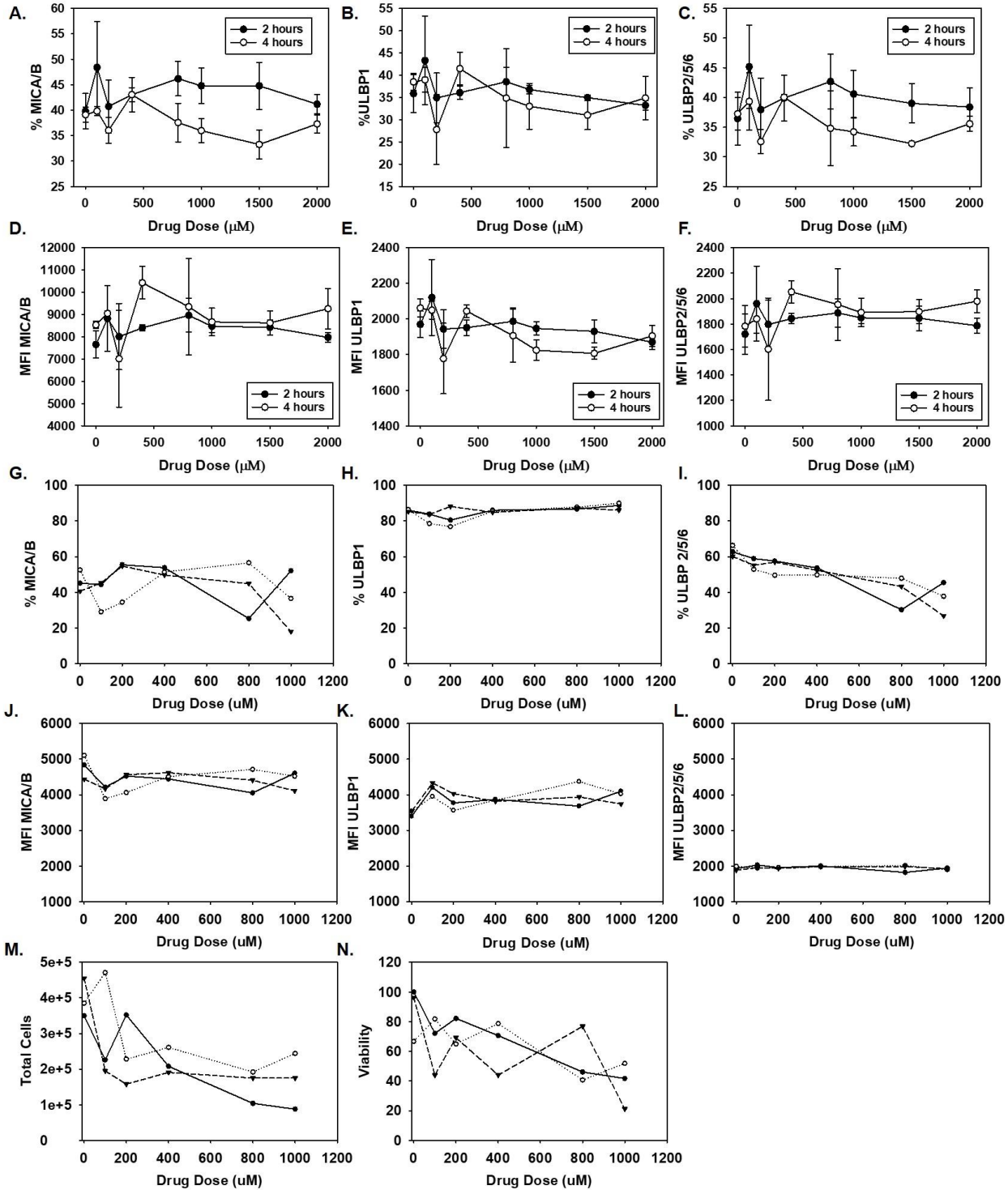
not a significant difference in the percentage of human cells within the tumor when comparing non-treated or TMZ+DTX treated mice, suggesting homing is not increased.

Supplemental Figure 2.3: Persistence of naïve γδ T cells



Supplemental Figure Legend 2.3: Naïve NSG mice were administered γδ T cells on day 0. Mice were bled on days 1, 3, and 8, and flow cytometry was used to detect human CD45 cells. Panel A shows the percentage of human cells in peripheral blood for 3 mice injected with 5-15 x 10^6 cells over 8 days as a function of the percentage measured on day 1. One mouse per group was sacrificed on day 1, 3, and 8 and spleen (C) and bone marrow (D) were harvested. Flow cytometry results are pre-gated on live cells, and the percentage of human CD45 is shown for each individual mouse.

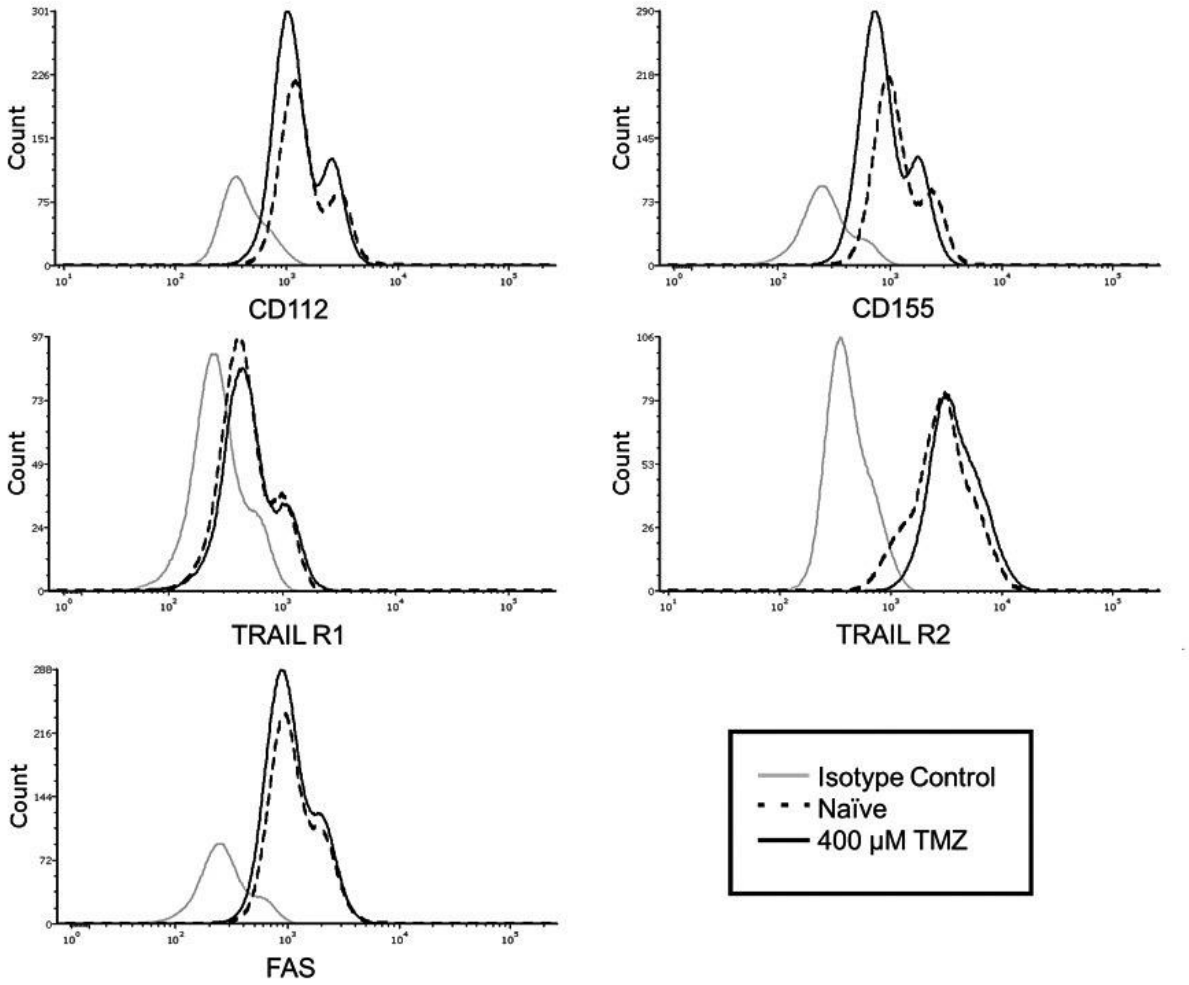
Supplemental Figure 2.4: Upregulation of stress antigens by TMZ *in vitro*



Supplemental Figure Legend 2.4: A-F IMR5 cells (250,000) were plated 24 hours prior to TMZ treatment. Cells were incubated with drug doses from 0-2000 μM. Cells in A-F were treated for 2 hours or 4 hours

prior to flow cytometry analysis. G-N are the average of biological triplicates with each symbol representing an experimental triplicate of cells incubated with varied doses of TMZ for 8 hours. A-C and G-I represent the percentage of positive cells for MICA/B, ULBP1, and ULBP2/5/6 and D-F and J-L show the mean fluorescence intensity (MFI). M and N are the total number of cells and viability by trypan blue exclusion after each drug treatment of 500,000 cells.

Supplement Figure 2.5: Innate cytotoxicity ligands on surface of IMR5



Supplemental Figure Legend 2.5: Key surface ligands for innate cell cytotoxicity were measured by flow cytometry on IMR5 cells after treatment with 400 μM TMZ. No inducible differences were noted in CD112, CD155, TRAIL-R1 and R2, or FAS expression with TMZ compared to naïve cells. Although there was not a measurable increase in these markers after the chemotherapy challenge, they all exhibited elevated expression compared to the isotype control.

Chapter 3

Novel ligand-based CAR targets MPL receptor in vitro and in murine xenograft acute myelogenous leukemia models

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Jaquelyn T. Zoine designed cloning constructs and experiments, conducted all experiments, and wrote the paper

Chengyu Prince assisted with preliminary experiments and human T cell isolation

Jamie Y. Story assisted with cell culture and mouse experiments

Allison M. Lytle designed cloning constructs

Andrew Fedanov produced all lentiviral vectors

Gianna M. Branella and Jordan E. Shields assisted with mouse experiments

Chris Porter provided feedback and insight to St. Jude PeCan and TCGA bioportal data sets

Christopher B. Doering, H. Trent Spencer, and Shanmuganathan Chandrakasan conceived the experiments and edited the paper

3.1 Abstract

The expansion of chimeric antigen receptor (CAR) T cells to diseases other than CD19+ malignancies has remained a challenge. While leukemias have seen the most success with CAR T cell therapy, the limited number of available single chain variable fragments (scFv), antigen loss and relapse remains a problem. Due to the limited number of antibodies against tumor associated antigens, strategies for diversifying CAR T cell engagement with tumor cells are necessary. To address the limited number of scFvs, CAR T cells were designed using a ligand binding domain instead of an scFv. We designed a ligand-based CAR to target stem-like leukemia cells, as well as normal stem cells. Thrombopoietin (TPO), the natural ligand to the myeloproliferative leukemia protein (MPL), was used as our antigen binding domain to engage the MPL receptor found on stem cells and erythropoietic and megakaryocytic acute myeloid leukemias (AML). We tested our CAR *in vitro* against AMLs with varied MPL expression to test the specificity. As expected, the TPO-CAR was specifically activated and cytotoxic against MPL+ leukemias. Additionally, we tested two versions of the TPO-CAR, a codon optimized (CO) and non-codon optimized (NCO). Though the CO TPO-CAR had greater expression by CD3ζ western blotting, a survival benefit was not measured. However the CO TPO-CAR depleted MPL+ leukemia cells in this model. Interestingly, the NCO TPO-CAR successfully extended survival *in vivo* in two xenograft models of MPL+ leukemias. Employing a ligand-based CAR on-target off-tumor side effects are better predicted because of better understanding a receptor ligand interaction. Therefore, we hypothesized the TPO-CAR would be toxic against long-term hematopoietic stem cells (LT-HSCs) because of the high MPL expression in the bone marrow. *In vivo*, the NCO TPO-CAR caused bone marrow suppression with mild conditioning. Additionally, with no prior conditioning the NCO TPO-CAR T cells induced minimal engraftment at 20-weeks post-transplant. The data collected demonstrate the preclinical potential of the TPO-CAR to be utilized in stem-like leukemia models for survival benefit and the continued testing and expansion of the CAR as a non-genotoxic conditioning regimen for bone marrow transplantation.

3.2 Introduction

The use of T-cell based therapies for the treatment of cancer has largely advanced to the engineering of T cells to generate profound tumor specific immune responses in patients. Chimeric antigen receptors (CARs) are recombinant receptors designed to bind tumor neo-antigens consequently activating the T cells while bypassing major histocompatibility complex (MHC) recognition and priming[370]. The majority of CARs are designed to recognize a specific antigen through the use of a single chain variable fragment (scFv), a variable heavy and light chain of a monoclonal antibody fragment joined by a linker sequence[371]. Three generations of CARs have been designed and tested, the second generation CARs—which contain one costimulatory domain and a CD3ζ TCR signaling domain—have proven particularly successful in eliminating CD19+ cancer malignancies[227-230, 232, 372-374].

The success of CARs have been restricted due to the limited number of tumor neo-antigens and available antibodies to them[375]. If there are antibodies to tumor specific antigens the ability to translate these into CARs is possible, though it does not necessarily translate into a successful and specific CAR. There are known obstacles in the CAR field related to CAR design which may cause scFv failure, including length of the hinge and linker region, aggregation, and immunogenicity against the scFv[56]. An alternative approach to engage a CAR is using a ligand based binding domain. Using a ligand binding domain to engage tumor cells by T cells can be advantageous because of its potential to expand the repertoire of binding domains and enhance CAR specificity. There are predictable toxicities when using a ligand-based CAR and the use of an endogenous human ligand should decrease the immune response associated with mouse chimera scFvs[309]. Furthermore, in cases where a tumor downregulates a receptor, a ligand binding domain is advantageous because ligands often bind multiple receptors[308, 376, 377]. While it is possible, these multiple interactions can result in on-target off-tumor side effects, these interactions will be better predicted and possibly preventable due to a better understanding of the binding between natural ligands and their receptors compared to an scFv.

Thrombopoietin (TPO) is a hematopoietic growth factor and natural ligand to c-MPL (myeloproliferative leukemia) receptor[378]. TPO is able to drive megakaryocytes and their progenitor's proliferation and differentiation as well as bind MPL to facilitate hematopoietic stem cell (HSC) self-renewal and maintenance[379-381]. The majority of TPO can be found in the liver, kidneys, and bone marrow niche. Activation of c-MPL results in downstream activation of pathways including JAK2/STAT, PI3K/Akt, and Raf1/MAP kinase, which are commonly implicated in hematological malignancies[381].

Acute myelogenous leukemia (AML) is a cancer is composed of myeloblasts. AML accounts for 20% of pediatric leukemias and is the most common leukemia in adults[382]. Additionally, similar to hematopoiesis, for AML there exists leukemia stem cells (LSC), capable of self-renewal and propagation of the leukemia[383]. LSCs are characterized by CD34+ CD38- cell populations and have been reported to be difficult to target because they are resistant to chemotherapy[384-386]. Also similar to HSCs, which depend on the c-MPL/TPO pathway for survival[387], leukemias such as megakaryocytic and erythropoietic leukemias have been reported to have high MPL expression[388]. For these reasons, we designed a CAR targeting the MPL receptor.

Here we show successful transduction of naïve T cells with a lentiviral TPO-CAR construct. We demonstrate TPO-CAR is capable of specific activation by MPL positive cell lines, is responsive to long term-HSCs, effectively kills MPL+ cancer cells in mixed populations, and extends survival compared to non-transduced T cells in multiple xenograft models. As predicted, hematopoietic suppression due to targeting MPL positive HSCs was observed.

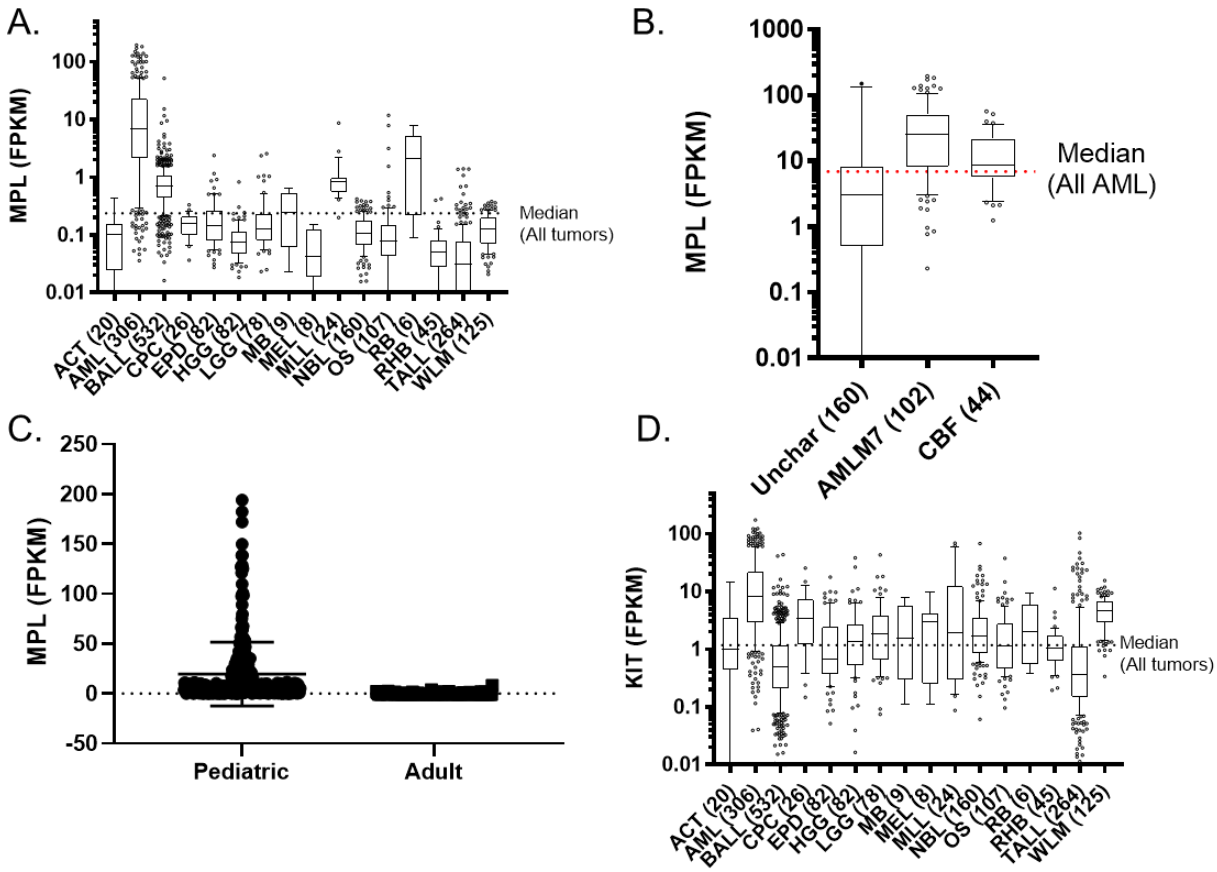
3.3 Results

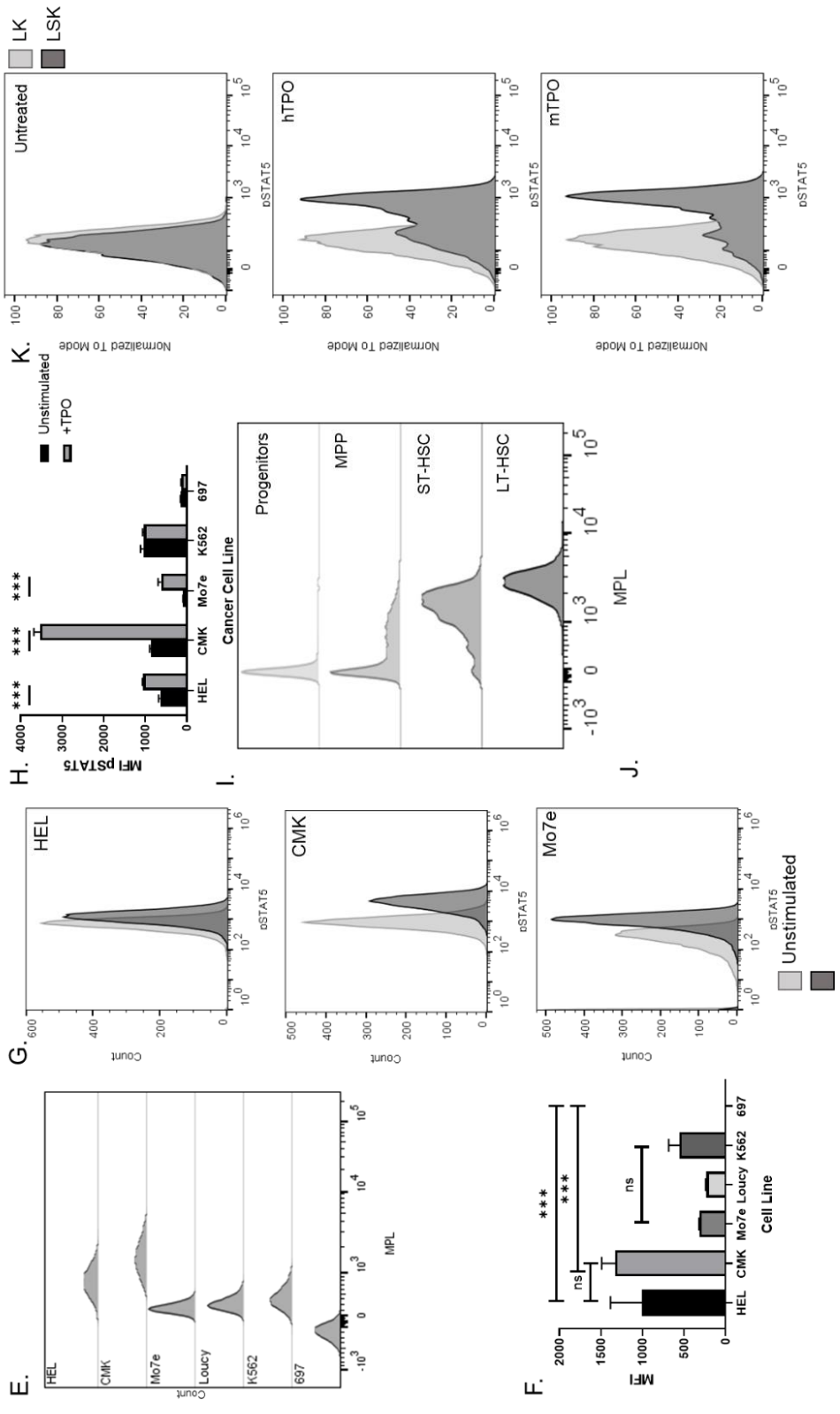
Detection of MPL on LT-HSC and leukemia cells

The function of MPL in stem cell populations is becoming increasingly better understood. Data from the St. Jude PeCan Data Portal[389-395] database suggests many pediatric malignancies have MPL expression;

however, acute myeloid leukemias (AML) stand out as highly expressive (**FIG 3.1A**). Specifically, pediatric AMLs are characterized as megakaryoblastic (AMLM7) and core binding factor (CBF) have higher than average expression (**FIG 3.1B**). Interestingly, adult AMLs do not express the same level of MPL according to available TCGA data (**FIG 3.1C**). Furthermore, we analyzed the St. Jude PeCan data portal for data on the c-kit receptor (**Fig 3.1D**), which is expressed on HSCs and targeted as a mechanism of bone marrow/stem cell depletion[396] and, as expected, RNA expression was high in multiple pediatric cancers as well as healthy tissue (**Table 3.1**). To establish the presence of MPL on various AML cell lines we measured surface expression of MPL using flow cytometry (**FIG 3.1E-F**). This data verified RNA expression levels available from the Cancer Cell Line Encyclopedia (CCLE) (**Table 3.2**) demonstrating high MPL surface expression on human erythropoietic leukemia (HEL) cells and acute megakaryocytic leukemia, CMK cells, and low/undetectable expression on Mo7e, an acute megakaryoblastic leukemia line. To verify these cell lines were responsive to TPO we stimulated HEL, CMK, Mo7e cells as well as control cell lines K562 and 697 for 45 minutes with mouse or human TPO, then fixed, permeabilized, and stained internally for pSTAT5 (**FIG 3.1G-H**) . Once stimulated with TPO, a significant increase in pSTAT5 compared to unstimulated was observed in HEL, CMK, and Mo7e cells and each cell line showed a significant increase in mean fluorescence intensity (MFI) (FIG 1H, $p<0.001$, 2-way ANOVA) compared to their unstimulated counterparts. Additionally, this shift was not observed in control cell lines K562 (chronic myelogenous leukemia) and 697 (B cell leukemia). We additionally tested the MPL and phosphorylated STAT5 (pSTAT5) expression in both mouse and human bone marrow samples. Our data show the more stem-like cells/ long-term HSC (LT-HSC: 2681.8 ± 253.2) have greater surface expression of MPL and higher MFIs compared to more differentiated short-term HSC (ST-HSC: 1373.0 ± 234.3, $p\geq0.0001$), multipotent progenitors (MPP: 570.6 ± 122.5, $p\geq0.0001$) and most differentiated progenitors (165.2 ± 26.1, $p\geq0.0001$) (**FIG 3.1I-J**). We experimentally determined both human and mouse recombinant TPO were able to induce pSTAT5 expression in mouse bone marrow specifically in the LSK (lineage negative, c-kit positive, Sca-1 positive) cells (**FIG 3.1K**).

Figure 3.1: Establishing MPL as a target in cancer and stem cell biology




E.
HEL
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Cell Line
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Cancer Cell Line
Unstimulated
+TPO
I.
Progenitors
MPP
ST-HSC
LT-HSC
J.
MPL
K.
LK
LSK
Untreated
hTPO
mTPO
Normalized To Mode
pSTAT5

Figure Legend: A. Data was acquired from the St. Jude PeCan database. MPL RNA sequencing data was taken and formatted to show the expression across multiple pediatric subpopulations including adenocortical carcinoma(ACT), acute myeloid leukemia (AML), B cell acute lymphoblastic leukemia (BALL), choroid plexus carcinoma (CPC), ependymoma (EPD), high grade glioma (HGG), low grade glioma (LGG), medulloblastoma (MB), melanoma (MEL), mixed lineage leukemia (MLL), neuroblastoma (NBL), osteosarcoma (OS), retinoblastoma (RB), rhabdosarcoma(RHB), T cell acute lymphocytic leukemia (TALL), and Wilm's tumor (WLM). Box and violin plots are shown with median expression demonstrated by the dotted line for all tumors. B. Data from pediatric AML patients demonstrating the acute megakaryoblastic leukemic (AMLM7, N=102) and core binding factor (CBF, N=44) leukemias have a higher gene expression for MPL compared to the uncharacterized AML population (N=160). C. St. Jude PeCan data portal expression and TCGA gene expression AML data sets for MPL expression. Pediatric N=306, Adult N=173 D. Gene expression analysis from the St. Jude PeCan data portal for c-kit, a commonly targeted protein in stem cell research but shows high expression across multiple tissues and cancer types making it a problematic target. E. Representative flow cytometry analysis of cancer cell lines HEL (N=3), CMK (N=3), Mo7e (N=3), and Loucy (N=3) surface MPL expression. F. Mean fluorescence intensity of MPL surface expression analysis showed significantly higher expression in the HEL (1008 ± 378.4) and CMK (1330 ± 160.5) cell line compared to the Mo7e (316.7 ± 6.66) and Loucy (233 ± 8.66) lines. G. Cells were stimulated for 45 minutes with recombinant human thrombopoietin, fixed, permbealized and evaluated for pSTAT5 expression. Representative flow cytometry of the HEL, CMK, and Mo7e cell lines showed increased pSTAT5 expression after stimulation compared to non-stimulated controls. H. Mean fluorescence intensity of accompanied pSTAT5 stimulation with thrombopoietin. All cell lines were reactive. HEL, CMK, and Mo7e cells showed a significant increase in pSTAT5 when stimulated by thrombopoietin by 2-way ANOVA ($p<0.0001$) compared to the control cell lines K562 and 697's which showed no difference with thrombopoietin. I. Flow cytometry analysis for MPL surface expression was completed on whole mouse bone marrow (N=13) and separated into progenitor and stem like compartments. Representative flow cytometry (y-axis: count, x-axis MPL) showed the long term hematopoietic stem cells

(LT-HSC) having the highest MPL surface expression compared to short term hematopoietic stem cells (ST-HSC), multipotent progenitor (MPP), and progenitors. J. Mean fluorescence intensity of the MPL expression in each bone marrow compartment was evaluated. There was a significant difference in MFI by one-way ANOVA in the progenitor (165.2±26.1), MPP (570.6±122.5), ST-HSC (1373±234.3), and LT-HSC (2682±253.2). K. To test the cross reactivity of mouse and human recombinant TPO, mouse bone marrow was stimulated with TPO for 45 minutes, fixed, permeabilized, and stained for pSTAT5. Bone marrow was further delineated by the lineage- c-kit+ (LK) and lineage- c-kit+ sca-1+ (LSK) when checking for pSTAT5 expression. Data suggests the less differentiate LSK compartment was reactive to both mouse and human TPO compared to more differentiated LK compartment.

Development of ligand based CAR targeting MPL

The TPO ligand was truncated at the 176 amino acid position and the cDNA encoding the protein was cloned into a second generation lenti-viral CAR construct (**FIG 3.2A**). Human T cells were isolated from PBMCs and activated for 24 hours. T cells were then transduced with vector and incubated for 18 hours. Five days post transduction cells demonstrated between 22-43% transduction efficiency by GFP (**FIG 3.2C**) with vector copy numbers ranging from 0.20- 0.96 (**FIG 3.2D**). T cells transduced with the TPO-CAR and coincubated with the Mo7e cell line showed significantly increased percentages of CD69 (early activation marker, 74.0% ± 6.2, $p<0.0001$), CD38 (long term activation marker, 76.3% ± 9.4, $p<0.0001$), and CD107a (degranulation, 21.3% ± 4.1, $p=0.027$) when co-cultured with Mo7e cells (**FIG 3.2E**). However, when TPO-CAR lysates were harvested and compared to lysates from CD19 CAR T cells and non-transduced (non-transduced) T cells, there was little protein expression compared to the CD19 CAR (**FIG 3.2F**). Further analysis demonstrated proper sequencing of the genomic DNA and no significant difference in mRNA by northern blot between the CD19 CAR and TPO-CAR (data not shown). To analyze whether there was an issue with the protein sequence and construct design we substituted our linker sequence with the CH3 domain of IgG1 and codon optimized our CAR construct using a custom codon usage bias table. The *in silico* optimization using a commercial algorithm was made from a custom table termed the "lovely codon optimization" (CO) instead of species-specific or genome-based tables. Optimization parameters included removing the cis-acting motifs, destabilizing RNA structures, and minimizing GC content (**FIG 3.2B**). To evaluate the new construct, T cells were isolated, activated, and transduced at a multiplicity of infection (MOI) of 50 with the lenti-viral non codon optimized (NCO) bicistronic vectors eGFP P2A TPO CAR (NCO TPO-CAR) or eGFP P2A CD19 CAR (CD19 CAR) or the codon optimized monocistronic vector CO TPO-CAR. Lysates were harvested between days 5-7 post-transdction and CD3z expression was detected via western blot (**FIG 3.2F**). In multiple donors, the CO TPO-CAR had greater protein expression compared to the NCO TPO-CAR and CD19 CAR.

Figure 3.2: Generating a thrombopoietin ligand-based CAR to target MPL

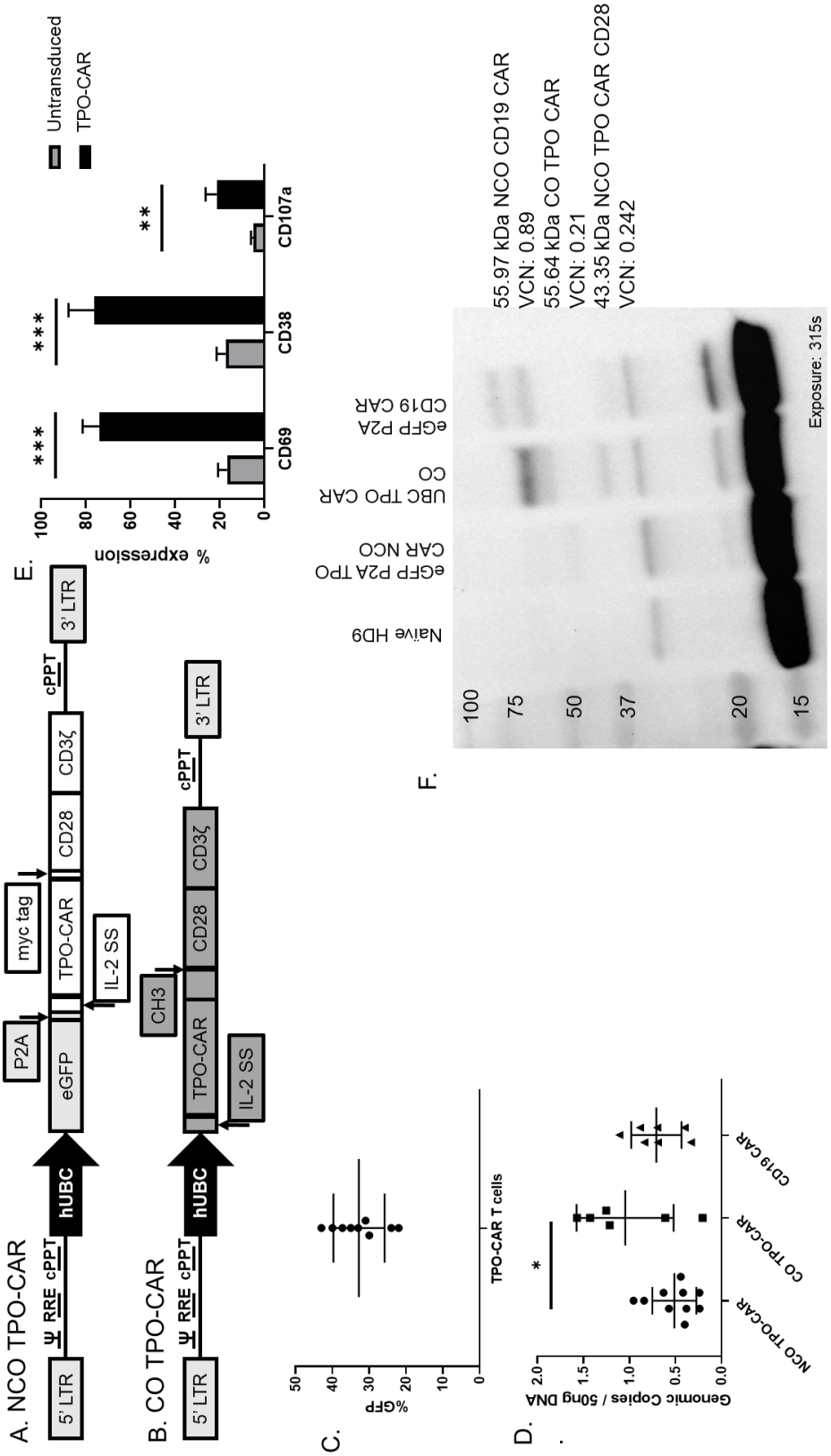
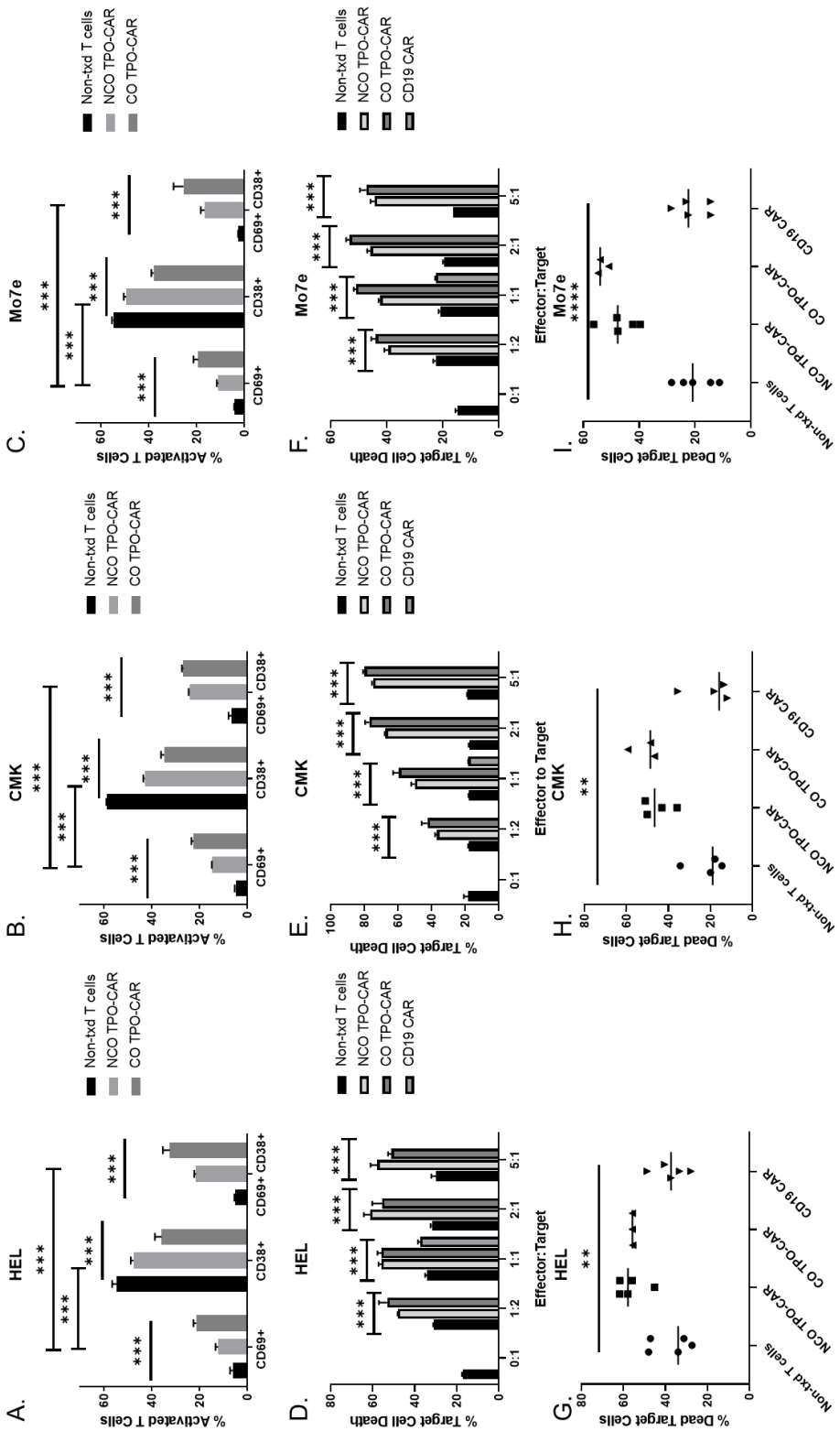


Figure Legend: A: Schematic of the NCO TPO-CAR bicistronic transgene sequences used for expressing enhanced green fluorescent protein (eGFP) and the TPO-CAR using a P2A sequence. It includes a 5′ long terminal repeat (LTR), human ubiquitin C promoter (hUBC), eGFP sequence, P2A sequence, an interleukin-2 signal sequence (IL-2 ss), the TPO-CAR, a myc epitope tag, the CD28 region, the CD3ζ intracellular domain and a 3′ LTR. B. Schematic of the CO TPO-CAR codon optimized transgene sequence. In contrast to the NCO-TPO-CAR this construct contains no myc tag epitope, is entirely codon optimized from the IL2 ss to the end of the CD3z sequence, and contains a CH3 hinge domain instead of the CD28 sequence. C: Primary T cells from 9 healthy donors were transduced 24 hours post isolation and activation at multiplicity of infection of 50. GFP percentage of the NCO TPO-CAR construct are displayed between 22-40%. D: Genomic DNA was isolated for RT-PCR and vector copy analysis demonstrated significantly higher VCN in the CO TPO-CAR construct compared to the NCO TPO-CAR construct but not significantly different to the CD19 CAR control. E. A 4 hour co culture experiment was set up with the NCO TPO-CAR and Mo7e target cell line to measure activation of the GFP+/- populations. After four hours cells were washed and stained for flow cytometry analysis. Significant increases by 2-way ANOVA ($p<0.001$) were measured in the transduced TPO CAR population in the percentages of CD69 (16.67±4.16 vs 74.0±7.55, early marker of activation), CD38 (17.33±4.16 vs 76.33±11.5, long term marker of activation), and CD107a (5±1 vs 21.3±5.03, degranulation). F. Protein cell lysates were collected and 40 ug were loaded into an SDS PAGE gel for western blot analysis using CD3z and HRP for detection. After 315s of exposure, CD3z bands were detected in Naïve T cells (HD9), the NCO TPO-CAR (CAR band: 43.35 kDa), CO TPO-CAR (CAR band: 55.64 kDa), and the control CD19 CAR (CAR band: 55.97 kDa). Vector copy number of the cells are reported to the right to demonstrate similar copy numbers. Data shows more CAR in the CO TPO-CAR compared to the CD19 CAR and undetectable NCO TPO-CAR.

Specific activation and cytotoxic targeting of MPL+ cells

CAR activation induced by HEL, Mo7e, and CMK cells was measured after 12 hours of co-incubation by flow cytometry for CD69 and CD38 surface expression. CO TPO-CAR is significantly activated compared to the NCO TPO-CAR and non-transduced T cells with all three cell lines ($p \leq 0.001$). Additionally, the NCO TPO-CAR is significantly activated compared to non-transduced T cells against all three cell lines ($p \leq 0.001$) (**FIG 3.3A-C**). *In vitro* cytotoxicity of the NCO and CO TPO CAR was evaluated against 3 MPL+ leukemia cell lines as well as a T cell acute lymphoblastic leukemia (ALL) cell line, Loucy, and B cell leukemia cell line, 697. Target cells were stained with VPD450 proliferation dye or CFSE dye. Co-cultures were established and incubated for 12 hours and subsequently stained for CD3, CD69, CD38, MPL, Annexin V, and PI for flow cytometry. Both the NCO and CO TPO-CAR significantly killed HEL, CMK, and Mo7e cells at effector to target ratios of 1:2, 1:1, 2:1, and 5:1 compared to non-transduced T cells and CD19 CAR T cells (**FIG 3.3D-F**). Comparison of the data from the 1:1 data from multiple donors showed consistent killing of the CAR-modified cells compared to controls (**FIG 3.3G-I**). As a test of the specificity, the remaining live HEL and CMK target cells were screened for cell surface MPL expression. We analyzed the HEL (**FIG 3.3J**) and CMK **(FIG 3.3K)** cells after the cytotoxicity assay for remaining target cells with MPL. Interestingly, there was a clear decrease in live MPL positive cells after co incubation with the NCO or CO TPO-CAR compared to targets incubated with non-transduced T cells. To further ensure the specificity of killing, TPO-CAR T cells were mixed with HEL target cells and the MPL- cells, K562 or 697 cells, at a 1:1:1 ratio. The NCO TPO-CAR demonstrated minimal toxicity against K562 (15.7 ± 1.6) and 697 (21.4 ± 0.5), while achieving cytotoxicity against the HEL cell line when coincubated at 57.8 ± 1.3, $p<0.0001$ and 41.9 ± 0.4, $p<0.0001$ (**FIG 3.3L**). This was repeated with the CMK target cell line and similar results were achieved: K562 11.7 ± 0.6 and 697 25.0 ± 5.74 versus CMK cell line 83.6 ± 8.6, $p<0.0001$ and 76.1 ± 1.0, $p<0.0001$ (Fig 3.3M). Again, our data demonstrated significant killing of MPL+ cells compared to either MPL- cell line. Finally, we tested a competitive cytotoxicity assay for 12 hours. Effector cells and target cell lines, HEL, CMK, and Mo7e were coincubated with or without recombinant human TPO. Our data shows that the TPO was able to significantly impact cytotoxicity with the NCO and CO TPO-CAR T

Figure 3.3: Activation, cytotoxicity, and specificity of the TPO CAR



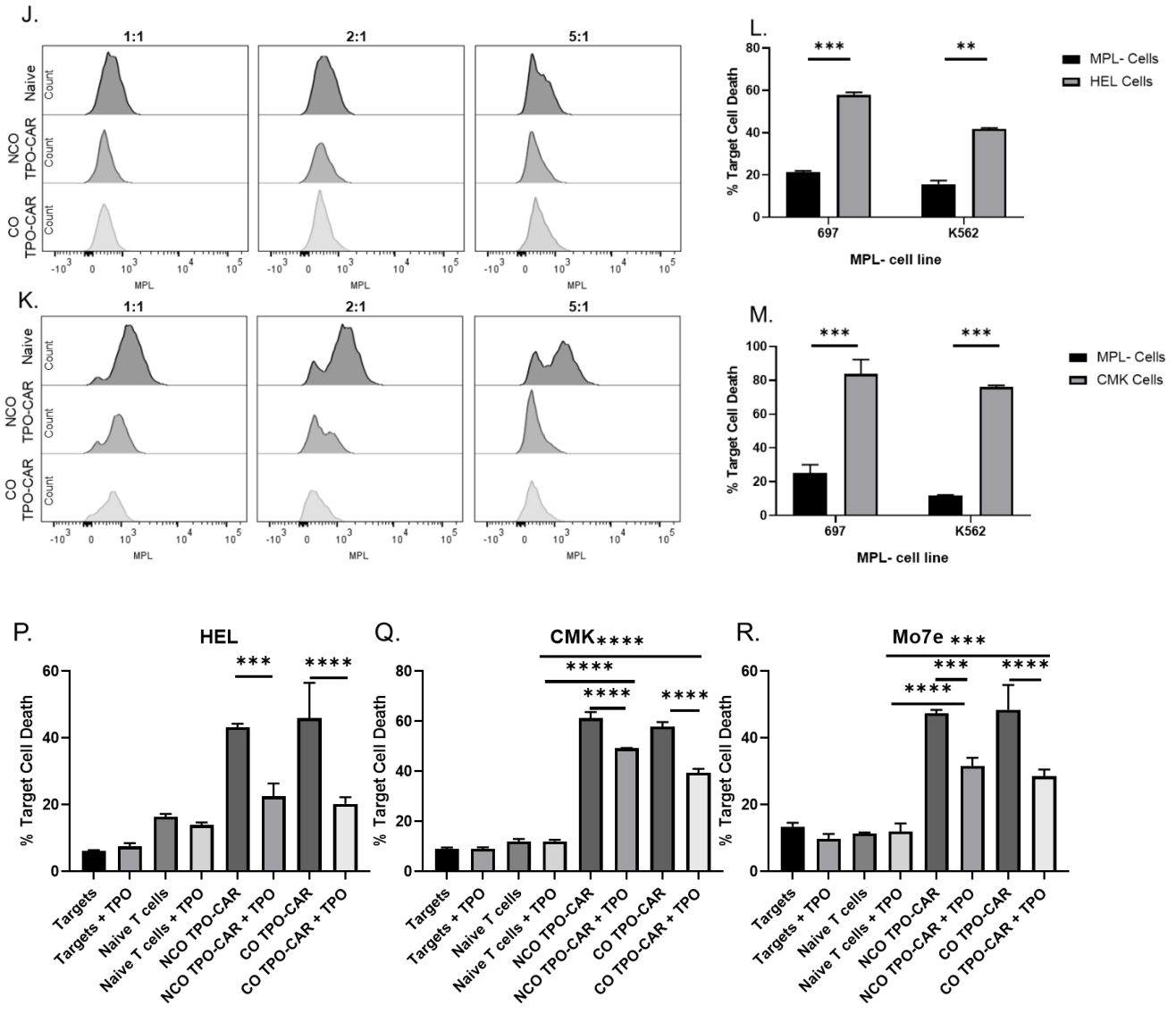


Figure Legend: A-C: Activation after a 12 hour co-culture experiment was measure when cells were co incubated with HEL (A), CMK (B), or Mo7e (C) cells. Activation was measured by CD69, CD38, CD69+ CD38+. Data is demonstrating one T cell donor with experimental triplicated, however, data is representative across donors. Significant increases in activation were seen across all cell lines and in all measures of activation compared to non-transduced T cells. ***p<0.001 D-F: The TPO CAR cytotoxic potential was measured against HEL (D), MO7e (E), CMK (F) cells in 12 hour co culture assay. Increasing effector to target ratios (E:T) were tested including 0:1 (stained target cells alone), 1:1, 2:1, and 5:1 (y-axis) with non-transduced T cells, NCO TPO-CAR, and the CO TPO-CAR. The CD19 CAR was only tested at

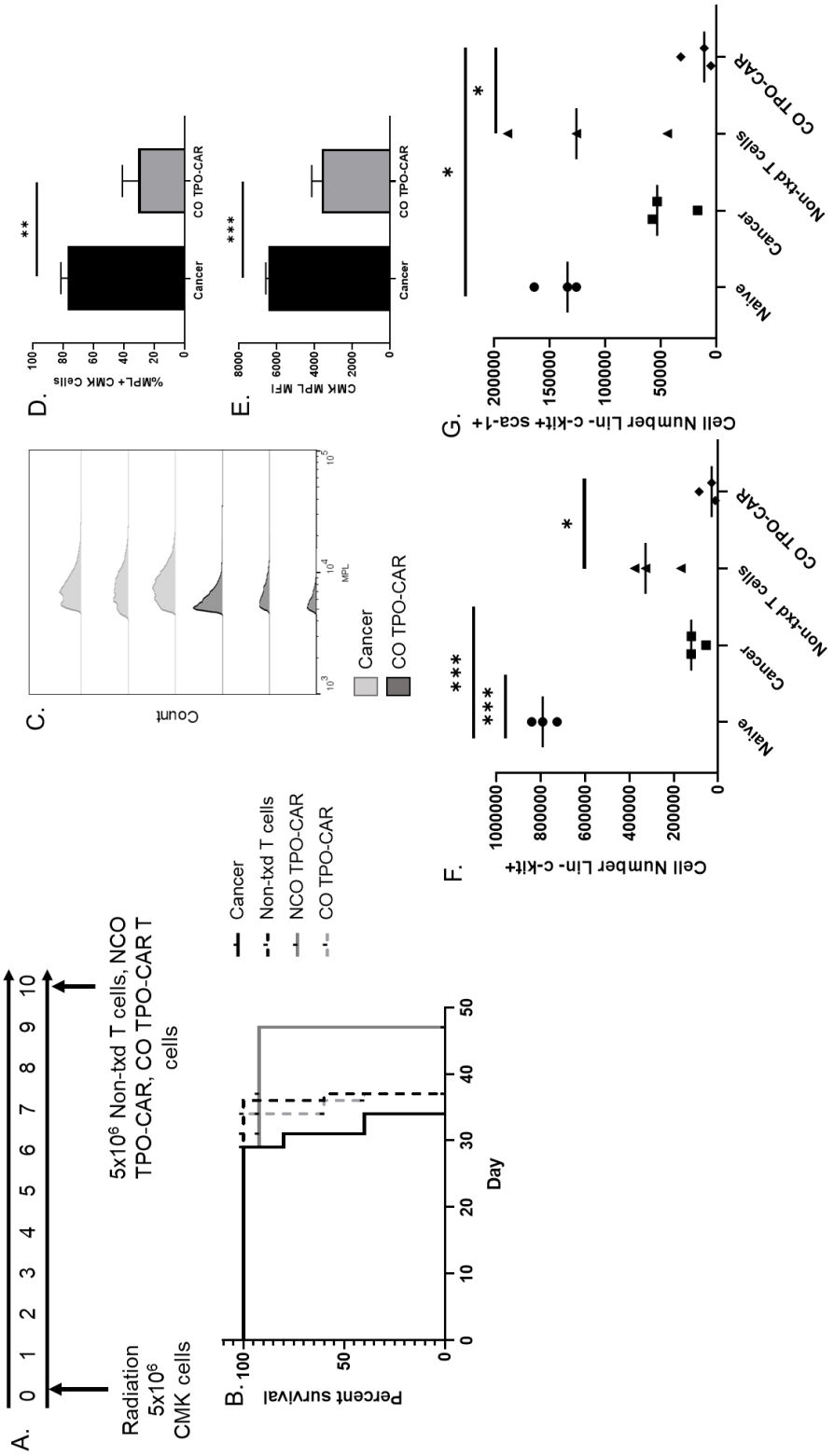
the 1:1 E:T ratio. Significant cell death was seen in the NCO TPO-CAR and CO TPO-CAR co culture conditions compared to the non-transduced and CD19 CAR transduced T cells in all target cell lines. ***p<0.001 G-I Cytotoxicity assays from multiple donors were pooled and the effector to target ratio 1:1 was compared within each cell line. The NCO TPO-CAR and CO TPO-CAR significantly killed the HEL (G), CMK (H), and Mo7e (I) significantly better than the non-transduced T cells and CD19 CAR T cells in all donors. **p<0.01 ***p<0.001. J.-K After the coculture cytotoxicity experiment, the remaining living target cells in the HEL (J) and CMK (K) cell lines were evaluated for remaining MPL expression. In the TPO-CAR conditions there was an appreciable decrease in surface MPL expression on remaining target in cytotoxicity data as efector:target ratios increased and further there was less MPL surface expression compared to target cells treated with naïve T cells. L and M. MPL – cells were stained with CFSE and HEL and CMK cells were stained with VPD450. One hundred thousand cells from 697 or K562 and HEL or CMK cell lines were mixed together and incubated with 100,000 TPO-CAR transduced T cells. Cytotoxicity was measured within the MPL- cells and MPL+ cells. The HEL cells and CMK cells showed significantly higher death than MPL- cells 697 or K562. **p<0.01 ***p<0.001. P-R. Target cells HEL (P), CMK (Q), and Mo7e (R) were cocultured +/- 400 ng/mL of TPO with naïve T cells, NCO TPO-CAR T cells, or CO TPO-CAR T cells at a 1 to 1 ratio for 12 hours. TPO cause a significant reduction in cytotoxicity in all three cancer cell lines when coincubated with the NCO TPO-CAR or the CO TPO-CAR. The CMK cells and the Mo7e cells cultured with the NCO TPO-CAR or CO TPO-CAR with TPO showed significantly greater cell death when compared to naïve T cell killing. Ordinary one-way ANOVA, **** p<0.0001, *** p<0.001

cells suggesting there was competition for the engagement of the MPL receptor (**Fig 3.3P-R**). However, cytotoxicity with the CAR T cells against the Mo7e and CMK cell lines in the presence of TPO was significantly greater than cytotoxicity from naïve T cells ($p<0.001$).

Utilizing the TPO-CAR for extended survival in leukemia xenografts

We tested the NCO and CO TPO CAR *in vivo* against the CMK cell line (**FIG 3.4A)**. NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG) mice were irradiated with 100 Rads and injected intravenously with a luciferase positive CMK cell line. Ten days after $5x10^6$ non-transduced T cells (N=4), NCO TPO-CAR (N=4), or CO TPO-CAR (N=4) transduced cells were intravenously injected. The mice were imaged and weighed regularly to evaluate cancer progression and general health. One mouse from the NCO TPO-CAR group died because of an experimental procedure prior to imaging on day 29. The remainder of the mice were not sacrificed due to illness until day 47, which was significantly longer than mice receiving non-transduced T cells. Interestingly, mice treated with non-transduced T cells and the CO TPO-CAR succumbed between days 34-37 (**FIG 3.4B**). We hypothesized these results could be due to the shedding of TPO from the CAR vector acting as a survival advantage for the cancer cells or CO TPO-CAR was too toxic to the stem cell compartment, resulting in no survival benefit. To test these hypotheses we first harvested media from transduced T cells and naïve T cells and performed a pSTAT5 activation assay to demonstrate if shed TPO would cause activation of our cancer cells (Supplemental Figure 3.1A-I). The data shows there was a significant activation of pSTAT5 by the T cell media of the NCO TPO-CAR T cells in the HEL and CMK cell line and the CO TPO-CAR T cells in the CMK cell line suggesting that shedding may be a significant problem when utilizing ligand-based CARs. To analyze bone marrow suppression, at the time of euthanasia from the in vivo experiment, the bone marrow cell counts in two femurs in mice received non-transduced compared to CO TPO-CAR T cells in the LK (lineage-, c-kit+) compartment was $4.46x10^4 \pm 2.3\ x10^4$ vs $1.99x10^4 \pm 1.8\ x10^4$ and LSK (LK, Sca-1+) was $3.22x10^4 \pm 9.2\ x10^3$ vs $1.85x10^4 \pm 1.6x10^4$. These results suggest the CO TPO-CAR is more potent and thereby demonstrated greater on-target off-tumor toxicity;

Figure 3.4: Testing *in vivo* effectiveness of TPO-CAR in leukemia xenografts



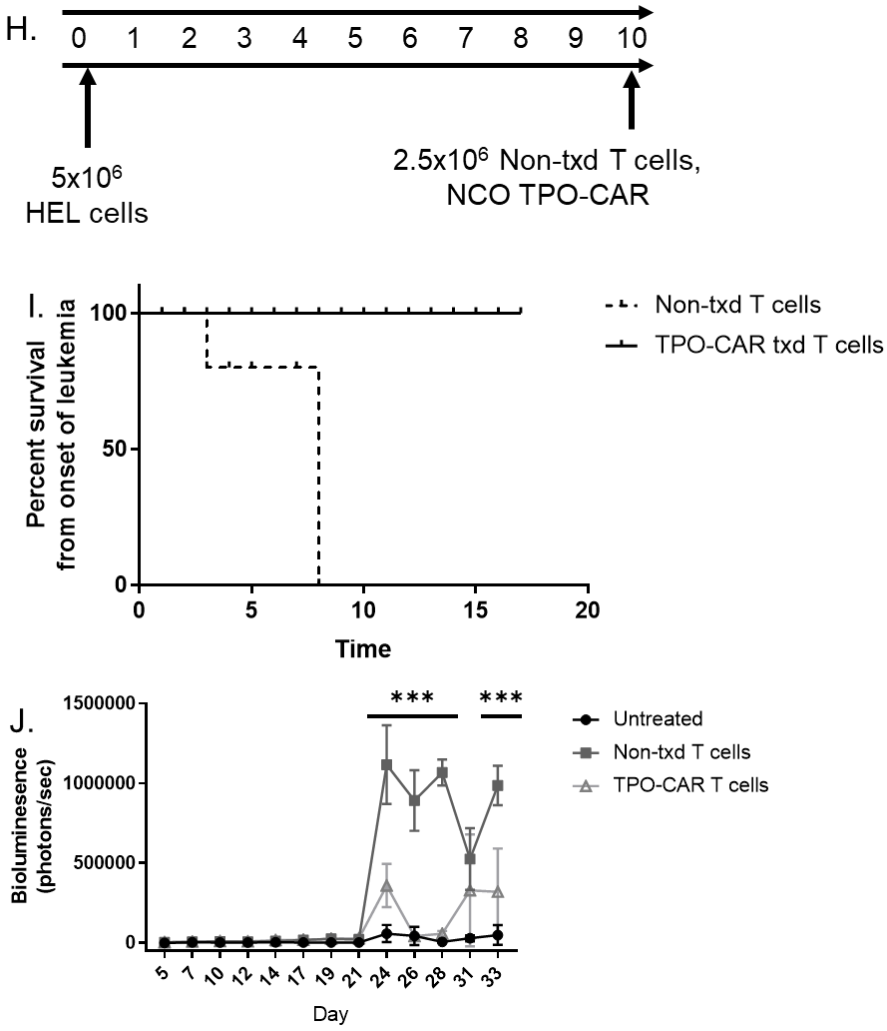


Figure Legend: A. Schematic of NSG mouse experiment to test the Non-transduced T cells (non-txd), NCO TPO-CAR T cells, and CO TPO-CAR T cells against the CMK cell line. Mice received low dose radiation to facilitate engraftment of CMK cells and were administered $5x10^6$ CMK cells modified with luciferase. Ten days after mice received $5x10^6$ T cells. B. Survival curves to day 47 from the start of treatment, shows a significant survival advantage among animals that received the NCO TPO-CAR T cells (N=4) to non-transduced (non-txd) T cells (N=4) and CO TPO-CAR T cells (N=4) in a CMK leukemia model (log-rank $p<0.001$). C-E. Splenocytes from cancer mice and LCO TPO-CAR mice were evaluated for remaining MPL expression because these were the only two groups that demonstrated there was a significant tumor burden in the spleen. In the TPO-CAR conditions there was an appreciable decrease in surface MPL expression (F) and reduction in MPL MFI (G) on remaining cancer compared to control mice. The bone marrow was

analyzed for LK (F) and LSK (G) bone marrow compartments. The data demonstrated significant differences in the LK compartment in Naïve vs cancer only mice, non-transduced T cells, and LCO TPO-CAR T cells ($p<0.0001$). In addition, non-transduced T cell treated mice had significantly more cells in the LK compartment compared to mice treated with the LCO TPO-CAR ($p=0.02$). Significant differences in the LSK compartment were noted in the LCO TPO-CAR treated mice compared to the naïve NSG mice ($p=0.01$) and the non-transduced T cell treated mice ($p=0.04$). H. Schematic of NSG mouse experiment to test the Non-transduced T cells (non-txd) and NCO TPO-CAR T cells T cells against the HEL cell line. Mice were administered $5x10^6$ HEL cells modified with luciferase. Seven days after mice received $2.5x10^6$ T cells. I. Survival curves to day 15 from the first detectable leukemia signal, shows a significant survival advantage among animals that received the NCO TPO-CAR T cells compared to non-transduced (non-txd) T cells (N=4) in an HEL leukemia model (N=4) (log-rank $p<0.001$). J. A follow up experiment measured the bioluminescence over time in the HEL leukemia model. Bioluminescence was significantly reduced by 2-way ANOVA ($p<0.001$) in mice administered the NCO TPO-CAR (N=4) compared to animals receiving non-transduced T cells (N=4). Mice that received no cancer (no treatment N=3) were included to show background luminescence.

however, we repeated the experiment outlined in figure 3.4A with a new donor, but sacrificed the mice at day 30 to evaluate cancer burden and the bone marrow compartment.

Mice were euthanized on day 30, after blood was drawn to evaluate overall health in complete blood counts (Supplemental Figure 3.2A-M). Spleens were dissociated for flow cytometry analysis. We analyzed the spleens from the cancer only mice and the mice that received the LCO TPO-CAR for the amount of MPL expression on the remaining cancer cells. Cancer cells were defined by human CD3- and CD33+. Animals receiving the LCO TPO-CAR compared to cancer alone showed a significant reduction in MPL surface expression (30.3% ± 10.7 vs 77.1% ± 4.3, p=0.002) and MFI (6421 ± 151 vs 3601 ± 535, p=0.0009) (**Fig 3.4C-E**). These results confirmed our in vitro data demonstrating the TPO-CAR is specific for MPL+ cells. In addition, we tested the bone marrow for depletion by taking two femurs and running flow to measure the LK and LSK compartments. The data suggests that all mice receiving cancer cells had some reduction in the amount of bone marrow in the LK compartment (**FIG 3.4F**, $p<0.001$). Mice receiving LCO TPO-CAR T cells compared to mice injected with non-transduced T cells showed a significant reduction in the LK and the LSK compartment (**FIG 3.4G**, $p<0.05$).

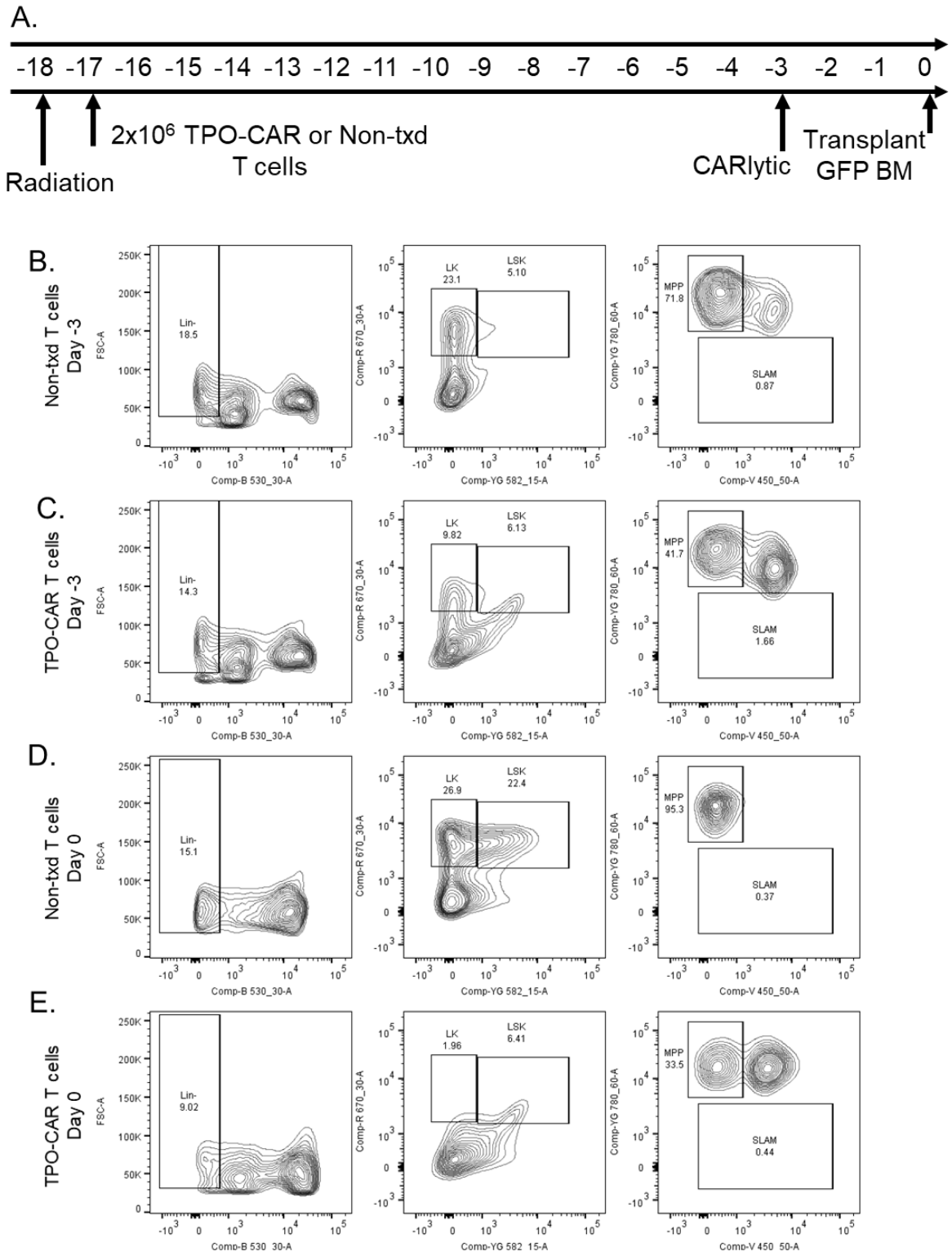
To further test the effectiveness, the NCO TPO-CAR, mice were intravenously injected with $5x10^6$ HEL cells modified with luciferase and 7 days later injected with $2.5x10^6$ non-transduced T cells or NCO TPO-CAR modified T cells (**FIG 3.4H**). Two experiments were performed in this model one demonstrating survival and additional experiment to monitor the progression of a luciferase signal by IVIS imaging. The mice treated with the NCO TPO-CAR showed a significant survival benefit from onset of leukemia by IVIS imaging compared to mice receiving non-transduced T cells (**FIG 3.4I**, $p<0.001$). Additionally, there was a significant reduction in bioluminescence in the mice treated with the NCO TPO-CAR (**FIG 3.4J**, $p>0.01$). Taken together, these data shows the NCO TPO-CAR has a significant impact on MPL+ tumor growth.

However, constructs with robust TPO-CAR expression, as shown with the CO TPO-CAR construct, can have lethal consequences if a rescue strategy is not employed.

Targeting LT-HSCs and stem cell populations for long term bone marrow engraftment

As anticipated and demonstrated with the CO construct, the TPO-CAR appears to target MPL+ bone marrow cells, specifically HSCs. To further test this, NSG mice were administered a low dose of radiation to facilitate T cell homing to the bone marrow, followed by administration of non-transduced or TPO-CAR transduced T cells (**FIG 3.5A**). Fourteen days after the injection of TPO-CAR T cells the percent reduction of bone marrow cells per femur in mice received non-modified compared to TPO-CAR modified T cells in the LK (lineage-, c-kit+) compartment was 80.1% and LSK (LK, Sca-1+) was 62.0% (**FIG 3.5B-C**). To eliminate CAR+ cells, mice were treated with a CARlytic (low dose fludarabine, cyclophosphamide, and alemtuzumab), and 3 days later the bone marrow compartment was analyzed and showed a 96.6% reduction of LK cells and 98.0% reduction of cells in the LSK compartment (**FIG 3.5D-E**). All mice were transplanted with bone marrow from a GFP+ transgenic mouse and at week 2 post-transplant there was a significant difference in GFP+ derived granulocyte engraftment between the non-transduced, 8.72% vs the TPO-CAR modified treated mice, 72.52% (data not shown). At 4 weeks post-transplant, all mice demonstrated 100% engraftment. We hypothesized this was due to the GFP+ bone marrow was able to outcompete the NSG bone marrow reconstitution or the pre-conditioning allowed for engraftment in both models. We repeated this experiment with no pre-conditioning prior to infusion of the T cells and transplanted mice with RAG1$^{-/-}\gamma c^{-/-}$ bone marrow (**FIG 3.5F**). Using a methocellulose-based assay, there was a significant difference in the multi-potential granulocyte, erythroid, macrophage, megakaryocyte progenitor cells colonies formed per 80,000 bone marrow cells treated with non-transduced T cells vs TPO-CAR T cells were preCARlytic 8.67 ± 1.0 vs 3.00 ± 1.0 and postCARlytic 16.00 ± 4.4 vs 4.33 ± 0.6, respectively (**FIG 3.5G-H**). Twenty weeks post-transplantation, engraftment of donor cells was evaluated. The mean engraftment of non-transduced T cell treated mice vs TPO-CAR T cell treated mice were compared and showed the LK compartment was 28.1 ± 9.4 vs 40.18 ± 15.7 ($p=0.22$, **FIG 3.5I**), LSK compartment was 6.6 ± 1.6 vs 13.0 ± 5.3

Figure 3.5: Evaluating on-target off-tumor side effects of NCO TPO-CAR on mouse bone marrow



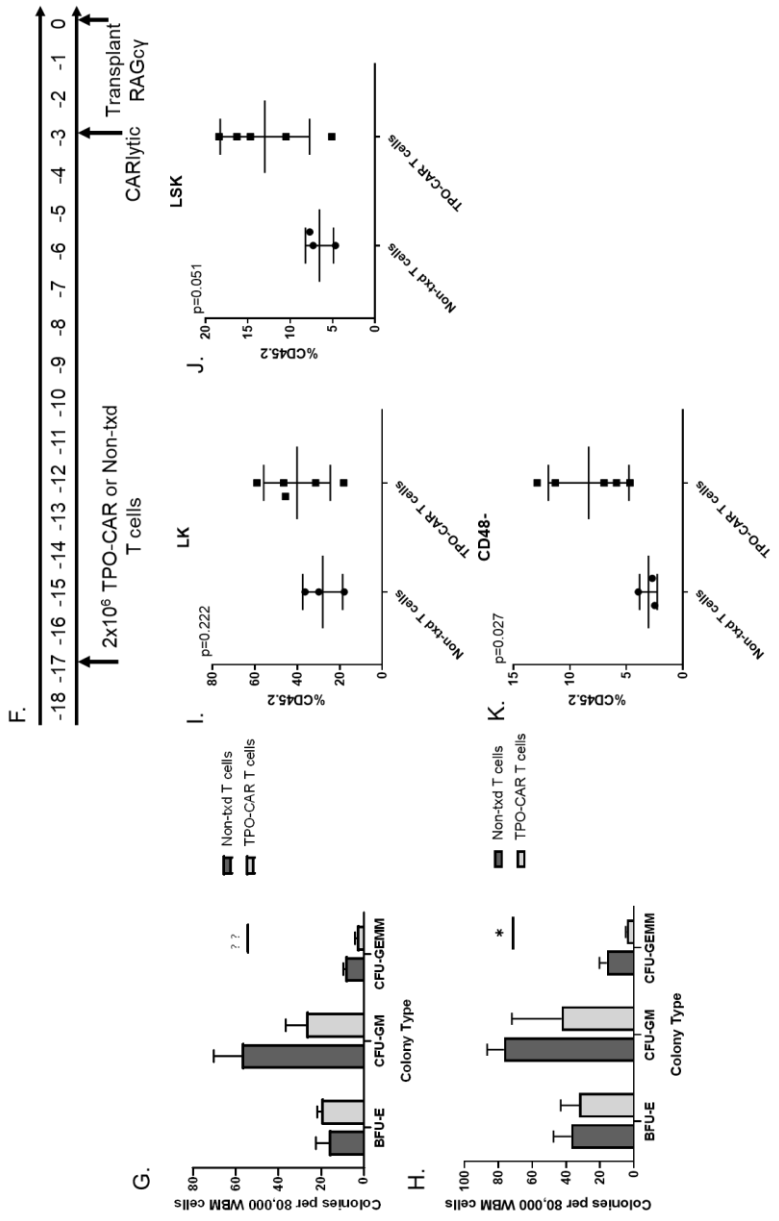


Figure Legend: A. Schematic of NSG mouse treatment with the NCO TPO-CAR. Mice received low dose radiation on day -18, day -17 mice were injected with non-transduced or TPO-CAR T cells. A low dose chemotherapy combination was termed CARlytic to remove the CAR so mice could be transplanted on day 0 with GFP bone marrow. B-E Mice treated with non-transduced T cells (B and D) and TPO-CAR T cells (C and E) were sacrificed on day -3 (B and C) or day 0 (D and E) and the bone marrow compartment was analyzed for Lin-, LK, LSK, MPP, and SLAM compartments. F. Schematic excluding prior conditioning

to CAR administration with a RAG cg transplant instead of GFP black 6 mouse. G and H Colony formation assays comparing colonies formed from a methocult assay were used to determine the impact the TPO-CAR had on the stem cells on day -3 (G) or day 0 (H). BFU-E and CFU-E showed no significant impact in colony formation. On day -3 and 0 the CFU-GEMM showed a significant reduction in colonies in the TPO-Car treated animals compared to naïve NSG mice and mice treated with non-transduced T cells. 20 week engraftment was evaluated in mice treated with the non-transduced T cell (N=3) and TPO-CAR T cells treated mice (N=5). The percentage of CD45.2 was measured in the bone marrow compartment to measure the percentage of engraftment in the NSG mice (CD45.1+). In the more differentiated compartments there were was less engraftment ie LK (I) and LSK (J) compartments whereas the LSK CD48- (K) population of cells showed significant engraftment.

(p=0.09, **FIG 3.5J**), and LSK CD48- compartment was 3.1±0.8 vs 8.4±3.5 (p=0.049, **FIG 3.5K**), respectively.

3.4 Discussion

To date, there are few ligand-based CARs in preclinical and clinical development including the interleukin 13R2, interleukin 11, adectin, follicle stimulating hormone receptor, and granulocyte-macrophage colony stimulating factor[310-314]. With the boom of CAR therapy it has become evident there is a lack of targets and antibodies to these targets. Therefore, we explored alternative mechanisms to engage surface receptors. Due to the extensive body of literature on understanding receptor ligand interaction, we used this foundation to explore and expand the repertoire of antigen binding domains for CAR technology through the use of ligand-based CARs. We hypothesized due to better understanding of ligand-receptor interactions, we could better predict the on-target, off-tumor side effects, which can help to anticipate and exploit side effects for clinical benefit[309].

We designed a ligand-based CAR targeting the MPL receptor for cancer stem cell clearance[380, 397]. Our reason for pursuing a cancer stem cell population is due to their chemo resistance and ability to self-renew making it an ideal candidate to prevent relapse[398]. We chose MPL as our candidate as opposed to the more common target c-kit in stem cell research because of its limited expression on healthy tissues, making the on-target off-tumor side effects more predictable and manageable (The GTex Portal). Furthermore, we were able to expand the use of this CAR targeting MPL to AML. MPL is abundantly expressed on erythropoietic and megakaryocytic leukemias which typically have fewer treatment options and afflict a unique population of patients with Down's syndrome[399-402]. Taken together, there was an unmet need to target this subset of leukemias as well as cancer stem cells. This is the first report of a CAR designed to target MPL and further using a novel ligand-based approach.

Upon CAR design challenges presented with decreased expression of our protein by western blot compared to the standard CD19 CAR despite significant functional output. We used a common approach in gene therapy to enhance protein expression, known as codon optimization, which robustly enhanced CAR expression[403]. We continued our analysis using both CARs to pursue specific cytotoxicity and activation of MPL positive populations of cells. We exhausted *in vitro* methods including activation by multiple indicators and cytotoxicity experiments with multiple cell lines including a CD19 CAR comparison. We recognized we were specifically targeting the MPL population by looking at the remaining living cells after the cytotoxicity assay to ensure the MPL+ cells were no longer remaining. It was essential to establish our CAR is specific since we are developing a novel target for immunotherapy.

Our *in vivo* testing of both the NCO and CO TPO-CAR was tested in an immune incompetent amegakaryocytic leukemia model. Interestingly, we observed a phenomenon which was also observed with the CD19 CAR[404]. We found the CO TPO-CAR treated animals were succumbing shortly after the cancer only treated animals. We hypothesized this was due to on-target off-tumor side effects. Due to the CO TPO-CAR being more highly expressed by western blot compared to the NCO TPO-CAR, we expect the CO construct more rapidly cleared the bone marrow due to higher expression leading the mice to fatal bone marrow failure. While we anticipated the CAR would have effects on the bone marrow, we expected the cancer would clear just as rapidly. However, we did establish long term survival in our megakaryocytic model using our NCO TPO-CAR. These data are consistent with our *in vitro* data. We ruled out the hypothesis the lack of survival in the CO TPO-CAR was due to loss of antigen because of the survival and bioluminescence data seen with the NCO construct. We observed that when the NCO TPO-CAR treated mice did succumb to their disease, it was due to an ablated bone marrow compartment. The NCO TPO-CAR was perhaps more successful *in vivo* because of the gradual selective expansion of the T cells. This can be partially due to the CAR clearing the bone marrow but could also be an effect of the human T cells activated and proliferated in the mouse bone marrow compartments determining their lethality. In addition to T cell crowding, activated cytokine milieu of human T cells in mouse marrow could have resulted in

bone marrow suppression. To manage this a suicide switch could be introduced into the CAR or an alternative immune cell source such as a γδ T cell or NK cell could be used in the place of αβ T cells.

We challenged the NCO TPO-CAR in a second *in vivo* model of erythropoietic leukemia with the HEL cell line. The HEL cell line did not have as strong induction of pSTAT5 or MPL surface expression compared to the CMK cell line, so we expected minor differences in the data. The NCO TPO-CAR again extended survival from leukemia onset and reduced bioluminescence. This highlights the lethality of the CAR to bone marrow compartment but also the capability of the CAR to clear erythropoietic leukemias.

To further understand the impact the NCO TPO-CAR was having on the bone marrow compartment two experiments were completed. One experiment gave mice mild conditioning and then evaluated the bone marrow compartments ability to grow colonies in a colony forming assay. We additionally assessed the depletion of the bone marrow. Unsurprisingly, compared to mice treated with non-transduced T cells mice administered the TPO-CAR had significant reduction in the amount of bone marrow the mice were left with and there was impaired functionality. We proceeded without any conditioning regimen and injecting unmodified or modified T cells to assess the 20 week engraftment status. We observed a trend in significance of engraftment in the more stem like compartment of Lin-, c-kit+, sca-1+, CD48- being the compartment saw the greatest engraftment.

Fortunately, many patients presenting with this level of disease in the MPL+ leukemias are typically of a poor prognosis and will eventually need to continue after treatment to a bone marrow transplant[405]. While there are avenues for turning the CAR off to prevent toxicity to the bone marrow compartment, an alternative would be to allow the CAR to function as a non-genotoxic conditioning regimen and then turn the CAR off to bring patients to transplant. However, it is evident in a preclinical model of AML the NCO TPO-CAR was capable of eliminating cancer and extending survival. Our overall goal would be to extend the use of this CAR to all relapsed cancers that have MPL expression on the surface. These studies taken

together demonstrate the use of a novel CAR with some development will make an ideal model for stem-like malignancies.

3.5 Materials and Methods

Cell lines and cell culture

HEL, K562 (ATCC, Manassas, VA), and 697 cells were cultured in RPMI 1640 with L-glutamine (Corning cellgro, Manassass, VA) and 10% FBS and 1% Penicillin/Strep added. CMK cells were also cultured under previous conditions except with 20% FBS. Mo7e cells were cultured in IMDM (1x) with L-glutamine and 25mM HEPES and supplemented with 20% FBS and 1% Penicillin/Strep.

Primary Cells

Whole blood cells were purchased from the American Red Cross. PBMC were isolated after cells were isolated with Ficoll-Paque Premium sterile solution from GE Healthcare (Uppsala, Sweden). Leukocytes were washed with PBS and T cells were isolated using EasySep Human T cell Isolation Kit (Stem Cell Technologies, Cambridge, MA). Immediately after isolation, T cells were activated with CD3/CD28 Dynabeads (ThermoFisher Scientific, Waltham, MA) for 24 hours.

Cloning of CAR constructs

CAR sequences were cloned into a vector containing the necessary components for lentiviral production. The binding domain of thrombopoietin[406] was then used as the binding portion for the CAR. The CAR was a bicistronic vector to co-express GFP. The codon optimized construct was redesigned to contain a CH3 hinge domain and was not bicistronic but the entire CAR from the IL2 signal sequence to CD3ζ was optimized for human cell expression. All gene were obtained by gene synthesis from Genewiz (South Plainfield, NJ).

Lentiviral Production

Viral accessory plasmids CAR expression plasmids were transiently transfected in 293T-17 cells using a calcium phosphate transfection method to generate LV vectors pseudotyped with the VSVG envelope similar to the method described previously except for the utilization of the calcium phosphate transfection reagent (Sigma Aldrich, St. Louis, MO). Conditioned media was collected for 3 days beginning at 48 hours post transfection and passed through a 0.45-µm filter. Virus was concentrated by overnight centrifugation at 10,000 × g overnight, followed by filtration using a 0.22-µm filter. Viral concentrate titers were determined using quantitative real-time PCR analysis.

Lentiviral Transduction

Transduction of recombinant HIV lentiviral particles was carried out by incubating cells with virus in complete medium supplemented with 8 µg/ml polybrene (EMD Millipore, Billerica, MA). Eighteen hours after transduction, media was replaced. The transduced cells were then cultured for at least 5 days before being used in experiments.

Flow cytometry

Cells were washed with phosphate buffered saline (PBS) and centrifuged at 100xg. Supernatant was decanted and replaced with the appropriate antibody cocktail in PBS. The antibodies used from BD Biosciences (Franklin Lakes, NJ), include: BUV737 Mouse Anti-Human CD3 (Clone SP34-2), BUV496 Mouse Anti-Human CD38 (Clone HIT2), APC-Cy7 Mouse Anti-Human CD69 (Clone FN50), PE Mouse Anti-Human CD45, V450 Mouse Anti-Human CD3 (UCHT1), BV605 Rat Anti-Mouse CD16/32 (Clone 2.4G2), BV421 Rat Anti-Mouse CD150 (Clone Q38-480), PE-Cy7 Hamster Anti-Mouse CD48 (Clone HM48-1). Antibodies used from BioLegend (San Diego, CA) include: APC Annexin V, PE Anti-Human CD110 (S16017E), Propidium Iodide Solution, APC Anti-Human CD38 (HIT2), FITC Ms CD3/Gr-1/CD11b/CD45R(B220)/Ter-119 (“Lineage”), PE Anti-Mouse Ly-6A/E (Sca-1) (Clone D7), APC Anti-Mouse CD117 (c-kit) (Clone ACK2), PerCP/Cy5.5 Anti-Mouse CD34 (Clone MEC14.7). Cells were analyzed by flow cytometry using an LSRII (BD Biosciences, Franklin Lakes, NJ).

Cytotoxicity Assays

T cells were tested for cytotoxic potential by co-culture experiment. Target cells (CMK, Mo7e, HEL) were labeled with the Violet Proliferation Dye 450 (BD Biosciences, Franklin Lakes, NJ) and assessed in flow cytometry-based cytotoxicity assays. In mixing cytotoxicity experiments, target cells, K562 and 697, were stained with CFSE (Thermo Fisher Scientific, Waltham, MA) to separate MPL+/- target cells. Target cells were incubated with T cells at the varied effector to target (E: T) ratios: 0:1, 1:1, 5:1, 10:1 for 12 hours at 37C. Target cell death was analyzed via flow cytometry using dead cell stains Annexin V and PI and effectors were analyzed for activation markers CD69 and CD38. Remaining targets were additionally analyzed for MPL surface expression. Antibodies were incubated for 60 minutes with shaking at room temperature and data was acquired after 1 volume PBS wash.

Real time quantitative PCR

Genomic DNA was extracted using the Qiagen DNeasy Blood & Tissue Kit according to the manufacturer's recommended protocol (Qiagen, Germantown, MD). Oligonucleotide primers were designed for a 150bp amplicon of the Rev-response element (RRE). Real-time PCR was performed in an Applied Biosystems® StepOne™ System (Thermo Fisher Scientific, Waltham, MA).

In vivo mouse experiments

NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG) mice were purchased from Jackson Laboratory (Bar Harbor, ME) and were maintained in a pathogen-free environment. Mice were cared for according to the established principles of the Institutional Animal Care and Use Committee (IACUC), and all animal protocols were approved by the IACUC. Five-week-old mice were injected tail vein with $5x10^6$ HEL luciferase cells, CMK luciferase cells, or Mo7e luciferase cells. Tumor growth and mice health were monitored three times per week by weighing, IVIS (*In vivo* Imaging System, Perkin Elmer, Waltham, MA) imaging, and bi-monthly

complete blood counts. Luciferase was made fresh immediately prior to imaging. Luciferase was injected at 10 mL/gram intraperitoneal. Mice were imaged 10 minutes after injection and luciferase was quantified.

Colony Formation Assay

MethoCult™ GF M3434 (Stem Cell Technologies, Cambridge, MA) was thawed in a 37C water bath and alliquoted into 4 mL tubes. Whole bone marrow from mice was flushed, pelleted, washed, and counted using red cell lysis buffer and trypan blue exclusion. Eighty thousand whole bone marrow cells were added to the 4 mL and the tube was vortexed for 1 minute. The tube rested for 15 minutes to reduce the bubbles and then 1 mL was alliquoted per 35 mm^2 plate. Each plate was given 10 days and the colonies were counted and classified based on the appearance using a light microscope 20x magnification. Colonies formed included primitive erythroid progenitor cells (BFU-E), granulocyte-macrophage progenitor cells (CFU-GM, CFU-G and CFU-M), and multi-potential granulocyte, erythroid, macrophage, megakaryocyte progenitor cells (CFU-GEMM). Colonies were classified based on standard protocol by Stem Cell Technologies (Cambridge, MA).

Statistical Analysis

All statistical analysis and graphing was performed using Sigma Plot version 13 (Systat Software Inc,) and GraphPad Software Prism. The exact method, for example ANOVA, t-test, or log ranked Mantel-Cox test, are described for each experiment where they are used.

3.6 **Supplemental Figures, Tables, and Legends**

Table 3.1:. Comparison of KIT and MPL expression in healthy tissue

Tissue	KIT	MPL
Breast	25.5	0.6
Ovary	15.3	0.3
Thyroid	12.7	0.3
Brain	12.6	0.2
Salivary Gland	11.4	0.1
Skin	11	0.1
Stomach	0.7	0.1
Lung	9.9	0.1
Urinary Bladder	9.5	0.1
	High	Low

Table 3.2: MPL RNA expression from CCLE

Cell Line	MPL	Tissue	Description
HEL	45.665	M6 AML	JAK2p.V617F
CMK	22.130	M7 AML	T21
Mo7e	1.095	M7 AML	infant
Loucy	1.262	T-ALL	SET-NUP214

Supplemental Figure 3.1: Shedding of TPO causes pSTAT5 signaling in cancer cells

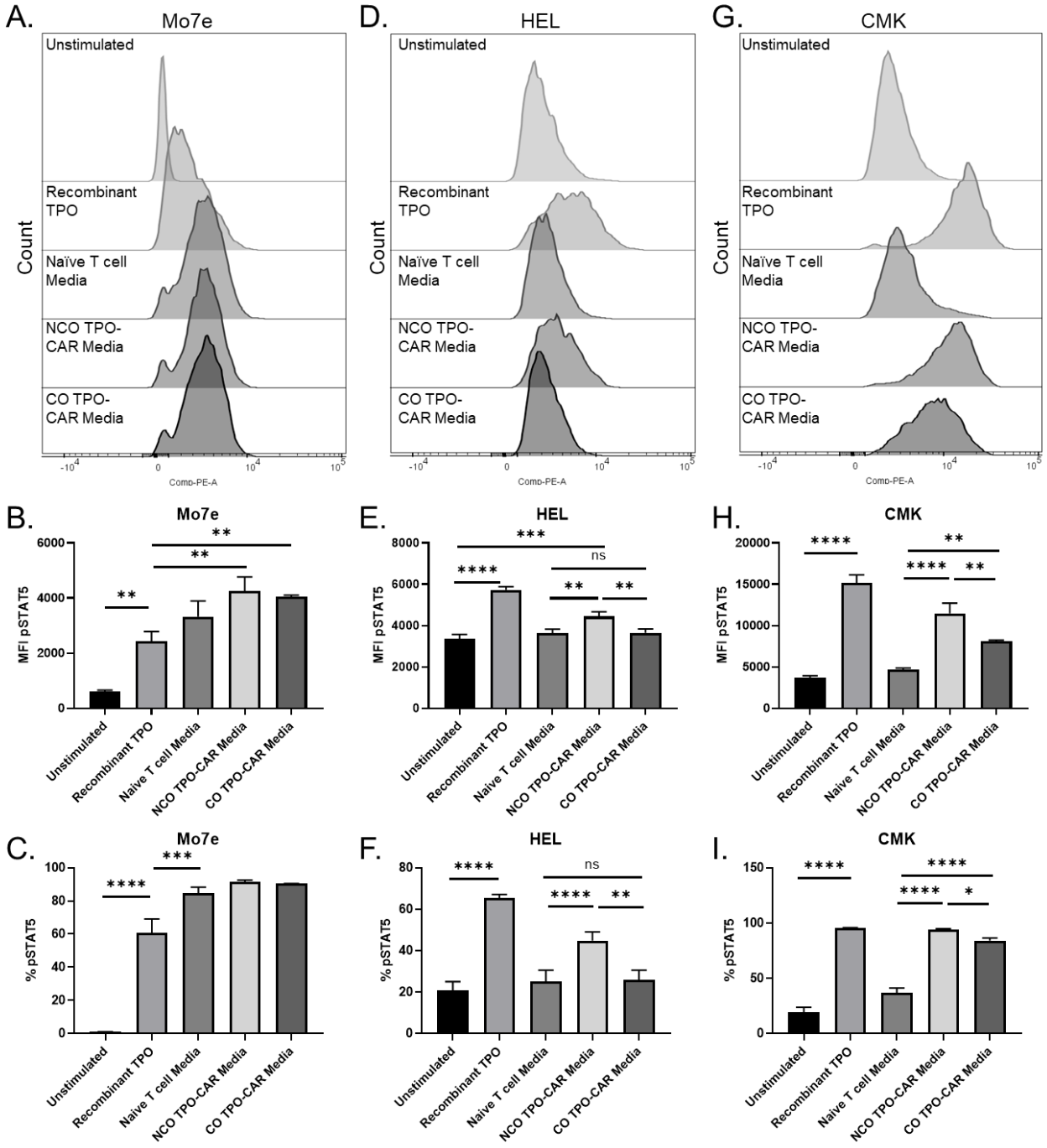


Figure Legend:A-I Cancer cells including Mo7e (A-C), HEL (D-F), and CMK (G-I) cells were stimulated with recombinant TPO, or media from naïve T cells, NCO TPO-CAR T cells, or CO TPO-CAR T cells. pSTAT5 expression was measured by flow cytometry and representative histograms are represented in A, D, and G, MFI of pSTAT5 in B, E, and H, and % of pSTAT5 in C, F, and I. Data demonstrates that there was significant increases in MFI and % from unstimulated cancer cells to cells stimulated with recombinant TPO. Mo7e cells were stimulated by naïve T cell media therefore, increases in pSTAT5 were not detected with NCO or CO TPO-CAR T cells. HEL cells demonstrated increases in MFI and pSTAT5 % with the NCO TPO-CAR T cells compared to naïve T cell media and CO TPO-CAR T cells. The CMK cell line demonstrated significant increases in pSTAT5 MFI and % with NCO TPO-CAR media and CO TPO CAR T cell media.

Supplemental Figure 3.2: Complete blood counts of CMK mice treated with CO TPO-CAR

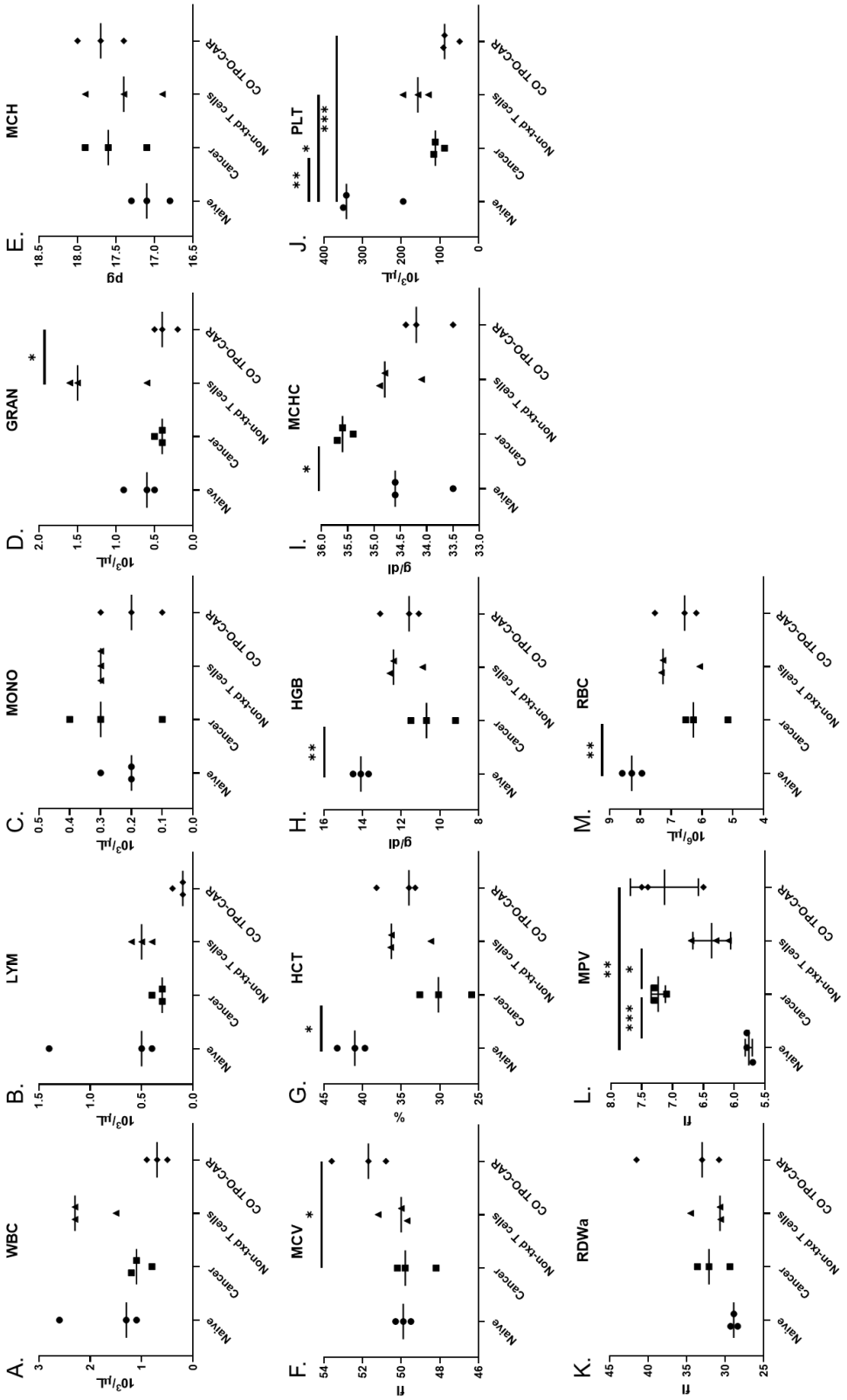


Figure Legend A-M. Complete blood counts were performed on peripheral blood. Blood contents and counts included white blood cells (WBC, A), lymphocytes (LYM, B), monocytes (MONO, C), granulocytes (GRAN, D), mean corpuscular hemoglobin (MCH, E), mean corpuscular volume (MCV, F), hematocrit (HCT, G), hemoglobin (HGB, H), mean corpuscular hemoglobin concentration (MCHC, I), platelet (PLT, J), red blood cell distribution width (RDWa, K), mean platelet volume (MPV, L), and red blood cell (RBC, M). Non-transduced (non-txd) T cells demonstrated significant difference compared to the CO TPO-CAR in granulocytes ($p=0.04$). Cancer only animals differed from the CO TPO-CAR in mean corpuscular volume (0.04) and mean corpuscular hemoglobin concentration ($p=0.0197$). Naïve NSG mice were significantly different from cancer mice in hemoglobin ($p=0.0034$), mean corpuscular hemoglobin concentration ($p=0.0432$), and red blood cells ($p=0.0058$). Naïve mice had higher platelet counts than all treatment groups and cancer only mice ($p<0.05$) and in mean platelet volume ($p<0.05$) with the exception of the non-transduced T cell treated animas.

Chapter 4

General Discussion

4.1 Summary of Results

Pediatric patients with resistant and recurrent cancers that have underwent surgery, chemotherapy, and radiation stand to benefit from immunotherapy. As immunotherapy becomes an increasingly viable option, pediatric patients can benefit from the reduction in long-term toxicities typically associated with current clinical strategies. Due to the fact that ACT is heavily reliant on αβ T cells, the majority of clinical results depend on the success of the expansion of αβ T cells. However, recent findings suggests that a clinical response is influenced by engagement of multiple arms of the immune system[407]. Additionally, tumors may not have specific antigens to be targeted or cancer cells may downregulate MHC I proteins, making the tumor undetectable to conventional adoptive cell transfer methods. CAR T cells have advanced ACT by allowing αβ T cells to bypass MHC processing and, in the presence of high antigen loads, to elicit a cytotoxic response against specific tumor associated antigens[41-43]. However, finding targetable tumor specific antigens remains a problem using a CAR T cell approach. Utilizing cells from another arm of the immune system, may be a more successful strategy for ACT.

Chapter 2 explores the *ex vivo* expansion of NB patient-derived γδ T cells using a serum-free protocol[408]. NB patients apheresis products were expandable using this protocol which allowed for a 25-310 fold expansion. These studies addressed if an apheresis product from a NB patient pre-exposed to chemotherapy would expand into cytotoxic γδ T cells that would result in a therapeutic benefit compared to current treatment strategies for NB patients. Given that γδ T cells are intrinsically anti-tumorigenic through multiple innate mechanisms and have high CD16 surface expression, we combined the cells with dinutuximab, a monoclonal antibody to GD-2. As predicted, γδ T cell's cytotoxicity *in vitro* was enhanced with dinutuximab against GD-2 high expressing cell lines. However, when the γδ T cells were tested *in vivo*, there was no significant reduction in tumor volume with or without dinutuximab. Nonetheless, γδ T cells and dinutuximab did elicit a survival advantage. To achieve a significant reduction in tumor volume, frontline chemotherapeutic for relapsed NB patients, temozolomide (TMZ) was introduced to induce the upregulation of stress antigens on the cancer cells and increase γδ T cell-mediated cytotoxicity. To reduce

potential toxicities introduced by chemotherapy, non-curative, low-dose TMZ was tested *in vivo*. When low doses of TMZ were used in combination with γδ T cells and dinutuximb, significant reduction in tumor volume and long-term survival was achieved. These studies confirmed that γδ T cells can be used as a foundational cell source for therapeutic benefit, as well as function successfully when combined with alternative immunotherapies and current chemotherapies.

In chapter 3, the limited number of single chain variable fragments (scFv) as antigen binding domains for CAR T cells was addressed by exploring a ligand-based approach[309]. With the limited number of tumor associated antigens with antibodies targeting them, strategies for diversifying CAR T cell engagement with tumor cells are necessary. The on-target off-tumor interactions are better predicted with a ligand-based CAR design, because they are not composed of chimeric proteins. Currently, leukemias have seen the most success with CAR T cell therapy, but antigen loss and relapse is still a problem and could potentially be a problem in or studies[113]. It is hypothesized that relapse occurs due to immune evasion of cancer stem cells[409]. To overcome this, we designed a CAR to target stem-like leukemia cells, as well as normal stem cells. Thrombopoietin (TPO), the natural ligand to the myeloproliferative leukemia protein (MPL), was used as our antigen binding domain to engage the MPL receptor found on stem cells and erythropoietic and megakaryocytic acute myeloid leukemias (AML). Therefore, we tested our CAR *in vitro* against AMLs with varied MPL expression to test the specificity. As expected, the TPO-CAR was specifically activating against and killing MPL+ leukemias. Additionally, we tested two versions of the TPO-CAR, a codon optimized (CO) and non-codon optimized (NCO). Though the CO TPO-CAR had greater expression by CD3ζ western blotting, this higher expression resulted in rapid toxicity to the bone marrow compartment *in vivo*. However, the NCO TPO-CAR successfully extended survival *in vivo* in two xenograft models of MPL+ leukemias. As mentioned, the on-target off-tumor side effects of the TPO-CAR were predicted due to MPL expression on long term hematopoietic stem cells (LT-HSCs). *In vivo*, the NCO TPO-CAR was able to induce bone marrow suppression with mild conditioning. To test the toxicity with no conditioning, a study was done to evaluate engraftment. NCO TPO-CAR T cells induced minimal engraftment 20-weeks

post-transplant, but these toxicities are notable. The data collected demonstrate the potential of the TPO-CAR to be utilized in a non-genotoxic conditioning regimen for bone marrow transplantation. These proof-of-concept studies will enable us to further develop strategies to use a TPO-CAR in cancer patients with MPL+ phenotypes.

4.2 Implications of Findings

The focus of this dissertation was to evaluate alternative strategies to pediatric cancer ACT. This was accomplished by studying a cellular source that did not have the same limitations as αβ T cells and by exploring other targetable cancer antigens using CAR technology. While both approaches have addressed gaps in pediatric cancer immunotherapy, each has their own set of unique challenges and advantages. The primary focus of ACT is to reinvigorate the natural immune response to target tumor cells. However, only recently has research been done to employ diverse strategies in immunotherapy.

Unfortunately, traditionally used αβ T cells lack mechanisms of activation outside of engagement by their TCR. Currently, research is being done to expand ACT using immune cells that activate and mediate cytoxicity through a variety of mechanisms, limiting evasion by cancer cells[407]. This includes, but is not limited to, iNKT cells, NK cells, macrophages, and γδ T cells. While the focus of this work has been on γδ T cells, NK cell immunotherapy has advanced the furthest and shown clinical promise as a substitute to αβ T cell based therapies. Briefly, there are 3 ongoing clinical trials with genetically modified NK cells (NCT03056339, NCT00995137, NCT01974479). Similar to γδ T cells, NK cells also function through the release of perforin and granzyme B and FasL-TRAIL-mediated pathways[410, 411]. γδ T cells and NK cells do not require prior antigen exposure to elicit cytotoxicity, have limited *in vivo* persistence, and do not elicit graft versus host disease (GVHD)[412, 413]. Limited *in vivo* persistence can facilitate minimal off-target side effects and long-term toxicities that CAR αβ T cells can elicit. Contrary to γδ T cells, NK cell activity can be disabled by intrinsic inhibitory receptors. The inhibitory receptors can limit NK cell-mediated

cytotoxicity, as they turn the immune response off[414]. Therefore, γδ T cells have all of the benefits of the NK cells, but lack the limitations of the NK cell-based immunotherapy.

The two groups within γδ T cells which are recognized as having immunotherapeutic benefit are characterized by their TCR. The Vδ1 cells are located in mucosal tissue, whereas Vδ2 cells are located in the peripheral blood[415]. We sought after these cells to provide an alternative to the persistent memory phenotype, as Vδ2 are the subset that lack the memory phenotype and have a limited lifespan *in vivo*. Part of the reason we wanted to explore the cytotoxic potential of these cells was to find a cellular based immunotherapy with limited, if any, negative implications. The preferential expansion of the Vγ9Vδ2 was ideal because of their ability to innately recognize tumor cells, successfully expand *ex vivo*, and implications in promising prognostic outcomes that results from γδ T cells infiltration from the tumor[349]. Additionally, γδ T cells are one of the few types of cells that do not cause GVHD, so there is potential to expand these cells into a universal donor cellular product. The premise of a universal donor can ultimately revolutionize the way ACT is approached. This would eliminate the ability of cancer cell contamination and remove any problems that could result from an inefficient number of T cells, which are two major issues with using autologous ACT in leukemia patients.

The expansion of γδ T cells is one of the greatest difficulties in their use clinically. Our studies suggest there is efficient for *ex vivo* expansion using serum-free products. The utilization of serum free products reduces the immunogenicity of a product that could be reinfused into a patient[352]. For example, cellular products are typically exposed to fetal bovine serum, which has been shown to elicit an immune response when given to patients. By utilizing a product devoid of serum, we reduce the potential induction of a complement mediated immune response. Additionally, studies have shown that IL-17 producing γδ T cells can be pro-tumorigenic[88, 416]. We extensively characterized the cells we were expanding and eliminated the possibility that IL-17 producing γδ T cells were contaminating the cellular product. Taken together, our

serum-free expansion resulted in robust and cytotoxic cells. This strategy further expands the possibilities of using γδ T cells as an effective ACT therapy.

Recently, research is being conducted to augment ACT. This dissertation pursues the use of a targeted monoclonal antibody to enhance homing, specificity, and functionality of the γδ T cells. While the combination of monoclonal antibodies has been extensively studied in NK cell models, there are few reports of the benefit of γδ T cells with dinutuximab in a NB model[358]. Additionally, the existing studies do not consider an autologous donor and pursue a third-party donor. While there was limited *in vivo* success with the γδ T cells and monoclonal antibody, the addition of chemotherapy led to a significant reduction in tumor burden. The chemotherapy facilitated a sensitized tumor stroma and microvasculature to induce T cell trafficking and cytotoxicity[417, 418]. Our results provide additional rational for developing combination protocols when using ACT.

Overall survival for high-risk NB is <50% at 5 years, with 80% of relapses occurring within 2 years of diagnosis despite aggressive chemotherapy and radiation therapy regimens[419, 420]. There is no clear optimal therapy for relapsed and high-risk NB. Further, MYCN amplified NB tumors progress more rapidly than non-MYCN amplified tumors[421]. All of the *in vivo* work in our NB models was with MYCN amplified xenografts, highlighting the effectiveness of our combination treatment. γδ T cells proved a therapeutically promising strategy when added to current treatment for high-risk NB patients. In addition, the amount of chemotherapy necessary to elicit therapeutic benefit was reduced when used in combination with immunotherapy. The reduction in chemotherapy dosage can facilitate the unnecessary toxicities that are associated with aggressive treatments for NB. Therefore, these studies provide necessary pre-clinical data to advance NB treatment, while also diminishing toxicities in current subpar regimens.

While this dissertation discusses one target for a ligand-based CAR, the use of a ligand-receptor interaction is promising to eliminate the concern of immunogenicity presented by scFv-based CARs[422]. Expanding the

repertoire of binding domains for CAR therapy will expand the number of cancers capable of benefiting from CAR therapy. However, on-target off-tumor side effects will be continue being a challenging aspect of a CAR approach. This is an inevitable outcome when a therapy is not targeting a tumor neoantigen, but there is a finite number of these antigens. It is noteworthy that on-target off-tumor effects are still prevalent in scFv CAR designs.

This dissertation highlights the effects the TPO-CAR has on the bone marrow compartment. Although bone marrow suppression was highlighted as a side effect of the CAR, the TPO-CAR's ability to target hematopoietic stem cells is a novel characteristic of a CAR. Using a CAR to target bone marrow was previously investigated using the c-kit receptor, however, this scFv based CAR had poor homing to the bone marrow and needed to be engineered with the CXCR4 receptor to facilitate migration[396]. Though sufficient suppression was reached once the c-kit CAR migrated to the bone marrow, mice had only 30% chimerism after receiving a transplant at 36 weeks, suggesting poor engraftment of the donor bone marrow. Although the goal of our study was not to use the TPO-CAR as a conditioning agent, we did see that both of our CARs were capable of greater bone marrow suppression by cell count in the femurs. This suggests the TPO-CAR would be an effective non-genotoxic conditioning regimen for bone marrow transplantation. Current conditioning regimens result in 10-20% morbidity prior to patients receiving the transplant[423, 424]. A non-genotoxic conditioning regimen, compared to a chemotherapy or total body irradiation approach, could minimize off-target side effects, decrease pulmonary and hepatic complications, decrease graft versus host disease, preserve fertility, and reduce the risk of secondary malignancies.

Several hypotheses have emerged on the role cancer stem cells play in tumorigenesis. Several have argued cancer stem cells are derived from stem cells or their descendent, whereas others suggest they originate from differentiated cells that reactivate stem cell machinery to create a pluripotent cell. Whatever their origins, the cancer stem cell hypothesis suggests that a small population of cancer stem cells drive tumor growth and maintenance[425]. Since the hypothesis was first suggested in 1994, cancer stem cells have been

identified in both solid and hematological tumors[426, 427]. Further, there is growing evidence that cancer stem cells are causative of cancer recurrence and relapse in breast cancer, glioblastoma, melanoma, and leukemias[427, 428]. This makes cancer stem cells an attractive target for novel therapies, with the goal of achieving long-term remission. Thus, the TPO-CAR could function in conjunction with frontline therapies to eliminate cancer stem cells and decrease the risk of cancer recurrence. Thereby, the TPO-CAR would have additional benefits across all cancers that recur and have MPL+ cancer stem cells.

The TPO-CAR showed promising efficacy in AML models, including acute megakaryocytic leukemia and erythropoietic leukemia. Acute megakaryocytic leukemia is a heterogeneous subtype of AML that has a poor prognosis and is more commonly diagnosed in children with Down Syndrome[429-431]. Our studies provide preclinical results and rationale for these patients to be treated with the TPO-CAR. Erythroid leukemia is characterized by erythroid hyperplasia with erythroid precursors being greater than 50% of the nucleated bone marrow cells[432-434]. This leukemia generally has a poor prognosis, but overall survival is improved with chemotherapy and bone marrow transplantation[435]. Taken together, erythroid leukemia could also benefit from the use of the TPO-CAR, given the limited available therapies able to lessen the aggressive nature of the disease. The data presented in this dissertation would indicate the TPO-CAR could clinically benefit patients in need of a bone marrow transplant or AML, though further preclinical optimization of the on-target off-tumor side effect is needed.

4.3 Limitations and Future Directions

The advancements being made in immunotherapy have revolutionized therapeutic options for cancer patients. However, an incredibly understudied area in ACT is the mechanisms that determine whether immune therapies are successful or not. Currently, work is being done to observe how changes to CAR design can affect the mechanism of action and function of the CAR. While we could have generated multiple TPO-directed CARs, the focus of these studies was to preclinically determine if TPO would be a

viable antigen to target MPL. Still, our studies do not mechanistically investigate why and how these alternatives work. In addition, more mechanistic studies as to what the TMZ was doing to the tumor environment would be beneficial to understand how chemotherapeutic combinations can positively contribute to tumor regression. With the excitement generated from the field, many investigators have been solving the obvious problems with current strategies. This leaves an opportunity for future scientists to divulge the mechanistic basis for how immunotherapy works, as well as how we can manipulate these mechanisms to better ensure success with immunotherapies for cancer patients.

As with most *in vivo* cancer studies in preclinical models, immune compromised animals are utilized to test human-derived cell lines and corresponding therapies. Both chapters in this dissertation relied on the NSG mouse to test the *in vivo* efficacy of the therapies. NSG mice are devoid of B and T cells, have non-functional NK cells, and have defective cytokine signaling. While this is not an ideal model, immune competent models are lacking for human T cell therapies due to GVHD. Mouse biology suggests that there is no exact match to human γδ T cells. Mouse γδ T cells behave more closely to DCs[86]; therefore, the next studies to be done to follow up on the use of γδ T cells in NB would be to create NB-patient derived xenografts. In this dissertation, we pursue an autologous donor γδ T cell product, but because γδ T cells do not result in GVHD, it would be interesting to compare an allogeneic/third party PBMC donor that had not been predisposed to chemotherapy. These studies are entirely feasible for follow up to our discovery and could expand the likelihood of creating a universal donor γδ T cells cell bank.

As mentioned, our goal for this dissertation using the TPO-CAR was to preclinically establish TPO, a ligand, as an antigen-binding domain for CAR therapy. However, we demonstrated the TPO-CAR was activated by both human and mouse tissue. Therefore, our studies could have been expanded to an immune competent leukemia mouse model. A limitation to our current TPO-CAR is that mouse T cells do not transduce well with a lentiviral vector, which would make studying the effect of immune cells on the TPO-CAR difficult. To overcome this, we attempted to make high-titer gamma retrovirus, but these efforts were

unsuccessful. In the future, generating a gamma retrovirus TPO-CAR vector to explore this immune competent mouse model would give insight into the immune response to the CAR.

As mentioned briefly above, to better understand the therapeutic impact of both of these therapies, it is essential to test against primary patient samples. In the NB model used, we anticipate the γδ T cells will be responsive *in vivo* to patient samples, as demonstrated by our *in vitro* data. Patients with high GD-2 surface expression would be ideal to establish xenografts from to test the combination of γδ T cells, dinutuximab, and TMZ. If the data suggests that the γδ T cells are successful when challenged with NB patient tumor samples, then there would be rationale to move this treatment forward into clinic. Additionally, the study of patient samples would progress the findings from chapter 3. We hypothesize that the TPO-CAR will be cytotoxic against MPL+ leukemia cells and MPL+ cancers. The study would take leftover patient samples, validate the MPL surface expression, and test *in vitro* the cytotoxic potential of the TPO-CAR. If the TPO-CAR successfully clears the leukemia cells, we would expand our studies to establish patient derived xenografts. It would be impactful to acquire samples from other cancer types and test the MPL surface and the ability of the patient's cancer cells to self-renew and form colonies *in vitro*. This data would support the hypothesis that the TPO-CAR would be effective in clearing cancer stem cells for a variety of cancers.

ACT relies on the quality and quantity of the cellular product. When considering an autologous γδ T cell product for NB patients, it highlights the need for a successful expansion to have a significant number of cells to result in a therapeutic benefit. This is a limitation in γδ T cell products because of their lack of persistence and memory phenotype. Therefore, we hypothesize that the cells will not expand *in vivo*. This would mean multiple injections of the γδ T cells would be necessary. Expanding a pediatric blood product may not allow for a sufficient number of cells. In addition, in CAR T cell therapies naïve (not triggered by antigen, more immature), stem-cell memory (TSCM, self-renewing central memory) or central memory (TCM, self-renewing memory cell where division can result in effector T cells, effector memory T cells, or central memory T cells), T cell subsets outperform effector (TE, result of antigen stimulation by naïve T

cell) and effector memory (TEM) T cells, despite TE and TEM having enhanced cytotoxic and cytokine-releasing potential of effector T cell subsets[232, 436-440]. Persistence and memory has been shown to be one of the major criteria for a successful CAR T cell therapy. However, these T cell subsets are also autologous in nature, but they have the potential to expand *in vivo*. Therefore, a major area of opportunity and development for cellular products is by finding a universal cell donor and source. For this reason, γδ T cells and NK cells are attractive because they do not elicit GVHD. Exploring the possibility of allogeneic donors with γδ T cells and NK cells for sufficient cell number or gene editing αβ T cells to not express HLA markers are two solutions for a sufficient, quality cellular product.

Chapter 2 of this dissertation highlights one of the major limitations in ACT, poor homing of T cells to the site of solid tumors. T cell homing and infiltration is still one of the major obstacles due to the competitive microenvironment that the T cells must traffic through. The studies presented highlight that 24 hours after injection, γδ T cells are not present in the tumor. While time points prior to this were not analyzed, we hypothesized the cells were clearing tumor cells and then dying because mice receiving γδ T cells showed longer survival and reduced tumor burden when compared to our controls. To improve this study, earlier time points would be collected and tested with multiple combinations of the drugs. Currently, research is being done to enhance T cell trafficking. For example, T cells are being engineered to express chemokine receptors to increase the number of T cells infiltrating the tumor including CXCR2, CCR2b, and CX3CR1[441]. The stroma of the tumor microenvironment in particular cancer associated fibroblasts favor a dense extracellular matrix through collagen secretion. This remodeling of the extracellular matrix network contribute to limited T cell trafficking. The cancer associated fibroblasts have been targeted using CXCR4 antagonists to block CXCL12 which is secreted by these fibroblasts[441]. This treatment is being tested in clinical trials currently to improve T cell infiltration (NCT01010880, NCT01359657, NCT01120457, and NCT02737072). Another method of tumors preventing T cell infiltration is through the lack of blood vessels penetrating the core of hypoxic tumors. Without a blood vessel system to reach the center of the tumor T cell infiltration is limited to the periphery and extravasation into the tumor mass. Anti-vascular endothelial

growth factor (VEGF) therapies have led to the normalization of the tumor vasculature resulting in greater T cell infiltration[441]. T cell infiltration has been associated with better prognoses in cancer patients which makes T cell trafficking and homing an important area for future development[364].

Our approach in chapter 3 to clearing the CAR using chemotherapy prior to transplanting bone marrow into mice was unconventional. While CARs have been powerful in clearing B cell leukemias, patients live without their B cells because there is currently no effective mechanism to turn the CAR off. Work has been done to create inducible CARs and CARs with suicide switches, but they are often not effective against 100% of the modified T cells. This is problematic because of memory T cells' ability to expand when reintroduced to the target antigen. Our solution to this problem, as well as reduce on-target off-tumor side effects, is to combine technology from chapters 2 and 3 of this dissertation.

Future approaches for the TPO-CAR would be to engineer γδ T cells to express the vector. TPO-CAR modified γδ T cells would be advantageous because γδ T cells do not have a memory phenotype. This would suggest that the γδ T cells would not persist to clear the bone marrow. Ideally, the modified T cells would preferentially engage cells with a cancer phenotype. γδ T cells' innate properties would also be beneficial in cases of leukemia where there is not 100% MPL+ leukemia. In addition to CAR targeting, the γδ T cells would be able to engage cancer cells through FAS-FASL interactions, NKG2D stress interactions, or secretion of proinflammatory cytokines. Additionally, modifying γδ T cells with a CAR directed towards neuroblastoma cells could enhance the trafficking and homing of the cells to the tumor. The future of pediatric immunotherapy lies in the creative strategies to engage the immune response through novel interactions and enhancing existing therapies through combination treatments to achieve maximum tumor clearance for long-term survival.

4.4 Conclusions

This dissertation investigated *in vitro*, *ex vivo*, and *in vivo* immunotherapeutic strategies for the treatment of pediatric cancer. The data in this dissertation demonstrate the successful use of γδ T cells in a NB model and a ligand-based TPO-CAR in leukemia and stem-cell models. This dissertation provides the relevant evidence for an autologous *ex vivo* expansion of γδ T cells from patient-derived PBMCs to elicit tumor regression when combined with frontline chemotherapy and monoclonal antibodies. While the data can be expanded into the development of a universal T cell product for ACT strategies, more work must be done to enhance the homing of the T cells to the site of the tumor. Additionally, this dissertation provided one of the few ligand-based CARs to successfully engage its target. The TPO-CAR effectively eliminated MPL+ leukemias in two models of AML. However, more discovery is necessary to manage the on-target off-tumor side effects. While both strategies were tested in a single cancer type, the results suggest optimization of protocols using γδ T cells and the TPO-CAR in pan-cancer modeling will result in favorable prognostic outcomes.

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