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T-CELL TREATMENTS FOR SOLID AND HEMATOLOGICAL TUMORS

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by

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A

DISSERTATION

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DOCTOR OF PHILOSOPHY

by

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DEDICATION

This dissertation is dedicated to one of my namesakes. He was a man I never met but have been told I would have liked, who is one of only two people in my family to have received a doctorate degree and said "much obliged" to those whom he encountered. To Robert James Caldwell, D.D.S. (Pictures enclosed)

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Many people have contributed to this work in the areas of experimental assistance, training, guidance, mentorship, cheerleading, praying, financial contributions, or combinations thereof. This list could never be totally complete, so please excuse me for any omissions.

First and foremost, I need to express my sincere appreciation for the mentorship provided by Dr. Laurence JN Cooper. He has funded, developed, supported, and fought for each of the ideas presented here and has made them into real therapies for patients with cancer. My gratitude to him is not measureable. I knew that I wanted to work for Dr. Cooper while still in my Master of Science degree program when I first heard about CARs and T cells killing tumors in my Translational Sciences class. Using the immune system to target cancer seemed like a logical and diplomatic strategy, and I was mesmerized by Dr. Cooper's enthusiasm to the field (as most people are, I believe). One rotation later, I joined the Cooper lab and have been growing T cells ever since. During my time in his lab, he taught me how to write grants, which has had its benefit in the recent past and will serve me throughout my career as a biomedical researcher, and I will remember our strategy sessions on how to translate our ideas into the clinic fondly. I can admit that the ROR1-specific T cell project was mired in roadblocks that made it seem impossible at times, but Dr. Cooper was determined to make it into a therapy for cancer patients and encouraged me to stick it out. Now it seems that these T cells will have broad applicability as cancer treatments. In the same light, I had a hair-brained idea to try and grow $\gamma\delta$ T cells, which was not my project at the time, and Dr. Cooper

was supportive of me pursuing this line of questioning. A few pilot experiments eventually resulted in two chapters of this dissertation. Last but certainly not least, he introduced me to my future boss Dr. Steven A. Rosenberg during his precious time visiting with him MD Anderson that resulted in accepting a post-doctoral fellowship at the NIH. For these things and many more – thanks!

I have also been blessed to have worked for two other mentors. First was Dr. Robert A. Davey, who was tasked with teaching me how to use the things I had learned in college to become a professional scientist. Simple practices were key to success: (i) making stock solutions to cut down on pipetting and have internal control of experiments, (ii) why moving tubes up and down on a rack allows you to remember what you've added, (iii) ask the right questions, (iv) the details matter, and (v) write everything down in detail because you will forget and you need to know. After leaving Dr. Davey's lab, I was privileged to earn my MS degree in Cancer Biology under the mentorship of Dr. Madeleine Duvic. Now I am the master of the Western blot. She asked me one day after I started my Ph.D. work in Dr. Laurence Cooper's lab, "You know what would be really great? Make one of those T cell treatments for my patients who have T cell lymphoma." That was the start of the γδ T cell project, which is a major focus of this dissertation. The idea was that γδ T cells could kill malignant αβ T cells. Although this never really happened *per se* as I had imagined it, it led to countless opportunities and may one day be a viable option for cancer treatments in lymphoma and leukemia, as well as non-hematological tumors. Ten years after first working at UTMB, I am now poised to graduate with a Doctor of Philosophy degree from the Cooper lab.

There are many people in Dr. Cooper's lab (both past and present) who have contributed to this work. Dr. Sourindra Maiti developed the NanoString assays and pushed using NanoString to detect Vδ and Vγ TCR usage, which was incredibly useful in these studies, and he also physically performed and assisted with a number of experiments. He was also instrumental in "thinking outside-the-box" about $\gamma\delta$ T cells, in particular. Dr. Kirsten Switzer and Tiejuan Mi helped design, implement, and supervise the mouse experiments, including tumor and T cell injections and bioluminescence imaging (BLI). Dr. Amer Najjar also helped design mouse experiments and performed dissections of mice with established ovarian cancer xenografts. Lenka Hurton developed a membrane-bound interleukin-15 (mIL15) fused to its receptor IL15Rα, and descriptions for introduced mIL15 herein are from that construct. She also helped process mouse tissues and with BLI. Denise Crossland and I discussed the projects at length and she fed my cells for me when I was not able to come to the lab. Hillary Gibbons Caruso edited papers and grants and also discussed the projects. Janani Krishnamurthy also conversed with me about the projects, especially at our Thursday afternoon informal writing sessions with other graduate students led by Dr. Dean A. Lee. Big thanks to David Rushworth, who drove back with me from Washington D.C. to Houston, TX in 26 hours to escape Super Storm Sandy so that he could sell his house and I could get married. Dr. Harjeet Singh was the main person to test the CD28 and 41BB chimeric antigen receptors (CARs) with CD19-specificity, which were later made into the ROR1-specific constructs. He also helped with flow cytometry, growing T cells, and many other tissue culture issues. Simon Olivares created the Cooper lab *Sleeping Beauty* transposition system (SBSO), of which many of the CARs, co-

stimulatory molecules, cytokines, antigens, etc., were cloned into and expressed on primary cells and established cell lines. Also, he was very helpful with cloning strategies and molecular biology techniques. Dr. Sonny Ang brought hypoxia to the Cooper lab, assisted early work with CARs targeting ovarian cancer, and cloned CD86 and CD137L for aAPC deconstruction experiments. Dr. Marie Forget and I talked about γδ T cells in melanoma and I hope to continue our collaboration while at the NCI Surgery Branch. Dr. Brian Rabinovich made lentiviral vectors that were used to make *ffLuc*-mKate virus-like particles for transduction of CAOV3 (ovarian cancer) and Kasumi-2 (B-cell ALL) cell lines. Radhika Thokala established the Kasumi-2-*ffLuc*-mKate cell line and helped with BLI. Dr. Pallavi Renkata-Manuri taught me how to do my first electroporation and was generous with early guidance on growing T cells. Dr. Hiroki Torikai helped me extensively with testing allogeneic reactivity, growing monocyte-derived dendritic cells, and experiments with umbilical cord blood. Dr. Bipulendu Jena created an anti-CD19-specific CAR idotypic antibody that was used for sorting experiments. He and Rineka Jackson constructed the Appendix M of the ROR1-specific T cell Phase I trial. Dr. Colleen O'Connor imparted that flicking a cell pellet was the best way to prime cells for resuspension, and she talked football with me during monotonous tissue culture periods – that is a major bonus for a Texas-born native. Margaret Dawson Johnston and Matthew Figliola processed almost all blood used in these studies and did uncountable other tasks around the lab to keep it running smoothly. Ling Zhang, Cuiping Dai, and Gary Ye were also vital for lab upkeep and providing lab-wide services. Dr. Kumar Pappanaicken helped with flow cytometry for ROR1 staining and will likely do pilot studies combining ROR1-specific T cells and γδ

T cells for pancreatic cancer treatments. I would also like to thank Linda Lopez, Barbara Liddle, Ruby Robinson, Tiffany Tran, Beverly Smith, and Cha Davis for their assistance. It is only appropriate to end with Helen Huls – the heart and soul of the Cooper lab. Without her we are a mess – literally at times. If you need to know how to do anything in regards to tissue culture, translating lab protocols for clinical trials, or how to manage a workforce, then she is your lady. Again, it would take too long to write down everything Helen has done for me in my professional development, but suffice to say that I would not be at this place without her. To very briefly summarize, the Cooper lab has been a great place to work.

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On a personal note, there are three people I need to mention from my childhood who have molded me into the man I am today and without whose influence I would not be writing this document today. When I was 14, I had a soccer coach, Stefan, who taught me exhaustion was not a time to quit – it was at that time that you pushed harder to get what you wanted. This is a crucial skill doing lab work and keeping a high level of concentration for hours on end with no food and endless distractions. The second person was my cardiologist, Dr. Chris Wyndham, who performed a largely experimental ablation surgery to cure me of supraventricular tachycardia. Without his help I would not have been able to do exercise or compete athletically – both of which are a huge part of my personality and my education. Moreover, the experience was the primary piquing of my interest in medicine. Third, Coach Monty was my high school football coach and he made me cry many times. He wouldn't allow me to be "just a kicker" for the team and made me play offensive line in practice with guys twice (and sometimes three) times my size. Two years later, I was an all-district left tackle at a major high school in Texas standing 5 foot, 10 inches tall and weighing 150 pounds. Natural size, strength, and ability will only take you so far. The rest is up to determination and taking risks – especially when you are up against something bigger and stronger than you. What better analogy for someone trying to cure cancer?

Last, but certainly not in the list of contributors, is my family. My parents have always been very supportive and loving. They will not understand much of this document, but that has never stopped them from asking with genuine interest. My

younger brother, Holt, is my best friend, and I am indebted to him for more than I can ever put down on paper. Thanks for keeping me sane. I am also blessed to have two older brothers, Will and Todd, who I would like to give special thanks for piquing my scientific curiosity at a young age and for hanging out with Holt and me when we really needed it, respectively. My loving step-father, Carl Sigrist, is a champion cook and a man of few words. Thanks for making my mother happy (and being her chef – hehe). Moreover, I am blessed to have loving "in-laws" who support and love me like one of their own. Cheers to Donald, Debbie, and Lindsey Dobbs. It is only appropriate to end with my amazing wife, Kristen Leigh Dobbs Deniger. I love you dearly and thank you immensely in supporting me in writing this document. Sorry for acting like a crazy person while preparing it. I am so glad the internet existed so that I could find you.

T-CELL TREATMENTS FOR SOLID AND HEMATOLOGICAL TUMORS

Publication No. ____________

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Supervisory Professor: Laurence Cooper, M.D., Ph.D.

Cell-based therapies have demonstrated potency and efficacy as cancer treatment modalities. T cells can be dichotomized by their T cell receptor (TCR) complexes where αβ T cells (95% of T cells) and γδ T cells (<5% of T cells) express α/β and γ/δ TCR heterodimers, respectively. γδ T cells have inherent anti-tumor immunity, but their use in the clinic is hampered by a lack of clinically-relevant expansion protocols. In contrast, αβ T cells do not have predictable anti-tumor immunity so they can be re-directed to specific molecules on the tumor surface through introduction of tumor-specific molecules such as chimeric antigen receptors (CARs) for reproducible tumor killing. CARs are constructed with the extracellular specificity of a monoclonal antibody to a tumor antigen, e.g. CD19 or receptor tyrosine kinase-like orphan receptor-1 (ROR1), fused to intracellular T cell signaling domains (CD3ζ, CD28, CD137). A comparative study was done between αβ T cells re-directed with ROR1-specific CARs signaling through CD3ζ and either CD28 (ROR1RCD28) or CD137 (ROR1RCD137) in the first specific aim of this dissertation. CAR^+ T cells proliferated to clinically significant numbers and $ROR1^+$ tumor cells were effectively targeted and killed by both ROR1-specific CAR^+ T cell populations, although

ROR1RCD137 were superior to ROR1RCD28 in clearance of leukemia xenografts *in vivo*. The second specific aim focused on generating bi-specific CD19-specific CAR^{+} γδ T cells with polyclonal TCRγδ repertoire on $CD19^{+}$ artificial antigen presenting cells (aAPC). Enhanced cytolysis of $CD19^{+}$ leukemia was observed by CAR^{+} γδ T cells compared to CAR^{neg} γδ T cells, and leukemia xenografts were significantly reduced compared to control mice *in vivo*. The third specific aim looked at the broad anti-tumor effects of polyclonal γδ T cells expanded on aAPC without CAR^{+} T cells, where Vδ1, Vδ2, and Vδ3 populations had naïve, effector memory, and central memory phenotypes and effector function strength in the following order: Vδ2>Vδ3>Vδ1. Polyclonal γδ T cells eliminated ovarian cancer xenografts *in vivo* and increased survival compared to control mice. Thus, translating these methodologies to clinical trials will provide cancer patients novel, safe, and effective options for their treatment.

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ABBREVIATIONS

aAPC: artificial Antigen Presenting Cell

αFR: α–Folate Receptor

αGalCer: α–galactosylceramide

APC: Antigen Presenting Cell

Ab: Antibody

ADCC: Antibody-dependent cell-mediated cytotoxicity

Ag: Antigen

ALL: Acute Lymphoblastic Leukemia

AML: Acute Myeloid Leukemia

ATCC: American Type Culture Collection

BCR: B-cell Receptor

BLI: Bioluminescence Imaging

CAIX: Carbonic anhydrase 9

CAR: Chimeric Antigen Receptor

CCL: CC Chemokine Ligands

CCR: CC Chemokine Receptors

CD: Cluster of Differentiation

CD19RCD28: CD19-specific CAR with CD28 and CD3ζ endodomains

CD19RCD137: CD19-specific CAR with CD137 and CD3ζ endodomains

CDR: Complementarity Determining Regions

CEA: Carcinoembryonic Antigen

cGMP: current Good Manufacturing Practices

CLA: Cutaneous Lymphocyte Antigen

CK1ε: Casein Kinase-1ε

CLL: Chronic Lymphocytic Leukemia

CML: Chronic Myeloid Leukemia

CMV: Cytomegalovirus

CRA: Chromium Release Assay

CREB: cAMP Response Element-Binding protein

CSF2R: Colony-Stimulating Factor 2 Receptor

CTLA4: Cytotoxic T-Lymphocyte Antigen 4

DC: Dendritic Cell

DTEA: Direct TCR Expression Array

eGFP: enhanced Green Fluorescent Protein

EGP-2: Epithelial Glycoprotein 2

EBV: Epstein-Barr Virus

EGFR: Epidermal Growth Factor Receptor

FACS: Fluorescence Activated Cell Sorting

FBP: Folate Binding Protein

FBS: Fetal Bovine Serum

FDA: Food and Drug Administration

ffLuc: *Firefly Luciferase*

FRA: oligonucleotides marking transposons signaling through CD137

GvHD: Graft-versus-Host Disease

HLA: Human Leukocyte Antigen

HIV: Human Immunodeficiency Virus

HSC: Hematopoietic Stem Cell

ICOS: Inducible T-cell Co-Stimulator

ICS: Intracellular Cytokine Staining

IFNγ: Interferon-γ

Ig: Immunoglobulin

IL: Interleukin

IL15/IL15Rα: fusion protein of IL15 to IL15Rα

IND: Investigational New Drug

IPP: Isopentenyl pyrophosphate

IRB: Institutional Review Board

L1-CAM: L1-Cell Adhesion Molecule

LAC: Leukocyte Activation Cocktail

LCA: Lymphocyte Code-set Array

LCL: Lymphoblastoid Cell Line

mAb: monoclonal antibody

MDACC: MD Anderson Cancer Center

MFI: Mean Fluorescence Intensity

MHC: Major Histocompatibility Complex

MICA/B: MHC Class-I Chain-related A/B

MIP1α: Macrophage Inflammatory Protein-1α

MIP1β: Macrophage Inflammatory Protein-1β mKate: red fluorescent protein

MRD: Minimal Residual Disease

NCI: National Cancer Institute

NHL: Non-Hodgkin's Leukemia

NIH: National Institutes of Health

NKT cells: Natural Killer T cells

NSG: Mice with the NOD.*scid*.$\gamma_c^{-/-}$ genotype

OvCa: Ovarian Cancer

ORF: Open Reading Frame

PaCa: Pancreatic Cancer

PBMC: Peripheral Blood Mononuclear Cells

PCR: Polymerase Chain Reaction

PD1: Programmed Death-1

PI3K: Phosphoinositide 3-Kinase

PKC: Protein Kinase C

PMA: Phorbol 12-Myristate 13-Acetate

polyA: polyadenylation tail for mRNA transcripts

pSBSO: *Sleeping Beauty* transposon plasmid

PSCA: Prostate Stem Cell Antigen

PSMA: Prostate-specific Membrane Antigen

RAC: Recombinant DNA Advisory Committee

RANTES: Regulated on Activation, Normal T cell Expressed and Secreted

ROR1: Receptor tyrosine kinase-like Orphan Receptor-1

ROR2: Receptor tyrosine kinase-like Orphan Receptor-2

ROR1RCD28: ROR1-specific CAR with CD28 and CD3ζ endodomains

ROR1RCD137: ROR1-specific CAR with CD137 and CD3ζ endodomains

RPMI: Roswell Park Memorial Institute medium

SB: *Sleeping Beauty*

scFv: single-chain variable fragment

SCID: Severe Combined Immunodeficiency

SIM: oligonucleotides marking transposons signaling through CD28

STAT: Signal Transducer and Activator of Transcription

TAG72: Tumor-associated Glycoprotein 72

T_C: Cytotoxic T cell

T_H: Helper T cell

T_H1: Type 1 $CD4^+$ Helper T cell

T_H2: Type 2 $CD4^+$ Helper T cell

T_H17: Type 17 $CD4^+$ Helper T cell

T_{REG}: Regulatory $CD4^+$ Helper T cell

T_{CM}: Central memory T cell

T_{EFF}: Effector T cell

T_{EM}: Effector memory T cell

T_{EMRA}: Effector memory RA T cell

T_M: Memory T cell

T_N: Naïve T cell

TAA: Tumor-associated antigen

TCR: T-cell Receptor

TGFβ: Transforming Growth Factor-β

TIL: Tumor-infiltrating Lymphocytes

TFNα: Tumor Necrosis Factor-α

UCB: Umbilical Cord Blood

UCSD: University of California at San Diego

UPenn: University of Pennsylvania

Vδ1: γδ T cell expressing TCRδ1 isoform

Vδ2: γδ T cell expressing TCRδ2 isoform

Vδ3: γδ T cell expressing TCRδ3 isoform

VEGFR2: Vascular Endothelial Growth Factor Receptor-2

WBC: White Blood Cell

ZFN: Zinc Finger Nuclease

Zol: Zoledronic acid (Zometa)

CHAPTER I

INTRODUCTION

I.A. Cancer

Cancer is caused by the uncontrolled and abnormal growth of cells that leads to disease and remains the second most common cause of death in the United States of America behind heart disease.(1) It is more prevalent in women than men where the median time at diagnosis is in their 60's and 70's, respectively.(2) Overall, the median age at diagnosis is 66 years old for all cancer types and more than 1.5 million people are estimated to have been diagnosed with cancer in 2012, according to the most current statistics from the National Cancer Institute (NCI) Surveillance Epidemiology and End Results (SEER; http://seer.cancer.gov/statistics). Of these diagnoses, >200,000 are represented from *each* of the three most common cancers: prostate, breast, and lung. The other groups of cancers, therefore, affect roughly 900,000 people per year in the United States, and some of the diagnoses carry dismal chances for survival. For example, roughly 22,000 women are expected to have a new diagnosis of ovarian cancer in 2013 where only 44% of them are expected to survive 5 years, and over 186,000 women are currently estimated to have a history of ovarian cancer in the United States. Similarly, greater than 48,000 new leukemia diagnoses, with 5-year overall survival rate of 56% are predicted for 2013, and more than 287,000 people in the United States have leukemia at present. Cancers can either arise from either (i) the hematopoietic compartment, i.e. bone marrow, blood, and lymphatic system, giving rise to hematological tumors or (ii) tissues outside of the hematological systems that are generically termed solid tumors. Despite the many treatments that exist for cancer, novel therapies are desperately needed to decrease the mortality and morbidity of this disease.

I.A.1. Hematological Tumors

Hematological cancers are delineated by their hematopoietic differentiation status and the tissue from which the tumor arises. In regards to leukemia, the different types are separated first by either myeloid or lymphoid lineages and then into acute or chronic stages. Thus, they are classified as (i) acute myeloid leukemia (AML), (ii) chronic myeloid leukemia (CML), (iii) acute lymphoblastic leukemia (ALL), or (iv) chronic lymphocytic leukemia.(3) Immunotherapy targeting tumor associated antigens (TAA), e.g. CD19 or Receptor tyrosine kinase-like orphan receptor-1 (ROR1), have potential to lead to tumor regressions and, in some cases when targeting CD19, complete responses have been observed in the clinic.(4-7) The main focus of this dissertation is on developing immunotherapies for the lymphoid subsets of leukemia.

I.A.1.a. B-cell Acute Lymphoblastic Leukemia

The most common pediatric malignancy known is ALL but also affects many adults.(8-10) The median age at ALL diagnosis in 2012 was estimated to be 13 years old.(2) For B-cell ALL (B-ALL), tumors typically arise from the pro-B cell stage and retain primitive characteristics without undergoing further differentiation.(11) A common subtype of B-ALL halted in normal B cell development is t(1;19) ALL, where the translocation results in an E2A-PBX1 fusion protein that functions in promoting developmental arrest and oncogenic transformation simultaneously.(12) Therapies are being actively sought after for treatment of this B-ALL group by targeting unique or dysregulated proteins resulting from aberrant E2A-PBX1 gene regulation.(13) Cytogenetics and flow cytometric staining of the tumor cell surface molecules are two key tools in the diagnosis of B-ALL, which has clinical presentation consistent of common ailments, i.e. fever, bleeding, pain, fatigue, and lethargy, but is commonly first detected due to high white blood cell counts (WBC).(14, 15) Aggressive treatment, including chemotherapy, radiation therapy, and hematopoietic stem cell transplantation (HSCT), has dramatically improved overall survival, but long-term health problems frequently arise following therapy particularly amongst children.(16, 17) More specifically, children in remission commonly develop secondary malignancies later in life, and most commonly develop AML.(18) Unfortunately, few effective treatments exist for AML. Incomplete eradication of the primary tumor can result in minimal residual disease (MRD) of the primary tumor and is also a common cause of malignancies later in life that are usually resistant to conventional therapies.(16, 19) Thus, it is of paramount importance that safe and effective therapies are developed for B-ALL patients in order to fully remove their primary tumor, reduce risk the for development of secondary tumors, and improve their expected quality of life as adults.

I.A.1.b. T-cell Acute Lymphoblastic Leukemia

T-cell ALL (T-ALL) accounts for less than 25% of ALL cases and has a dismal prognosis relative to B-ALL.(20) The differentiation stage of T-ALL has importance as more immature T cells are correlated to more aggressive disease.(14, 21) Diagnosis and treatment are, in general, similar to those for B-ALL, although one unique and common clinical manifestation of T-ALL is a large mediastinal mass causing shortness of breath.(20, 22) Prognostic indicators for T-ALL response to therapy are widely sought after but are not yet predictive of response. However, particular emphasis on NOTCH mutations and chromosomal translocations has generated much enthusiasm for being able to stratify patients into potential responders and non-responders.(23, 24) As with B-ALL, MRD is a primary concern as it contributes to relapse in many cases and can be diagnosed by amplification of specific TCR alleles.(25) Currently, no adoptive T cell therapies directly targeting their neoplastic T cell counterparts exist for T-ALL. Therefore, development of T cells capable of fratricide may improve the outcomes for T-ALL patients in dire need of therapeutic intervention.

I.A.1.c. Chronic Lymphocytic Leukemia

In contrast to ALL, CLL occurs much later in life and is not as aggressive as ALL.(26) CLL often arises from activated or memory B cells and progresses slowly but is deadly nonetheless with a 5-year median survival.(27) Furthermore, a CLL profile with (i) alterations in chromosomes 11 or 17, (ii) unmutated immunoglobulin heavy chain (IgV_H) genes, (iii) expression of zeta-chain associated protein kinase-70 (ZAP70), (iv) expression of CD38, (v) rapid doubling time of tumor lymphocytes, or (vi) increased serum β_2-microglobulin, soluble CD23, and thymidine kinase activity have been correlated with a more aggressive disease status and markedly decreased median survival.(28) CLL is generally asymptomatic and high WBC commonly results in early diagnosis that is later corroborated with cytogenetics and flow cytometry. Most current therapies are not curative and often require palliative care, but some strategies, e.g. chemotherapy, antibody therapy, and stem cell transplant, can extend survival up to multiple years.(29) T cell immunotherapy is an actively pursued therapy for CLL due to the many targetable TAA, e.g. CD19, CD20, CD23, CD52, and CD40, and monoclonal antibody therapies directed at these TAA have resulted in objective clinical responses in CLL treatments.(30) Furthermore, mAbs can be also adapted to T cell therapies in the form of chimeric antigen receptors (CARs) by linking a single chain antibody specific for the TAA to T cell intracellular activation domains.(31) Indeed, several clinical trials with CAR-based T cell therapies targeting CD19 have generated complete responses in both B-ALL and B-CLL (discussed further in **Chapter I.D.3.**).(4-7, 32) Because CLL can be sensitive to immunotherapy, it is a prime disease target for T cell treatments.

I.A.2. Solid Tumors

There are many different types of solid tumors but this dissertation will focus on generating T cell therapies for two model cancers with hopes of future applications to other solid tumors. Ovarian and pancreatic cancers were chosen because of (i) their poor prognostic outcome, (ii) lack of efficacious T cell immunotherapies, and (iii) favorable responses targeting these tumors in initial pre-clinical tests.

I.A.2.a. Ovarian Cancer

Ovarian cancer (OvCa) is commonly referred to as “the most common gynecological malignancy.”(33) The median age at diagnosis is 63 years old, and most patients are diagnosed in late stage (III or IV) which has a 5-year overall survival rate of 27%.(34-36) OvCa typically arises from the ovary, fallopian tube, or peritoneal cavity, and is unique in that traditional metastasis is not common outside of the intraperitoneal cavity.(37) Growth within the intraperitoneal cavity can grossly impact the ability of surrounding organs to function properly and, in some case, can be sites for local metastases. The most useful prognostic indicator for OvCa is CA125, also known as mucin 16 (MUC16), which is shed into the bloodstream and is predictive of progressive OvCa disease status.(38) Standard of care for women facing OvCa treatment is surgical resection and aggressive chemotherapy.(39, 40) Many immunotherapy approaches have been tried with few objective clinical responses.(41-44) Even though OvCa appears to have sensitivity to immunomodulation, a cell-based therapy that results in objective clinical responses has yet to be developed. As the survival rate is dismal for advanced OvCa, novel therapies are urgently needed to combat this disease.

I.A.2.b. Pancreatic Cancer

Pancreatic cancer (PaCa) is one of the worst cancer diagnoses because 1-year and 5-year overall survival rates are 20% and 5%, respectively.(45) It is commonly differentiated based on the anatomical location of the tumor where the tail, neck, and head of the pancreas are distinct locations and the pancreatic head is the most common site where tumors arise.(46) Similar to many of the cancer types discussed above, common health ailments, i.e. pain, weight loss, and appetite-related problems, are used in diagnosis, and patients are usually asymptomatic until metastases have already developed thereby limiting the ability of surgery to cure PaCa.(47) Diabetes is also a common diagnostic tool and is one of many risk factors, in addition to smoking, pancreatitis, genetic predisposition, and nutritional status.(46) Tumor resection dramatically improves outcome, but most cases involve metastases (liver and lymph nodes commonly) that are very difficult to control and treat with standard care.(48) Radiation and Gemcitabine is the standard of care for PaCa but elicits limited efficacy outside of palliative care.(49) Combinational approaches with other chemotherapies were also tested in clinical trials with some promising results but were not curative.(50) Perhaps the most promising results that have been generated are with vaccines (peptide, tumor lysate, or dendritic cells (DCs)) to boost resident immune responses to PaCa.(51, 52) Clinical data support that PaCa is sensitive to T cell responses and suggests that direct adoptive transfer of PaCa-reactive T cells could result in robust clinical responses.

I.B. Tumor Associated Antigens

The choice of which tumor associated antigen (TAA) to target is crucial for the success of the immunotherapy.(53, 54) The ideal TAA is not expressed on any normal tissues but highly expressed on the tumor cell surface. Most TAAs known thus far are cell surface glycoproteins that are involved in tumor growth or survival, e.g. growth factor receptors, that drive proliferation of the tumorigenic cells. Furthermore, optimal TAAs are often required for the growth of tumor cells meaning the cancer is dependent on the TAA, and removal or inhibition of the TAA or elimination of cells expressing the dependent TAA can lead to effective treatment. Dependence on the TAA is sought after in order to avoid antigen escape of tumor cells, i.e. no longer expressing the targeted TAA but continuing to proliferate, which can lead to relapse and disease progression.(55) Ideally, the TAA would exist on multiple tumor types to allow for targeting of many cancers with a single therapy. With these considerations in mind these studies focus on two TAAs, CD19 and ROR1, which have great promise as targets for cellular immunotherapy.

I.B.1. CD19

CD19 is a B-cell lineage-specific protein not expressed on other tissues and is, therefore, an ideal TAA for B-cell malignancies because B cells are not required for survival.(4, 6, 31, 56, 57) Similar to T cells, B cells have a B cell receptor (BCR) expressed on the cellular surface specific for a single cognate Ag.(58) Upon BCR/Ag binding, the B cell will proliferate and produce antibodies with specificity identical to that of the BCR that are secreted into the circulation for opsonization and pathogen clearance.(59) The BCR complex is crucial for signal transduction, and is composed of CD19, CD21, and CD81, where CD19 is crucial for intracellular signaling.(60-62) CD19 is expressed from the early pro-B cell stage until memory stage and is lost as B cells differentiate into plasma cells. Because of its importance in B-cell function and persistence throughout B cell development, almost all (95%) of B-cell non-Hodgkin's leukemia (NHL) express CD19.(31) Successful removal of $CD19^{+}$ tumors results in B-cell aplasia, which can be treated with serum immunoglobulin infusions to restore humoral immunity.(4, 6, 32, 56, 63) Thus, targeting CD19 has proven to be safe and effective means for eliminating B-cell neoplasms, albeit with diminished quality of life.

I.B.2 Receptor Tyrosine Kinase-like Orphan Receptor-1

In contrast to CD19, much less is known about ROR1, but what is known is that ROR1 (i) is a cell surface protein involved in Wnt5a signal transduction, (ii) plays a critical role in development, (iii) is no longer expressed post-parturition and is not found on almost all adult tissues, and (iv) has aberrant expression later in life on tumor cells making it a candidate TAA target.(64-67) ROR1 and its redundant partner in development, ROR2, were originally cloned and named neurotrophic tyrosine kinase receptor-related-1 and -2 (NTRKR1 and NTRKR2), respectively.(68) An analysis of the ROR1 protein structure reveals that it consists of signal peptide trailed by extracellular Ig-like C2 domain, Frizzled cysteine-rich domain (Fz-CRD), and Kringle domain that are followed by transmembrane (TM) alpha helix, intracellular protein kinase, serine/threonine-rich domain, and proline-rich domains (**Figure 1a**). Sequence alignment shows that ROR1 is 57% identical and 81% homologous to ROR2 where there is homology in signal peptide (62%), Ig-like C2 (85%), Fz-CRD (93%), Kringle (90%), TM (95%), protein kinase domain (90%), serine/threonine-rich (87%), and proline-rich (54%) domains between the two proteins (**Figure 1b**). Single and double knockout mice for ROR1 and ROR2 were established that had multiple developmental problems leading to death shortly after birth.(69, 70) More specifically, $ROR1^{-/-}$ mice died of respiratory distress following birth, while $ROR2^{-/-}$ mice died of more advanced cardiovascular problems as well as skeletal abnormalities, and $ROR1^{-/-}ROR2^{-/-}$ double knockout mice had exacerbated disease including transposition of the great arteries, pubic bone dysplasia, and sternal defects. Furthermore, ROR2 continues to be critical for skeletal development during life as autosomal recessive diseases resulting in bone dysmorphia and have been mapped to ROR2 gene mutations (chromosome 9q22) but not ROR1 gene (chromosome 1p32-31).(71-74) To date, ROR1 has not been linked to inherited genetic disease in adults, indicating that its major roles are only in fetal development. In 2008, three independent investigators published reports of ROR1 expression in tumors, and each described ROR1 expression in ~95% of CLL patients with confirmation of absent expression on most normal tissues.(65, 75, 76) Subsequently, ROR1 has been detected in breast cancer, pancreatic cancer, ovarian cancer, melanoma, gastric carcinoma, non-small cell lung cancer, t(1;19) B-ALL, and mantle cell lymphoma, but some reports indicate that cytosolic expression of ROR1 exists in some tissues and that there may be surface expression on hematogones (normal B cell developmental precursors), the pancreas, and adipose tissue.(13, 66, 67, 77-81)

The discovery of ROR1 expression on tumor cell lines enabled a number of biochemical studies to determine the role of ROR1 in neoplastic transformation. IL6 leads to transcriptional activation of signal transducer and activator of transcription-3 (STAT3) that then increases gene expression of ROR1 transcripts, which may give insight to a potential autocrine or paracrine loop for oncogenic transformation and/or disease progression.(82) Wnt5a binding of ROR1 (presumably to the Fz-CRD) leads to casein kinase-1ε (CK1ε) activation of phosphoinositol-3 kinase (PI3K) that phosphorylates Akt and results in activation of the transcriptional activator cAMP-response-element-binding protein (CREB), which upregulates genes important for proliferation and, thus, is likely to result in oncogenic transformation (**Figure 1c**).(67, 79) The discovery of ROR1 on tumor cells is relatively new, so other signaling pathways have not been elucidated and direct targeting of ROR1 in humans has not been tested to date. Nonetheless, all indications suggest that ROR1 is an ideal TAA target for cellular immunotherapy with broad applicability, and immunotherapies targeting ROR1 in humans will be the ultimate test of its safety as a TAA.

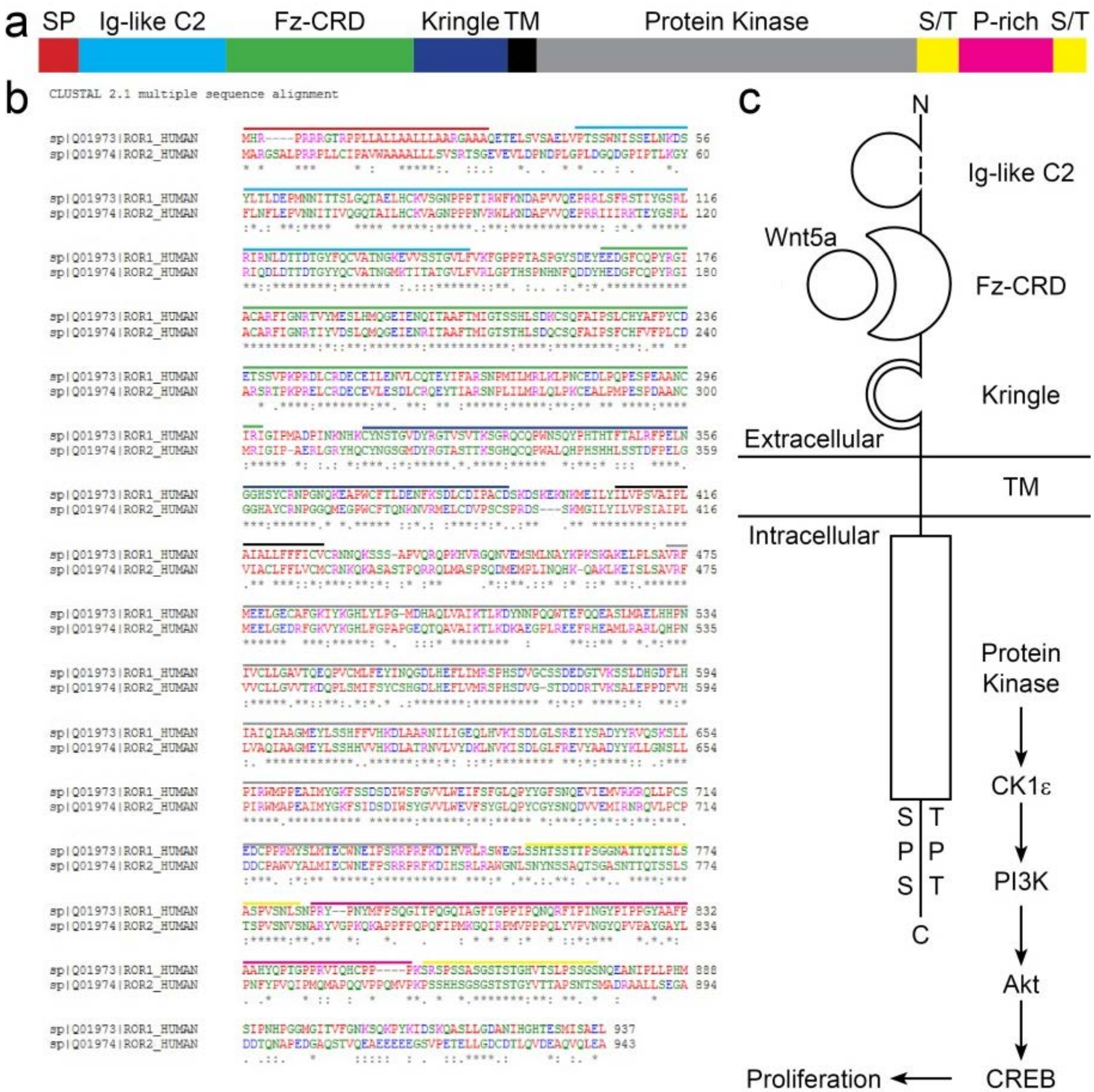

Figure 1. ROR1 Protein Structure. (**a**) Diagram of protein sequence of ROR1 protein domains where abbreviations are as follows: SP; signal peptide, Fz-CRD; Frizzled cysteine-rich domain, TM; transmembrane alpha helix, S/T; serine/threonine-rich domain, P-rich; proline-rich domain. (**b**) Sequence alignment between ROR1 and ROR2 proteins by ClustalOmega (http://www.ebi.ac.uk/Tools/msa/clustalo/). Lines above text correspond to colors in (**a**), (*) describes identical amino acids, (:) denotes analogous closely related amino acids, and (.) describes similar amino acids. (**c**) Diagram for ROR1 protein structure in the cellular membrane where Wnt5a binding Fz-CRD leads to the following signal transduction pathway: casein kinase-1ε (CK1ε) → phosphoinositol-3 kinase (PI3K) → Akt → cAMP-response-element-binding protein (CREB) → transcriptional activation of genes for proliferation.

I.C. T cell Immunity

The immune system is critical for pathogen clearance and prevention of disease. It is broadly partitioned into innate and adaptive immune systems, but interplay between innate and adaptive immunity is essential to an effective immune response.(83-86) The innate immune system is composed of many cell types, e.g. macrophages, natural killer (NK) cells, that have broad ranges of specificity to pathogens to remove them upon their primary encounter, and therefore serve as the first line of defense.(87) In contrast, the adaptive immune system is highly specific for a particular part of a pathogen and develops as a secondary and long-lasting response to a individual pathogen. The two major sections of adaptive immunity are the cellular and humoral immune systems.(88) B cells mediate humoral immunity primarily through the production of antibodies (Ab), which coat the surface of pathogens to label them as foreign for direct lysis through complement activation, which forms holes in the membrane thereby destroying the target cells, or by phagocytosis and elimination during the process known as opsonization.(89) In contrast, T cells mediate cellular immunity through direct contact with their target and either directly or indirectly mediate destruction of the pathogenic cell. T cells are typically dichotomized into helper (T_H) or cytotoxic/killer (T_C) T cells based on their expression of CD4 and CD8, respectively.(90) The combined interaction of these components of the adaptive immune system allow for its unique characteristics of (i) generating highly specific responses to pathogens, (ii) memory formation for more rapid and stronger responses to pathogens upon a repeated or secondary exposure, and (iii) adaptation to increase sensitivity through maturation.(88) Because T cells can exert direct cellular cytotoxicity and create memory responses, they have been used successfully to target and kill cancer cells.

I.C.1. αβ T cells

The quintessential T cell lineage is the αβ T cell subset, which comprises up to 95-99% of circulating T cells, and are the object of most canonical T cell paradigms.(58) In addition to staining for either CD4 or CD8, these T cells are typically identified by co-staining with CD3 and their αβ T-cell receptor (TCRαβ). Effector functions are endowed upon αβ T cells through an extensive educational process that results in a unique specificity to an antigen and a corresponding response in the form of T cell help (CD4) or cytolysis (CD8). Therefore, it is important to understand the nuances of αβ T cell development and education in order to maximize their impact in adoptive immunotherapy.

I.C.1.a. T-cell Receptor Genetics

TCRs are subjected to genetic rearrangement events during development to randomly arrange distinct gene segments into an extremely high number of combinations and thus corresponding antigen affinities.(91) Four TCR loci, i.e. TCRα, TCRβ, TCRγ, and TCRδ exist in the human genome, which lead to two distinct T cell lineages based on TCR pairing.(92) More specifically, the αβ T cell lineage is defined by the pairing of TCRα and TCRβ chains whereas the γδ T cell lineage is defined by T cells expressing

TCRγ and TCRδ heterodimers. Each TCR allele is further compartmentalized into variable (V), diversity (D), junction (J), and constant (C) regions.(93) TCRα and TCRγ genes have V and J regions while TCRβ and TCRδ genes have V, D, and J regions and all TCRs contain C regions (**Figure 2**). Each specific region is termed based on its region and origin, i.e. Vα describes the variable region from the alpha locus or Jδ describes the junction region from the delta locus. The V regions contain complementarity determining regions (CDR) that confer high degrees of antigen specificity, and are therefore important for defining T cell affinity.(94) These V, D (where applicable), J, and C segments are recombined into unique combinations in each T cell during T cell development in a process known as V(D)J recombination.(95, 96) The TCRγ (Gene ID: 6965) and TCRβ (Gene ID: 6957) loci are in distinct locations at 7p14 and 7q34, respectively, but TCRδ locus (Gene ID: 6964) exists within the TCRα (Gene ID: 6955) locus at 14q11.2 (**Figure 2**). Upon V(D)J recombination of the Vα and Jα, the entire δ-chain locus is deleted from the T cell genome in a T-cell receptor excision circle (TREC).(97) Thus, once the α-chain locus has recombined for a particular T cell, it can no longer become a γδ T cell. Programmed mutation of the T cell germline DNA allows for unbiased generation of many TCR specificities for extremely high combinational probabilities (at least 10^{16} possible combinations for αβ T cells) for binding any potential foreign pathogen.(98) It is in this random genetic process through which T cells acquire exquisite abilities to mediate cellular immunity.

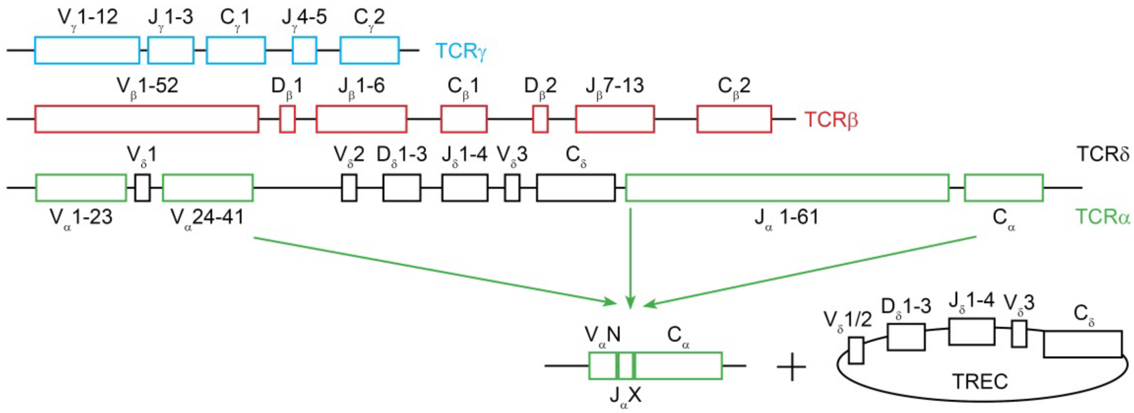

Figure 2. Genetic Loci for TCR alleles. Simplified schematic of exons encoding V, J, and C regions with D regions for β and δ chains for TCRγ (blue), TCRβ (red), TCRα (green), and TCRδ (black). V(D)J recombination of Vα, Jα, and Cα results in excision of the TCRδ locus in a T cell Receptor Excision Circle (TREC).

I.C.1.b. αβ T cell Development

The thymus is crucial for T cell development as it is the location for both V(D)J recombination and thymic selection. Thymic selection is important for maintaining central tolerance by eliminating poorly-reactive T cells and over-reactive T cells from the T cell pool by neglect and negative selection, respectively, following V(D)J recombination.(99, 100) Positive selection only allows for T cells with intermediate reactivity to their antigen to be released into the periphery.(101, 102) Thymic selection is carried out by thymic cortical epithelial cells which express high levels of major histocompatibility complex (MHC) molecules along with a wide array of proteins, including self-antigens, that are then processed and presented in the context of MHC on the epithelial cell surface.(103, 104) Both MHC Class-I (MHC-I) and Class-II (MHC-II) are expressed by the thymic cortical epithelial cells to stimulate CD8 and CD4 T cells, respectively. The developing T cells express both CD4 and CD8 in the thymus, and based on their TCRαβ binding affinity to either MHC-I or MHC-II and subsequent TCRαβ signaling they will become single positive for either CD8 or CD4, respectively.(105, 106) In this way, both affinity and peripheral T cell function is acquired in the thymic cortex.

I.C.1.c. αβ T cell Activation

T cells need to escape the thymus, encounter their corresponding antigen, and have a licensing event towards the antigen in order to become functionally responsive. At least two signals are required for T cell activation but 3 total signals are ideal for full T cell activation.(107-109) Signal 1 comes from TCRαβ interaction with MHC/peptide complexes mediated by CD4 or CD8 co-receptors.(110) However, the intracellular domain of TCR is very short and not able to generate its own intracellular signal. Signaling comes from CD3 molecules that are bound to TCR in the transmembrane through non-covalent interactions.(111) A complex of CD3 subunits surrounds the TCR composed of CD3γ/CD3ε and CD3δ/CD3ε heterodimers and CD3ζ/CD3ζ homodimer. Each of the CD3γ, CD3δ, and CD3ε subunits has an immunoreceptor tyrosine activation domain (ITAM) motif and the CD3ζ subunit has three ITAM motifs for a total of ten ITAMs surrounding each TCR. Upon TCRαβ binding to peptide/MHC complex, co-receptors (CD4 or CD8) bind to the constant regions of MHC and begin the signaling process through Lck and Fyn phosphorylation of tyrosine (p-Tyr) residues on the ITAMs.(112) Then ZAP70 can bind to p-Tyr through SH2 domains and becomes activated by Lck. Activated ZAP70 leads to a cascade of downstream activation events resulting in transcriptional and post-translational modifications for the molecules responsible for T cell proliferation and differentiation.(113) However, only receiving signal 1 will lead to functional unresponsiveness otherwise known as anergy.(114, 115) Therefore, the second signal is required and is termed co-stimulation. Examples of activating co-stimulatory molecules expressed on the T cell surface are CD27, CD28, and CD137 (41BB), which bind to CD70, CD80/CD86, and CD137L (41BB-L), respectively, expressed on the antigen presenting cell (APC).(116-118) Some co-stimulatory molecules are inhibitory, e.g. cytotoxic T-lymphocyte antigen-4 (CTLA4) and programmed death-1 (PD1), for immune regulatory purposes.(119) Dendritic cells (DCs) are professional APCs because of their ability to process and present a wide milieu of peptides, high expression of MHC molecules, and expression of co-stimulatory molecules.(120) DCs are present in tissues and following activation by the innate immune system to foreign antigens/pathogens, they migrate to secondary lymphoid organs to present their environmental data and license T cells to fight the pathogens.(121, 122) It is also important to note that cytokine support, e.g. interleukin-12 (IL12), IL15, and type I interferon (IFN), is generally regarded as signal 3 for T cell activation.(123) In summary, the combination of (i) TCRαβ engagement with MHC/peptide complex with appropriate co-receptor (CD4 or CD8) binding to MHC, (ii) co-stimulation, and (iii) cytokine support licenses T cells to find their corresponding antigen expressed on damaged or pathogenic cells and to eliminate those cells.

I.C.1.d. $CD4^+$ $\alpha\beta$ T cell Subsets

$CD4^+$ T cell subsets are numerous and typically described by the effector cytokines they release, and they can be stratified into T_H0 (naïve), T_H1, T_H2, T_H17, regulatory T cells (T_{REG}), and natural killer T (NKT) cells.(124) Naïve T_H0 cells can be polarized to differentiate based on environmental cues that then translate into distinct transcriptional programs and result in lineage commitment.(125) T_H1 encourage inflammation and help promote CD8 memory responses by producing IL2, IL12, interferon-γ (IFNγ), and tumor necrosis factor-α (TNFα) while T_H2 cells inhibit inflammatory T_C response and foster humoral immunity by secreting IL4, IL5, IL6, and IL10.(126) The primary role of T_H17 cells is to enhance neutrophil responses, and these cells are most often characterized by their ability to produce IL17.(127) There is plasticity between T_H17 cells and T_{REG} cells as both require transforming growth factor-β (TGFβ) but addition of IL6 polarizes towards T_H17 lineage. T_{REG} cells are infrequent and can exert strong blockades against other T cell effector functions through both cell-to-cell contact mechanisms and through production of IL10 and TGFβ.(128) Thus, they are critical for maintaining peripheral tolerance, and when dysregulated can contribute to diseases such as cancer (in the case of overactive T_{REGS}) or autoimmune disorders (in the case of underactive T_{REGS}). An extremely rare subset of $CD4^+$ T cells are NKT cells, which express invariant TCRαβ alleles, e.g. Vα24/Jα18 with Vβ11, and are known to produce both T_H1 and T_H2 cytokines.(129) The best described antigen for NKT cells is α-galactosylceramide (αGalCer) presented to NKT cells in the context of CD1d, a non-classical MHC molecule, which leads to NKT expansion and effector function, but the "natural" ligands for NKT in humans are not fully known to date.(130) Some NKT cells express CD8 instead of CD4 and others express neither co-receptor, but their roles are less well known. In aggregate, $CD4^+$ T cells are an important arm of the cellular immune response and can generate a wide range of effects towards eliminating pathogens.

I.C.1.e. $CD8^+$ αβ T cell Subsets

In contrast to $CD4^+$ T cell subsets, $CD8^+$ T cells subsets are usually defined in terms of their memory response from previous encounters with antigens.(131) As mediators of direct cellular cytotoxicity, $CD8^+$ T cell memory responses are commonly studied in the context of pathogenic infection or in the context of long-lived tumor-reactive T cells.(132-134) After antigen exposure, naïve T cells (T_N) proliferate rapidly and exert cytotoxicity as effector T cells (T_{EFF}). The large numbers of antigen-specific T cells then needs to be reduced as to not increase the total peripheral T cell pool each time a pathogen elicits a response, so there is a contraction phase marked by T_{EFF} sensitivity to extrinsic apoptosis. However, the numbers of antigen-specific cells surviving the contraction phase are greater than the initial antigen-specific T cell pool so that exposure to the same pathogen will result in a faster and stronger attack on the pathogen. These remaining cells are termed memory T cells (**Figure 3**). Three memory T cell subsets have been described and are called central memory (T_{CM}), effector memory (T_{EM}), and effector memory RA (T_{EMRA}) T cells.(135) T_N express CD45RA, CD27, CD28, and CCR7 where CD45RA expression is lost on both T_{CM} and T_{EM} but is re-expressed on T_{EMRA} without CD27, CD28, and CCR7. The T_{EM} and T_{CM} groups can be distinguished by CD28 and CCR7 where the former expresses neither and the latter expresses both. T_{CM} cells have the greatest proliferative capacity with limited effector functions and serve as long-lasting antigen-specific pools. In contrast, T_{EM} have immediate effector functions, limited replicative capacity relative to T_{CM}, and serve as the main memory cytotoxicity mediators.(136) Lastly, T_{EMRA} cells are terminally differentiated cells that have effector functions without much proliferative capacity. Even though $CD4^+$ T cells are not typically stratified in this manner, memory populations have been detected that could produce cytokines following subsequent antigen exposure.(137, 138) Furthermore, $CD4^+$ T cells are necessary for generating $CD8^+$ T cell memory, suggesting that even though they may not fit into clear subsets they are present and required for memory cytotoxicity.(139) The application of these groupings to cancer immunotherapy also comes with caveats due to the high degree of differences in their disease pathologies, i.e. virus versus cancer. CD27 expressed on T_N, T_{CM}, and T_{EM} was correlated with greatest responses in cancer immunotherapy, and can be used to predict therapeutic efficacy.(134) While immediate effector function towards cancer in adoptive T cell immunotherapies is desired, it appears that T_N and T_{CM} cells are better for this particular task.(131) Generation of persistent $CD8^+$ populations with memory to the tumor, therefore, is an important consideration for immunotherapy efficacy.

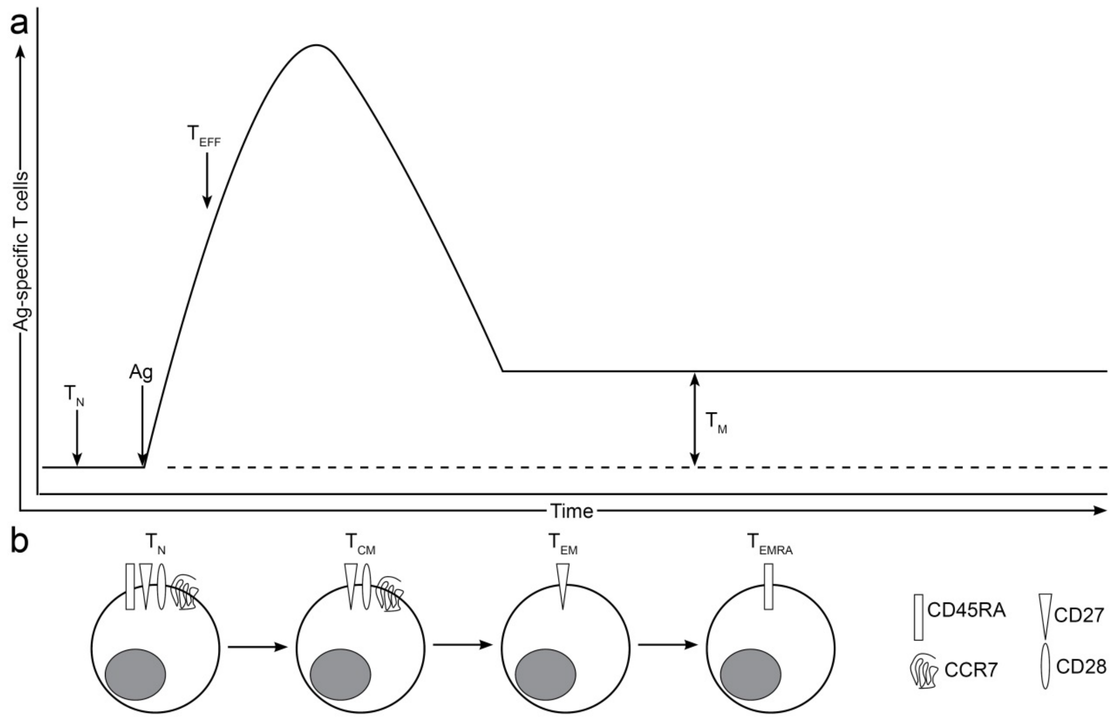

Figure 3. CD8$^+$ Memory T cell Subsets. (**a**) Limited quantities of antigen-specific naïve T cell (T_N) pool exist prior to exposure to antigen (Ag). Upon Ag contact, massive Ag-specific T cell proliferation occurs in the effector T cell (T_{EFF}), which is followed by apoptotic contraction phase. Memory T cells (T_M) are developed from the increase in Ag-specific T cell population relative to the T_N starting population. (**b**) Prior to Ag exposure T_N cells express CD45RA, CD27, CD28, and CCR7 where CD45RA expression is lost in the formation of T_{CM} and both CD28 and CCR7 are lost with T_{EM} cells. Terminally differentiated T_{EMRA} cells lose CD27 expression and express CR45RA again.

I.C.2. γδ T cells

γδ T cells are a completely separate T cell lineage from αβ T cells, and γδ T cells have both innate and adaptive immune cell functions.(140) In contrast to αβ T cells, γδ T cells have predictable inherent anti-tumor immunity mediated directly through their TCR.(141) However, γδ T cells comprise only 1 – 5% of the circulating T cell repertoire, making them difficult to work with because of a relative lack of robust protocols for polyclonal γδ T cell expansion and their infrequent quantities in peripheral blood.(142, 143) They are identified by co-expression of $CD3^{+}TCR\gamma\delta^{+}$ where expression of CD4 or CD8 is rare, and can be stratified into Vδ1, Vδ2, and Vδ3 subsets based on TCRγδ alleles.(144) Targets of γδ T cells include tumor cells, viruses, bacteria, mycobacteria, and cell stress-associated proteins.(145, 146) Therefore, γδ T cells are a promising T cell immunotherapy option despite their limited frequencies in blood if they can be expanded.

I.C.2.a. Unique Characteristics of γδ T cells

There are three variable TCRδ chains and 14 variable TCRγ chains expressed in humans, and fewer unique TCRγδ combinations are observed in γδ T cells compared to the immense combinational diversity seen with αβ T cells following V(D)J recombination.(92, 144) Expression of TCRγδ heterodimers on the T cell surface in the thymus inhibits recombination of β-chain locus during the $CD4^{neg}CD8^{neg}$ stage thereby committing the T cell to the γδ T cell lineage.(147) This double negative status is often maintained after exit from the thymus, most likely because TCRγδ recognizes antigens outside of MHC-restriction in many cases, making co-receptor expression dispensable for function and endowing them with an ability to recognize antigens outside of the signaling constraints imposed by classical thymic selection.(148) However, the thymus is not required for all γδ T cell development, as many of these γδ T cells take up residence in peripheral tissues and exhibit immediate effector functions against pathogens.(149) Resident γδ T cells can be found in the mucosa, tongue, vagina, intestine, lung, liver, and skin and can comprise up to 50% of the T cell populations in intestinal epithelial lymphocytes (IEL).(144, 150) In contrast, circulating γδ T cells can be found in the blood and lymphoid organs, and are canonically dominated by γδ T cells expressing Vδ2 TCR isotype (called Vδ2 cells) with few γδ T cells expressing the Vδ1 TCR isotype (called Vδ1 cells) that are more frequently associated with resident γδ T cells.(146) Moreover, Vδ2 cells most commonly pair with Vγ9, but Vδ1 and Vδ3 have broad γ-chain pairing potential.(141, 146) Therefore, the location of γδ T cells can lead to their subset diversity and effector functions that can be mediated through specific combinations of γ and δ TCR chains to recognize pathogens upon encounter in their resident or circulating locations.

I.C.2.b. Vδ1 γδ T cells

Vδ1 cells have a wide range of effector functions and are located in a variety of anatomical locations.(151) They can, theoretically, pair with any of the TCRγ chains, and there are a variety of known ligands for Vδ1 cells.(140) In fact, the crystal structure of Vγ1Vδ1 has been solved in combination with one of its antigens, MHC Class-I chain-related A (MICA).(152, 153) Cellular stress and/or viral infection result in MICA and its analog, MICB, to become expressed on the stressed/infected cell's surface, so MICA/B is commonly present on tumor cell surface.(154) MICA is also recognized by NKG2D, a receptor expressed by γδ T cells, NK cells, and, less frequently, αβ T cells.(155) Other non-classical MHC molecules and cell stress proteins are also recognized by γδ T cells. For instance, Vγ4Vδ1 T cells have been shown to have specificity towards heat shock proteins and the non-classical MHC molecule CD1d.(156) Heat shock proteins are commonly over-expressed in tumor cells to handle their high protein translation loads.(157) The CD1d molecule is best described in its ability to expand NKT αβ T cells, but γδ T cells have also been described to have direct NKT-like functions, enhance NKT αβ T cells reactivity to αGalCer, and have even been shown to have specificity to cardiolipin with CD1d.(158-160) Also, murine Vγ5Vδ1 cells are well described in their ability to serve as dendritic epidermal T cells (DETCs) with APC function.(161-163) Lastly, correlative studies have implicated Vδ1 T cells to have immunity towards cytomegalovirus (CMV) and human immunodeficiency virus (HIV).(164, 165) In aggregate, Vδ1 cells have immunity towards microbial pathogens, have antigen presenting capabilities, and can target proteins expressed on the tumor surface.

I.C.2.c. Vδ2 γδ T cells

The most extensively studied subset of γδ T cells is the Vδ2 lineage, which similar to Vδ1 cells, recognize microbial pathogens, serve as APCs, and target cell-stress proteins expressed on tumor cells.(141, 166) Bacterial alkylamines and *Listeria monocytogenes* are recognized by Vδ2 cells when paired with Vγ2.(167-169) In contrast to Vδ1, a strong preference towards Vδ2 heterodimerizing with Vγ9 has been well documented. Vγ9Vδ2 cells have been shown to react to phospho-antigens (isopentenyl pyrophosphate; IPP), F_1-ATPase expressed on the cell surface, and *Mycobacterium tuberculosis*.(170-172) Furthermore, Vγ9Vδ2 cells are reactive to cells treated with aminobisphosphonates, e.g. Zoledronic Acid (Zol), which is the only current means of propagating γδ T cells *ex vivo* in the clinic.(173, 174) Aminobisphosphonates inhibit cholesterol synthesis and build up intermediates in the mevalonate-CoA pathway, including IPP, which is a ligand for Vγ9Vδ2.(175) This process was serendipitously discovered when patients with bone disorders who were treated with aminobisphosphonates to resume bone growth experienced large *in vivo* expansions of Vγ9Vδ2 T cells, and aminobisphosphonates methods were subsequently translated into laboratory practice to expand Vγ9Vδ2 cells *ex vivo*.(176) Thus, Vδ2 cells are the *only* γδ T cells that have been used for adoptive T cell therapy. Utility of the Vδ1 and Vδ3 lineages is appealing, but there are no current means to rapidly expand them to clinically-significant numbers and the existing polyclonal γδ T cell population is too few in number for direct infusion. Nonetheless, numerous clinical trials treating cancer patients with (i) infusions of Zol for *in vivo* Vγ9Vδ2 expansions and/or (ii) infusions of *ex vivo* expanded Vγ9Vδ2 cells have generated objective clinical responses but complete responses have been unpredictable and have not always been directly correlated to the Vγ9Vδ2 cells.(177-182) Thus, the extensive work studying Vδ2 cells has generated much interest in using γδ T cells for adoptive immunotherapy.

I.C.2.d. Vδ3 γδ T cells

In contrast to Vδ1 and Vδ2 cells, very little is known about γδ T cells expressing Vδ3 TCR alleles (called Vδ3 cells). The limited quantities in peripheral blood and lack of commercially available reagents for Vδ3 inhibit attempts to study this subset. Vδ3 cells are indirectly correlated with CMV and HIV immune responses, but nothing is known about their anti-tumor immunity.(165, 183) Developing a means with which to study this lineage could have important scientific and clinical significance.

I.D. Chimeric Antigen Receptors

Chimeric Antigen Receptors (CARs) re-direct T cells to antigens independent of their endogenous TCR specificity.(184, 185) These recombinant molecules contain in order from N-terminus to C-terminus: (i) a single chain variable fragment (scFv) derived from a monoclonal antibody with specificity to a TAA, (ii) an extracellular stalk, (iii) a transmembrane domain, and (iv) T-cell signaling endodomains (**Figure 4**). Binding of the scFv to its corresponding TAA leads to T cell activation resulting in proliferation, cytokine release, and cytotoxicity.(186) Thus, CAR^{+} T cells are re-directed to TAA outside of their thymically-selected affinities.

I.D.1. CAR Generations

Successive modifications to the design of CARs have improved their ability to re-direct T cells to TAAs.(187) CAR technology was invented by Dr. Zelig Eshhar (Weizmann Institute of Science, Rehovot, Israel) in 1989, and the original CAR differed from the more modern CARs by (i) having only CD3ζ and (ii) TCR constant domain scaffold.(188) Second generation CARs have shown the most efficacy in re-directing T cells and are superior to first generation CARs by adding in a co-stimulatory endodomain, e.g. CD28 or CD137 (41BB), to supplement CD3ζ signaling strength present in both generations (**Figure 4**).(189-193) Third generation CARs, therefore, contain three endodomains, and the most common combination has been CD28, CD137, and CD3ζ.(194-196) The order of endodomains does appear to have importance in the ability to stimulate the T cell in both second and third generation CARs, where CD3ζ works best at a position most distal to the membrane.(192, 197) The scaffold sequence used has the most difference between investigators where IgG4 constant regions (used in this dissertation), CD8α, no stalk, and flexible spacers have been used successfully.(13, 32, 192, 193, 198, 199) Although there exist some differences between groups in their CAR-modified T cell products in tumor killing, CARs in general have been shown as a consistent and effective means to target desired antigens and change the T cell response outside of their endogenous specificity.

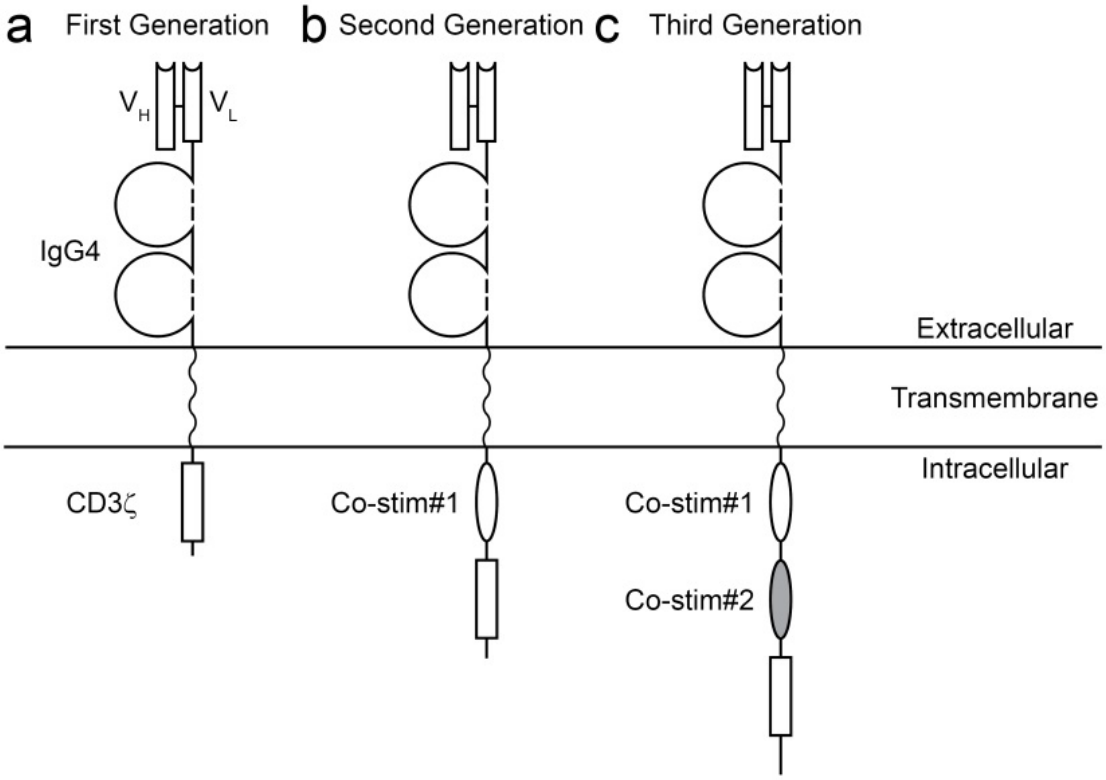

Figure 4. Schematic Representation of CARs. (**a**) First generation CARs were constructed with single chain variable fragments (scFv) composed of heavy (V_H) and light (V_L) variable fragments from monoclonal antibodies specific for TAA, followed by IgG4 constant region (CH2 and CH3 domains displayed), a transmembrane α-helix, and CD3ζ signaling endodomain. (**b**) Second generation CARs added a co-stimulatory domain, e.g. CD28 or CD137, between CD3ζ and transmembrane domain. (**c**) Third generation CARs use two co-stimulatory domains upstream of CD3ζ.

I.D.2. Tumor-associated Antigens Targeted with CARs

Effective targeting of different TAAs using CAR-modified T cells has generated enthusiasm around CAR-based immunotherapies. B-cell malignancies have been targeted with CARs specific for ROR1, κ-light chain, CD19, CD20, CD22, CD23, and CD30, which are all confined to the hematopoietic compartment and are not expressed on solid tissues.(57, 77, 200-208) Moreover, CD30 is also expressed on T cells, making CD30-specific CAR^+ T cells candidates for T-ALL therapy, but no T-ALL-specific CARs have been generated to date. Only one report of CARs targeting CML has been made thus far but the actual TAA was not examined.(209) CARs specific for CD33 and CD123 have been generated to target AML, but may have off-target effects due to the importance of CD33 and CD123 in hematopoiesis and viral immunity because of their expression on plasmacytoid dendritic cells that are critical producers of type-I interferons needed for viral clearance.(210-214) OvCa has been the target of multiple CARs including those specific for mesothelin, α-Folate Receptor (αFR), and folate-binding protein (FBP).(42, 215-219) Renal cell carcinoma has been targeted through the carbonic anhydrase IX (CAIX), which has minimal expression in normal tissues and is increased in hypoxia.(220-222) Carcinoembryonic antigen (CEA) is a developmental antigen absent on normal tissue and up-regulated in malignant cells, and CARs targeting CEA have been developed for pancreatic and colorectal cancers.(223, 224) Similarly, the oncofetal antigens h5T4 and ROR1 (discussed in **Chapter I.B.2**) are only expressed during development and CARs specific for these antigens can target multiple tumor types.(77, 199, 223) The differences between published ROR1-specific CAR T cells and the ones developed in this dissertation are discussed in detail in **Chapter II**.

Both CAR and mAb immunotherapies have had much success targeting human epidermal growth factor receptor-2 (EGFR2, HER2, or ERBB2), which is expressed highly in many cancers.(194, 225-228) However, there is low-level expression of HER2 on normal tissues, which caused an "on-target/off-target" toxicity in the only trial to date testing CAR^+ T cells specific for this TAA on breast cancer, thereby limiting its application.(229) Other EGFR members have been targeted with CARs, including EGFRvIII, which is uniquely expressed on glioblastoma.(230-232) Even glycoproteins (Lewis-Y antigen) can be targeted by CARs, and Lewis-Y antigens are typically studied in the context of EGFR family members.(233) The ganglioside GD2 and L1-cell adhesion molecule (L1-CAM) are common expressed on neuroblastoma, melanoma, and sarcoma (GD2 only), and CARs targeting these TAA were shown to control neuroblastoma growth.(234-239) In addition to GD2 and L1-CAM, high molecular weight melanoma-associated protein was used as a target for melanoma.(240) Melanoma is highly responsive to immunotherapy, and complete responses have been generated from a single infusion of tumor infiltrating lymphocytes (TILs).(241, 242) Prostate cancer has two specific antigens with limited expression outside of the prostate, prostate stem cell antigen (PSCA) and prostate-specific membrane antigen (PSMA), which were both targeted with CARs.(243-245) MUC1 was also another CAR target for both prostate and breast cancers.(246, 247) Other ubiquitous tumor markers, e.g. tumor associated glycoprotein-72 (TAG72) and epithelial glycoprotein-2 (EGP-2) have been targeted by CARs for multiple cancer therapies.(248, 249) Angiogenesis is even the target of a CAR via specificity for Vascular Endothelial Growth Factor Receptor 2 (VEGFR2), which is crucial for introducing new blood vessels into the tumors.(250,

251) However, there are major concerns of long-term persistence of these VEGFR2-specific cells in terms of regular vasculature growth. Lastly, receptors expressed on tumors can be targeted by "zetakines," which function like CARs but replace the scFv of the CAR with the ligand for a receptor of interest. For example, IL13-Receptor-α-2 (IL13Rα2) was targeted by an IL13 fused to T cell signaling domains to target glioblastoma multiforme and neuroblastoma.(252-255) As outlined, many tumor antigens have been targeted by CARs, highlighting the enthusiasm given to this immunotherapy.

I.D.3. Clinical Trials with CAR$^+$ T cells

Many of the CARs described above have been translated into T cell immunotherapies for cancer patients and have resulted in promising objective clinical responses.(200, 241, 242, 256, 257) The majority of the trials have been focused on CARs developed from the FMC63 mAb specific for CD19.(186, 258, 259) CD19-specific CAR$^+$ T cells have eliminated tumor from patients resulting in B cell aplasia, a litmus test for long-lived clinical responses.(4-7, 260) It was in this model that second generation CARs proved to have superior anti-leukemia effects compared to first generation CARs. Furthermore, long-lived persistence of CAR$^+$ T cells has been achieved by rendering them bi-specific to TAA and Epstein-Barr virus (EBV)-specific antigens through skewing TCR repertoire in *ex vivo* co-cultures with EBV-transformed lymphoblastoid cell lines (LCL).(206, 211, 212, 236, 237) The most striking clinical responses, including maintained complete responses, have been achieved with second generation

CD19-specific CAR$^+$ T cells signaling through CD137 and CD3ζ.(4, 7, 32) The exact reason why these cells out-performed other CARs signaling through CD28 and CD3ζ is unknown at present, and pre-clinical models have not shown many differences between CD28 and CD137 CARs.(5, 6) This is an active area of investigation and **Chapter II** focuses on this question directly with ROR1-specific CARs that are in the approval stages for a Phase I clinical trial. The focus of all Phase I clinical trials, of which most CAR trials have been, is safety and establishing a maximum tolerated dose. Unfortunately, there have been 2 deaths on CAR$^+$ T cell clinical trials. The first death followed administration of CD19-specific T cells to an elderly patient, who later died of complications not thought to be directly linked to the immunotherapy.(261) In contrast, the second death was directly attributed to the CAR$^+$ T cells. In this study, a third generation CAR (CD28, CD137, and CD3ζ) specific for HER2 (based on the monoclonal antibody trastuzumab) was used to treat breast cancer, and following infusion of 10^{10} T cells, the patient died of cytokine storm in response to basal levels of HER2 on the lungs.(229) This tragedy has heightened the safety concerns around CAR$^+$ T cell immunotherapy, and TAA choice, CAR design, and T cell dose are being closely monitored in current and future trials.(262) Nonetheless, clinical trials are currently accruing with CAR$^+$ T cells targeting HER2 for sarcoma (NCT00902044), glioblastoma multiforme (NCT01109095), and multiple cancer (NCT00889954) treatments (http://www.clinicaltrials.gov/). Clinical trials with CAR-modified T cells specific for αFR were not effective at treating advanced ovarian cancer, and the lack of efficacy was attributed to lack of persistence of T cells *in vivo*.(42) Other trials targeting solid tumors with TAA, e.g. GD2, L1-CAM, CAIX, and IL13Rα2, which are similar to

HER2 expression in that there is some expression on normal tissues, have been safe and sometimes effective at reducing tumor burden.(186, 220, 221, 235-237) Therefore, the safety and efficacy of a particular CAR$^+$ T cell clinical trial may vary from investigator to investigator due to nuance in a number of variables surrounding propagation and CAR design and/or from variability between individual patients.

I.E. *Ex Vivo* Propagation of T cells

Many platforms exist for the propagation of T cells *ex vivo*, and this dissertation focuses on the use of *Sleeping Beauty* (SB) transposition for gene transfer into T cells followed by propagation on artificial antigen presenting cells (aAPC). This non-viral system for propagating T cells can be contrasted to viral-mediated gene transfer in that the latter requires previous expansion, e.g. with agonistic antibodies or stimulating beads, in order to transduce cells with the transgene of interest and the former does not require previous expansion but rather propagates the T cells *ex vivo* following gene transfer. The SB/aAPC strategy has been translated into the clinic, and modification of the current SB/aAPC will be used to streamline translation of therapies developed in this thesis to the clinic.

I.E.1. *Sleeping Beauty* Transposition-mediated Gene Transfer

Non-viral gene transfer with SB transposition establishes stable transgene expression in human cells.(263, 264) SB genes are originally derived from fish that were undergoing active transposition in their evolutionary maturation and were adapted for transposition into human cells.(265) In short, a DNA transposon with flanking inverted repeats and direct repeats is ligated into the human genome at TA dinucleotide repeats by the SB transposase enzyme.(266) TA dinucleotide repeats are randomly distributed in the human genome, yielding potential for random integration into the genome and has shown to be safe in regards to transgene insertion in pre-clinical studies.(267-269) This is of particular importance in gene therapy as inappropriate integration at gene start sites or promoters, within exons, or even distal to genes within enhancers or repressors can cause cellular transformation. Lentiviruses and γ-retroviruses have higher efficiency in transgene delivery than SB, but these vectors are known to integrate near genes or within genes.(186) Moreover, this was a particular problem in gene therapy trials treating X-linked severe combined immunodeficiency syndrome (X-SCID) where roughly half of the patients receiving transduced cells later developed leukemia as a result of integration near the *LMO2* gene.(270, 271) In contrast, no preference towards a particular chromosome or gene "hotspot" has been detected with SB.(267) Application of SB to human T cells has worked as a two DNA plasmid system, where one plasmid contains the SB transposon with the transgene of interest, e.g. CAR, and the other plasmid encodes the SB transposase.(272) Electro-transfer of the DNA plasmids by Amaxa nucleofection into quiescent peripheral blood mononuclear cells (PBMC) results in transient expression of SB transposase that then ligates the CAR transposon into the genome. As soon as the SB transposase mRNA is degraded translation of SB transposase protein is halted, thereby limiting the chances of additional transposition events. CAR expression can be encouraged through the co-culture of T cells on aAPC that express cognate antigen for the CAR.(273) aAPC serve as feeder cells, and recursive stimulations with γ-irradiated aAPC promote CAR-specific growth. Typically, after 30 days of co-culture >90% of cells will express CAR (**Figure 5**). Thus, SB transposition is an efficient gene transfer modality in T cells and modified T cells can be expanded *ex vivo* by aAPC co-culture.

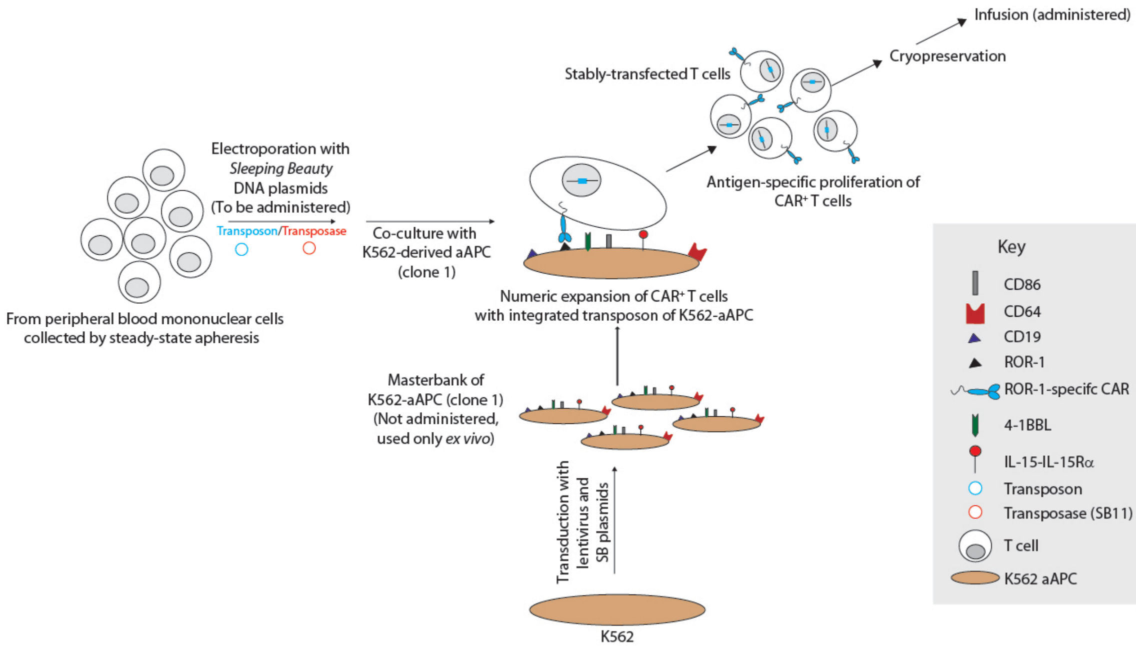

Figure 5. Schematic of CAR$^+$ T cells Expansion on aAPC. PBMC are isolated by Ficoll-Hypaque or steady state apheresis and are electroporated with plasmids encoding either (i) *Sleeping Beauty* transposase or (ii) *Sleeping Beauty* transposon containing CAR. Transient expression of CAR is observed the following day, and recursive stimulations with K562-derived aAPC are performed weekly with exogenous IL2 and/or IL-21. Pictured here are the clone#1 aAPC that expresses CD19, ROR1, CD64, CD86, CD137L, and IL15/IL15Rα. Following a month of co-culture on aAPC, stable CAR expression is achieved and clinically-relevant numbers of CAR$^+$ T cells are ready for cryopreservation and then infusion into cancer patients.

I.E.2 Artificial Antigen Presenting Cells

CARs stimulate T cells independent of their TCR specificity, and a primary aim of this propagation schema is to stimulate the CAR without affecting TCR repertoire by avoiding TCR/MHC interactions. Classical dendritic cells, thought of as "professional" APC, are infrequent in peripheral blood, laborious to manipulate, have limited replicative ability, and would need to be generated in the autologous setting for each immunotherapy patient. For these reasons, an alternative means for CAR-specific proliferation was sought after with the goal of serving as a global "off-the-shelf" bank of aAPCs to stimulate T cells independent of their MHC typing.

I.E.2.a Unique Features of K562 for Antigen Presentation

K562 has become an efficient aAPC line because it (i) lacks most MHC Class-I molecules, (ii) can be genetically modified easily, and (iii) proliferates robustly for easy cell banking and scale-up purposes.(273-276) The lack of MHC Class-I molecules (no A or B but limited C) on the K562 surface is advantageous because CD8-specific allogeneic reactivity is minimized or could be tailored to certain HLA restriction for TCR-specific responses.(277, 278) Expansion of T cells on aAPC has shown that polyclonal TCR repertoire is readily achieved, suggesting that the aAPCs do not skew endogenous TCR-response to a particular affinity or antigen.(263) Another important characteristic of using K562-derived aAPC is their susceptibility to further gene modification by either non-viral or viral mediated gene transfer. For instance, a master aAPC cell bank was modified with both IL15 fusion protein to IL15 receptor-α

(IL15/IL15Rα) and ROR1 antigen for memory formation and propagation of ROR1-specific T cells, respectively (**Chapter II**). Also, HLA-Cw3 was detected on K562 cells, so Cw3 was efficiently removed with zinc-finger nucleases to create HLA$^{-/-}$ K562 cells (Torikai H, Cooper LJN, and Lee DA, unpublished observations) in order to generate new aAPC completely devoid of HLA Class-I molecules. Thus, working cell banks can be easily re-tooled to ask biological questions regarding aAPC mechanics and/or maximize therapeutic cell output. Given the apparently unlimited proliferative capacity of K562 cells and their genetically modified counterparts, optimization of stimulations can be done easily and changed at will with options to use high ratios of aAPC to T cells. Furthermore, γ-irradiation of aAPC prior to co-culture with T cells is well tolerated by K562 in acute phases but eventually subjects the aAPC to death (typically 3 days) thereby eliminating most risk for unintended transfer of this tumor cell line into patients.(273) Therefore, K562 cells are an ideal source for antigen presentation and T cell stimulation.

I.E.2.b. Established aAPC Cell Banks and Clinical Trials with aAPC

As of now, four clinical trials have used K562-derived aAPC as T cell and NK cell expansion platforms at MD Anderson (NCT01653717, NCT01619761, NCT00968760, NCT01497184). Clone#4 aAPC generated at University of Pennslyvania (UPenn) was used successfully to expand CD19-specific CAR^{+} T cells in both autologous and allogeneic settings.(57, 263, 272, 273, 279-281) The surface phenotype of clone#4 is characterized by expression of: (i) CD19, (ii) CD32 (as an endogenous protein), (iii)

CD64, (iv) CD86, (v) CD137L, and enhanced green fluorescence protein (eGFP; surrogate marker for IL15 expression). Similarly, clone#9 aAPC was also generated at UPenn and has a surface phenotype of: (i) CD19, (ii) CD32 (as an endogenous protein), (iii) CD64, (iv) CD86, and (v) CD137L. Clone#9 aAPC was further modified to express membrane-bound IL21 for trials propagating NK cells.(275) Translation of expansion protocols into the clinic was readily achieved and validated this approach. Patients treated with aAPC-expanded lymphocytes did not show toxicity, suggesting that this is a safe approach (Cooper LJN, unpublished observations). Thus, aAPC will be used for the propagation of T cells in this dissertation for direct clinical application.

I.F. Dissertation Specific Aims

This dissertation has three major specific aims, which attempt to solve the gaps in the above knowledge and/or application of immunotherapy. More specifically, these aims are directed at either harnessing the inherent anti-tumor immunity of T cells for cancer therapy, modifying T cells with natural anti-tumor capacity with CARs for enhanced specificity, or re-directing T cells with unpredictable anti-tumor immunity to cancer through CAR expression. This multivariate approach has resulted in approval of one Phase I clinical trial and holds the potential to result in other clinical trials for treatment of both solid and hematological tumors.

<u>I.F.1 Specific Aim#1: To evaluate whether ROR1-specific T cells can target ROR1^{+} tumor cells while sparing normal tissues.</u> The *hypothesis* of this specific aim is that

ROR1-specific CARs will re-direct the specificity of T cells to target $ROR1^+$ malignancies and that CARs signaling through CD137 will be superior to CD28 in therapeutic efficacy. The *rationale* for this specific aim is that (i) ROR1 is a candidate TAA because it is expressed on a number of tumors but is not on most normal tissues, (ii) the 4A5 monoclonal antibody specific for ROR1 can be adapted to generate a CAR, (iii) CARs can re-direct T cells to TAA and empower them to kill TAA^+ malignancies, and (iv) cancer patients treated with CAR^+ T cells have achieved complete responses. *Sub-Aim 1.1. To generate ROR1-specific* CAR^+ *T cells.* Sequences from 4A5 antibody hybridoma will be constructed into second generation ROR1-specific CARs signaling through (i) CD28 and CD3ζ (ROR1RCD28) or (ii) CD137 and CD3ζ (ROR1RCD137), which will be part of SB transposons for stable CAR expression in T cells. CAR^+ T cells will be propagated on γ-irradiated $ROR1^+$ aAPC (clone#1), and CAR^+ T-cell numeric expansion will be monitored by inferred cell counts and flow cytometry for 28 days. *Sub-Aim 1.2. To phenotype ROR1-specific* CAR^+ *T cells.* Extended phenotyping for memory and homing markers will be performed by flow cytometry at the end of the co-culture period. Genotyping will also be performed with nCounter gene expression platform for TCR isotype expression and lymphocyte-associated genes. *Sub-Aim 1.3. To assess whether* CAR^+ *T cell function is specific for ROR1.* Cytokine production and 4-hour chromium release assay (CRA) will be used to evaluate CAR^+ specificity in responding to $ROR1^+$ targets with $ROR1^{neg}$ targets as negative controls. $ROR1^+$ leukemia xenografts will be established in immunocompromised mice which will be treated with CAR^+ T cells to evaluate tumor clearance *in vivo*.

I.F.2. Specific Aim#2: To assess whether a CD19-specific CAR expressed on γδ T cells will render them bi-specific to tumors through their TCR and CAR. The *hypothesis* of this specific aim is that enforced CAR expression on γδ T cells would stimulate them independent of their TCRγδ, thus leading to expansion of γδ T cells with polyclonal TCRγδ repertoire, and would amplify the anti-tumor effects from TCRγδ towards TAA^+ malignancies through the CAR. The *rationale* for this specific aim is that (i) γδ T cells have inherent anti-tumor immunity through a number of combinations of TCRγ and TCRδ pairings, (ii) the use of γδ T cells in the clinic is currently restricted to Vγ9Vδ2 even though other γδ T cell lineages have anti-tumor reactivity, (iii) CARs stimulate T cells independent of their TCR, (iv) electroporation of SB transposons containing the CAR can be achieved in quiescent PBMC with a polyclonal repertoire of γδ T cells, and (v) CD19-specific CAR transposon plasmids and $CD19^+$ aAPC are currently in clinical trials at MD Anderson and these reagents can be used to quickly translate findings from this specific aim into clinical trials. *Sub-Aim 2.1. To propagate CAR^+ γδ T cells on aAPC.* The second generation CD19-specific CAR (CD19RCD28) currently in clinical trials is available as highly pure DNA and will be used for gene transfer into quiescent PBMC from which CAR^+ γδ T cells will be propagated on $CD19^+$ aAPC. CAR expression and inferred cell counts will be used to evaluate CAR^+ γδ T cell numeric expansion. *Sub-Aim 2.2. To phenotype CAR^+ γδ T cells.* After a month of expansion on aAPC, CAR^+ γδ T cell surface phenotypes will be evaluated for T cell and memory molecules by flow cytometry and TCRγδ allele expression will be assessed by nCounter gene expression analysis. *Sub-Aim 2.3. To determine the ability of CAR^+ γδ T cells to* *functionally respond to tumors.* Cytokine production and 4-hour CRA assays will be tested against $CD19^{+}$ tumor targets with $CD19^{neg}$ targets serving as negative controls. Autologous CAR^{neg} γδ T cells will be used to compare CAR-specific responses to $CD19^{+}$ tumors. $CD19^{+}$ leukemia xenografts will be established in immunocompromised mice which will be treated with CAR^{+} γδ T cells to evaluate anti-tumor effects *in vivo*.

I.F.3. Specific Aim#3: To evaluate the inherent anti-tumor activity of aAPC-expanded γδ T cells against solid and hematological cancers. The *hypothesis* of this specific aim is that aAPC will expand polyclonal γδ T cells that will have broad anti-tumor immunity. The *rationale* for this specific aim is that (i) CAR^{neg} polyclonal γδ T cells proliferated in parallel to CAR^{+} γδ T cells described in specific aim#2 on aAPC, (ii) no current expansion protocols exist for polyclonal γδ T cells for the clinic, (iii) aAPC are currently in clinical trials and are available as a master cell bank in the manufacturing facility at MD Anderson, (iv) γδ T cells expressing Vδ1 are correlated with long-term remissions in cancer therapy but have not been directly infused as an adoptive immunotherapy, (v) γδ T cells expressing Vδ2 have shown anti-tumor effects as direct adoptive immunotherapies, (vi) γδ T cells expressing Vδ3 have not been described to have direct anti-tumor immunity leaving a gap in the field of knowledge, and (vii) a polyclonal approach to γδ T cell immunotherapy could target multiple ligands on the tumor through a diverse repertoire of TCRγδ. *Sub-Aim 3.1. To propagate γδ T cells on aAPC.* PBMC or UCB will be sorted for γδ T cells, and then co-cultured with aAPC used in clinical trials at MD Anderson. Flow cytometry and inferred cell counts will be used to evaluate proliferation of γδ T cells. Subsets of γδ T cells will also be sorted and expanded as co-cultures with clinical aAPC to assess differences in γδ T cell lineages. *Sub-Aim 3.2. To phenotype γδ T cells expanded on aAPC.* After one month of co-culture on aAPC, the surfaces of polyclonal or sorted γδ T cells will be evaluated for T cell and memory markers by flow cytometry and TCR allele expression will be assessed on nCounter gene expression platform. *Sub-Aim 3.3. To examine the range of killing capabilities by aAPC-expanded γδ T cells.* Polyclonal or sorted γδ T cells will be evaluated for their ability to produce cytokines in response to TCR stimulation or co-culture with tumor cells derived from solid and hematological cancers. Standard 4-hour CRA will be used to assess acute cytolysis and long-term co-cultures will evaluate durable killing abilities. Neutralizing antibodies will be employed to determine the specificity of killing. OvCa xenografts will be established in immunocompromised mice which will be treated with polyclonal γδ T cells to test their tumor clearance *in vivo*.

CHAPTER II

Clinical Implications for ROR1-specific T cells

II.A. Hypothesis and Rationale

The *hypothesis* of this chapter is that ROR1-specific CARs will re-direct the specificity of T cells to target $ROR1^+$ malignancies and that CARs signaling through CD137 will be superior to those signaling through CD28 in therapeutic efficacy. The *rationale* for this chapter is that (i) ROR1 is a candidate TAA because it is expressed on a number of tumors but not on most normal tissues, (ii) the 4A5 monoclonal antibody specific for ROR1 can be adapted to generate a CAR, (iii) CARs can re-direct T cells to TAA and empower them to kill TAA^+ malignancies, and (iv) cancer patients treated with CAR^+ T cells have achieved complete responses. This chapter describes pre-clinical testing of ROR1-specific T cells that have clinical implications as cancer immunotherapies.

II.B. Introduction

Current clinical trials use T cells expressing CARs specific for CD19, an antigen expressed on the surfaces of all B cells, to eliminate refractory B-cell malignancies.(4, 57, 184, 186) However, there is also loss of normal $CD19^+$ B cells in patients undergoing this therapy, which can result in serious health complications including loss of humoral immunity.(7, 32) Furthermore, loss of $CD19^+$ B cells in an elderly patient treated with CD19-specific CAR^{+} T cells resulted in death from an opportunist viral infection.(261) ROR1 is absent on most normal B cells and other healthy tissues (**Chapter I.B.2.**), but is expressed on many B-cell tumors (mantle cell lymphoma (MCL), ALL with t(1:19) translocations, and >95% of CLL) and solid tumors (lung and breast cancer, OvCa, PaCa, renal cell carcinoma, and melanoma) where ROR1 expression is required for cellular growth and survival.(13, 64, 66, 67, 75, 79, 80, 282) Thus, CARs targeting ROR1 instead of CD19 would allow for tumor elimination while sustaining the normal B cell repertoire, and ROR1-specific T cells have the potential for use in a number of solid tumors.

The design of the CAR is a source of debate at present. Striking clinical data, including complete responses, were observed in ALL and CLL patients treated with second generation CD19-specific CARs having CD137 (41BB) endodomain or the more frequently used CD28 region.(5-7, 32) However, the differences between the two CARs or their mechanisms of improved efficacy over other CAR clinical trials are unknown at present. CAR clinical trials targeting CD19 open at MD Anderson use the CD28 moiety (NCT01653717, NCT00968760, NCT01497184), but are being adapted to (i) directly compare CD28 to CD137 CARs and/or (ii) replace CD28 CARs with CD137 CARs. These trials, and those performed at other independent centers, will aim to validate these remarkable responses and determine whether CD28 or CD137 is the ideal co-stimulatory domain for CD19-specific CARs.

However, these results may not necessarily hold true for targeting different antigens due to differences in antibody affinity and/or antigen expression. Direct immunotherapy of ROR1-specific antibody (through clone 2A2) has been proposed as an option for leukemia and broader cancer treatment, but this antibody appears to have strong cytoplasmic staining in a number of normal tissues (despite absence of ROR1 mRNA expressed in these tissues) and directly binds to adipocytes that express small amounts of ROR1 mRNA.(77, 81, 283) CARs have been developed from the 2A2 (mouse) and R12 (goat) antibodies, and CAR^+ T cells were generated in central memory T cells (T_{CM}) that could then efficiently lyse $ROR1^+$ tumor, but their reactivity towards normal tissues outside of normal B cells was not evaluated.(77, 199) The optimal 2A2 and R12 CARs for expression in T_{CM} cells had short extracellular domains (14 amino acids) with CD137 and CD3ζ signaling endodomains. In contrast to other ROR1-specific antibodies, the 4A5 clone developed by Dr. Thomas J Kipps (Moores Cancer Center, UCSD) has not been shown to bind any normal tissues, except hematogones (dispensable B-cell precursors), but is highly reactive to a number of cancers, including leukemia, OvCa, and PaCa.(66, 67, 75, 79) Therefore, this clone was chosen for generation of ROR1-specific T cells in the expansion system developed at MD Anderson that has a number of differences to the previous studies, including (i) 4A5 antibody specificity, (ii) expression of CAR in polyclonal peripheral T cells containing naïve and T_{CM} reported to have maximal efficacy as CAR^+ T cells,(131) (iii) propagation of CAR^+ T cells on aAPC containing membrane-bound IL15/IL15Rα fusion protein for optimal cytokine signaling potency and memory formation, and (iv) expansion schema without the need for sorting steps that can complicate clinical translation. Thus, CARs developed based on this strategy are hypothesized to have efficient killing of $ROR1^+$ malignancies and could answer some of the same fundamental CAR questions in a broader set of peripheral T cells.

Clinical trials have not yet tested ROR1-specific CARs in humans, so this report of pre-clinical testing of ROR1-specific CARs aims to directly test CD28 and CD137 signaling CARs to streamline trial design and clinical efficacy for cancer treatments. "First-in-man" clinical trials open at MD Anderson translated (i) co-electro-transfer of CD19-specific CAR *Sleeping Beauty* (SB) transposon with SB transposase and (ii) expansion of CD19-specific CAR^{+} T cell on CD19^{+} aAPC into clinical manufacturing and were successfully transplanted into leukemia patients without toxicity or adverse event, suggesting that this is an effective and safe strategy (Cooper LJN, unpublished observation). This study builds upon these successes and adapts current (i) CAR plasmids, (ii) working aAPC cell banks expressing co-stimulatory molecules for endogenous co-stimulation of CD28 and CD137, and (iii) protocols for direct clinical application. A phase I clinical trial has been approved by the National Institutes of Health (NIH) DNA Recombinant Advisory Committee (RAC) based on the data herein and is currently under review at the MD Anderson Cancer Center Institutional Review Board (IRB). Thus, ROR1-specific CARs are close to being tested for the first time in cancer immunotherapy.

II.C. Results

II.C.1. ROR1 Surface Expression on Tumor Cells

Surface expression of ROR1 was detected on a number of leukemia cell lines, OvCa cell lines, and primary leukemia patient samples before proceeding with generating ROR1-specific CARs. The 4A5 monoclonal antibody has been shown to have high affinity binding to ROR1,(75) and it was provided by Dr. Thomas J Kipps (UCSD) for testing ROR1 expression at MDACC. EL4 is a murine T-cell lymphoma cell line with low cross-reactivity with human T cells most likely due to their differences in MHC molecules. This cell line does not express human ROR1, thus they were genetically modified to express ROR1 in order to assess CAR-specific responses independent of their TCR interaction with MHC (**Figure 6a**). Human B-cell ALL cell lines were readily accessible and were profiled for ROR1 expression. As expected, ROR1 was present on some, but not all, B-ALL cell lines. More specifically, NALM6 and Kasumi2 tested negative and positive for ROR1, respectively (**Figure 6b**). ROR1 was also expressed on most (11 of 12) OvCa cell lines tested, which are best exemplified by $ROR1^{+}$ EFO27 cells and the only $ROR1^{neg}$ OvCa cell line tested, A2780 (**Figure 6c**). ROR1 was originally described as a cancer antigen in B-cell CLL, so primary B-cell CLL patient samples were acquired for testing in parallel with LCL derived from healthy donor B cells immortalized with EBV. Indeed, CLL samples stained for ROR1 while LCL did not (**Figure 6d**). These results corroborated the previous literature and gave us confidence to go forward with generating a ROR1-specific CAR designed from the 4A5 antibody.

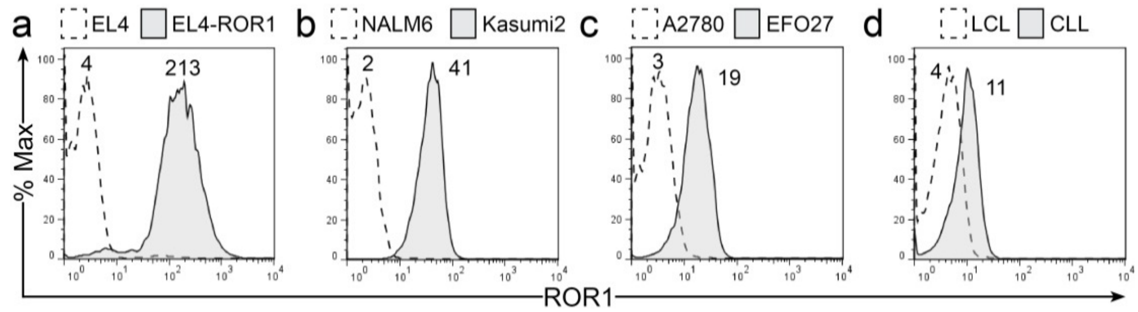

Figure 6. Surface Expression of ROR1 on Tumors. The 4A5 mAb specific for ROR1 was used to assess ROR1 expression on the surface of (**a**) EL4 parental (ROR1^{neg}) and genetically modified EL4-ROR1 cells, (**b**) B-ALL cell lines NALM6 and Kasumi2, (**c**) OvCa cell lines A2780 and EFO27, and (**d**) primary patient B-CLL cells or healthy donor LCL by flow cytometry. Mean fluorescence intensities (MFI) are displayed near corresponding histograms and legends are displayed above corresponding graphs.

II.C.2. ROR1-specific CAR Plasmid Construction

Two SB transposons were constructed with second generation ROR1-specific CARs for side-by-side comparison between the CD28 (ROR1RCD28) and CD137 (ROR1RCD137) endodomains (**Figure 7a and 7b**, respectively). CD19 constructs were prepared in parallel with the CD28 (CD19RCD28) and CD137 (CD19RCD137) endodomains as controls for current standard T cell therapy and were identical to ROR1-specific CARs except in two pieces. First, the single chain variable fragment (scFv) differ between the CD19 and ROR1 constructs where the FMC63 and 4A5 monoclonal antibodies specific for CD19 and ROR1 were used, respectively. Second, CD19 CARs use the colony-stimulating factor-2 receptor (CSF2R) signal peptide whereas ROR1 CARs use the murine IgGκ signal peptide. Human elongation factor-1α promoter was used to drive CAR expression of all CARs. Following the promoter, the CAR open reading frame was composed of (from 5' to 3'): signal peptide, scFv with Whitlow linker, modified extracellular IgG4-Fc stalk,(272) CD28 transmembrane domain, CD28 or CD137 endodomains, and intracellular CD3ζ containing three ITAM domains. Interspaced between the STOP codons and the polyadenylation (polyA) tail were unique oligonucleotides to distinguish the two CAR transposons by polymerase chain reaction (PCR). The CD28 constructs could be distinguished from CD137 constructs by the "SIM" and "FRA" oligonucleotides, respectively. Thus, detection of T cell persistence in patients undergoing ROR1-CAR T cell therapy can be monitored and can corroborate flow cytometry data. SB indirect repeats flanking the promoter (5' end) and the polyA tail (3' end) defined the CAR transposons to be integrated within TA repeats in the human T cell genome. Lastly, kanamycin resistance was used to selectively amplify CAR plasmids in bacteria to large quantities (0.5 – 1.0 mg), which were cleared for transfection after testing negative for endotoxin. In summary, these two ROR1-specific CAR plasmids mimic current plasmids used for CD19-specific CAR clinical trials at MD Anderson and should be directly translatable to the clinical setting.

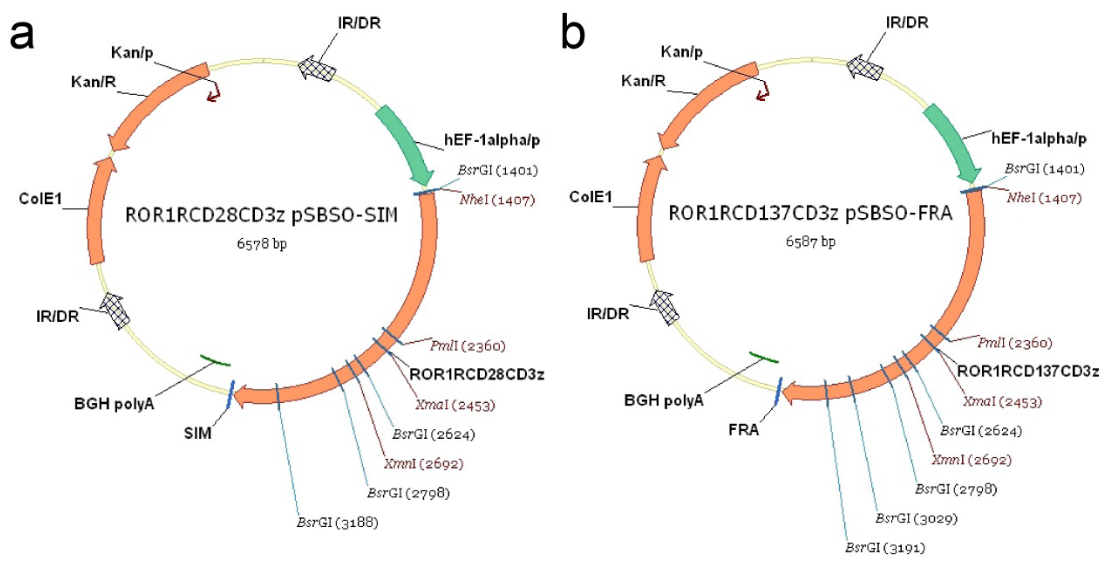

Figure 7. ROR1-specific CAR Transposons. DNA plasmid vector maps for (**a**) ROR1RCD28 and (**b**) ROR1RCD137. Abbreviations are as follows, IR/DR: *Sleeping Beauty* Inverted Repeat, hEF-1alpha/p: Human Elongation Factor-1 alpha region hybrid promoter, ROR1RCD28CD3z: Human codon optimized ROR1-specific scFvFc:CD28zeta chimeric antigen receptor, ROR1RCD137CD3z: Human codon optimized ROR1-specific scFvFc:CD137zeta chimeric antigen receptor, SIM: "SIM" PCR tracking oligonucleotides, FRA: "FRA" PCR tracking oligonucleotides, BGH polyA; bovine growth hormone polyadenylation sequence, ColE1: A minimal *E.coli* origin of replication, Kanamycin (Kan/R): Bacterial selection gene encoding Kanamycin resistance, Kanamycin promoter (Kan/p); Prokaryotic promoter. Digestion with BsrGI enzyme can distinguish the two plasmids, which have high degrees of similarity. The entire plasmid sequences were verified by Sanger-based sequencing techniques.

II.C.3. Development of ROR1^{+} aAPC (clone#1)

aAPC have been shown to propagate T cells *ex vivo* through (i) expression of cognate antigen or (ii) activation through membrane-bound antibody. However, current clinical K562-based aAPC cell banks at MD Anderson do not express ROR1. Therefore, a new aAPC was developed to express ROR1 and an IL15 fusion protein to the IL15 receptor-α (IL15/IL15Rα) along with the other molecules present on aAPC surfaces. Trans-presentation of IL15 by IL15Rα has been shown to have higher signaling potency than IL15 alone in other models.(284, 285) Clone#1 feeder cells were derived from the K562 cell line, which was previously made to express CD19 antigen, co-stimulatory molecules (CD86 and CD137L), and Fc receptors (endogenous CD32 and introduced CD64) for loading of agonistic anti-CD3 antibody (OKT3). Thus, the CAR^{+} T cells had the potential to receive co-stimulation through the CAR and from endogenous binding of CD28 and CD137 on the T cell to CD86 and CD137L, respectively, on the aAPC. Prior to co-culture, aAPC were γ-irradiated (100 Gy) and typically die within 3 days of co-culture. Clone#1 aAPC were phenotyped prior to co-culture to ensure that all markers were present at >80% (**Figure 8 right panels**). Negative and positive controls were parental K562 cells (**Figure 8 left panels**) and clone#4 aAPC (**Figure 8 middle panels**) used in CD19-specific CAR^{+} T cell clinical trials at MD Anderson, respectively. The expression of IL15 by clone#4 is detected with eGFP as a surrogate marker but IL15 was directly detected on the surface of the clone#1 cells. Cytokine support, co-stimulation, and antigen expression by clone#1 aAPC gave us confidence in its ability for use in CAR^{+} T cell propagation.

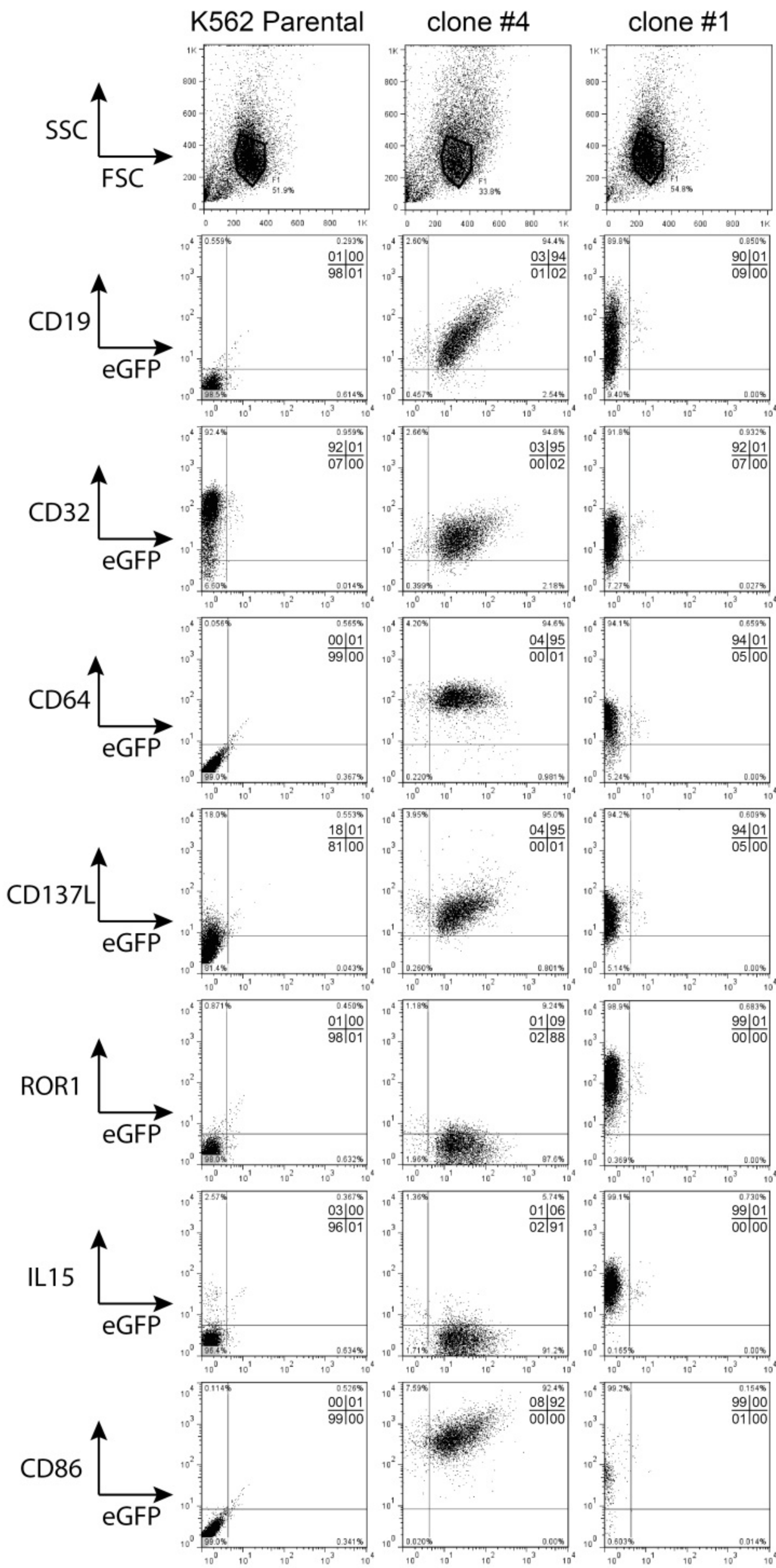
K562 Parental
clone #4
clone #1
SSC
FSC
CD19
eGFP
CD32
eGFP
CD64
eGFP
CD137L
eGFP
ROR1
eGFP
IL15
eGFP
CD86
eGFP

Figure 8. Surface Phenotype of Clone#1 aAPC Used for ROR1-specific T cell Expansion. Parental K562 (left), clone#4 aAPC (middle), and clone#1 aAPC (right) were stained for surface marker expression and were analyzed by flow cytometry. Top plots are forward scatter (FSC; x-axes) by side scatter (SSC; y-axes). Other plots were eGFP (x-axes) with the following on the y-axes from top to bottom: CD19, CD32, CD64, CD137L, ROR1, IL15, and CD86. Quadrant frequencies are displayed in the upper right corners.

II.C.4. CAR$^+$ T-cell Expansion on Clone#1 aAPC

Healthy donor PBMC were electroporated with (i) no DNA as a negative control for CAR expression, (ii) SB11 transposase and ROR1RCD28 transposon plasmids, or (iii) SB11 transposase and ROR1RCD137 transposon plasmids. The following day, cells were phenotyped for CAR expression on their surfaces where "no DNA" and isotype antibodies served as negative controls. Transient expression of CAR was detected in T cells at 41% ± 6% and 41% ± 8% (mean ± SD; n=3) for ROR1RCD28 and ROR1RCD137, respectively, as evidenced by co-staining for Fc (IgG4-Fc extracellular stalk of CAR) and CD3 (**Figure 9a**). Co-cultures were then initiated with γ-irradiated clone#1 aAPC and CAR$^+$ T cells at a 1:1 ratio. Similarly, γ-irradiated OKT3-loaded clone#4 aAPC and "no DNA" T cells were co-cultured at 1:1 ratio of total cells. Co-cultures were supplemented with IL21 (30 ng/mL) at the outset of co-culture and every 2-3 days thereafter. Recursive stimulations were performed every 7 days as above for four total stimulations, except that (i) IL2 (50 U/mL) was supplemented with IL21 starting at the second stimulation and (ii) NK cells were depleted from cultures with CD56 microbeads at day 15. At day 29, stable CAR expression was observed suggesting that clone#1 aAPC enforced CAR expression in T cells (**Figure 9b**). More specifically, CAR was expressed in T cells at 90% ± 3% and 79% ± 11% (mean ± SD; n=3) for ROR1RCD28 and ROR1RCD137, respectively, at the end of the co-culture. There was a difference between the transient and stable populations for ROR1RCD28 ($p = 0.006$) and ROR1RCD137 ($p = 0.009$), but the populations did not have significant differences in CAR expression ($p = 0.184$) following expansion. ROR1RCD137 had consistently lower mean fluorescence intensity (MFI) compared to ROR1RCD28 (51 ±

8 vs 102 ± 68, respectively) after expansion, but the reason for this is unknown at present. Recombinant rROR1 (rROR1; soluble extracellular domain) was purified and directly conjugated to a fluorescent marker (courtesy of Dr. Thomas J Kipps, USCD) for detection of antigen binding by CAR^+ T cells. CD19-specific CAR^+ T cells were expanded in parallel to serve as negative controls for rROR1 binding (**Figure 10a**). The CD19RCD28 had higher CAR expression than did ROR1RCD28, which could be explained by the differences in signal peptides used (human CSF2R and murine IgGκ, respectively). Nonetheless, ROR1RCD28 bound to rROR1, but CD19RCD28 and CAR^{neg} T cells did not bind to rROR1 (**Figure 10b**). Proliferation kinetics between the two ROR1 CAR populations was similar in total cells counts ($p = 0.66$; Two-way ANOVA) and in CAR^+ T cell counts ($p = 0.74$). Total cell proliferation closely coincided with CAR^+ T cell proliferation kinetics for both ROR1RCD28 and ROR1RCD137 (**Figure 11**). ROR1RCD28 resulted in an average of 2.5×10^9 total inferred cell counts (range 1.4×10^9 – 4.0×10^9) and 2.2×10^9 CAR^+ T cells (range 1.3×10^9 – 3.6×10^9), and ROR1RCD137 resulted in an average of 3.6×10^9 total inferred cell counts (range 3.7×10^9 – 8.2×10^9) and 2.9×10^9 CAR^+ T cells (range 2.4×10^9 – 6.7×10^9). Thus, SB transposition resulted in stable CAR expression and co-culture on clone#1 aAPC led to clinically-relevant numbers of ROR1-specific T cells.

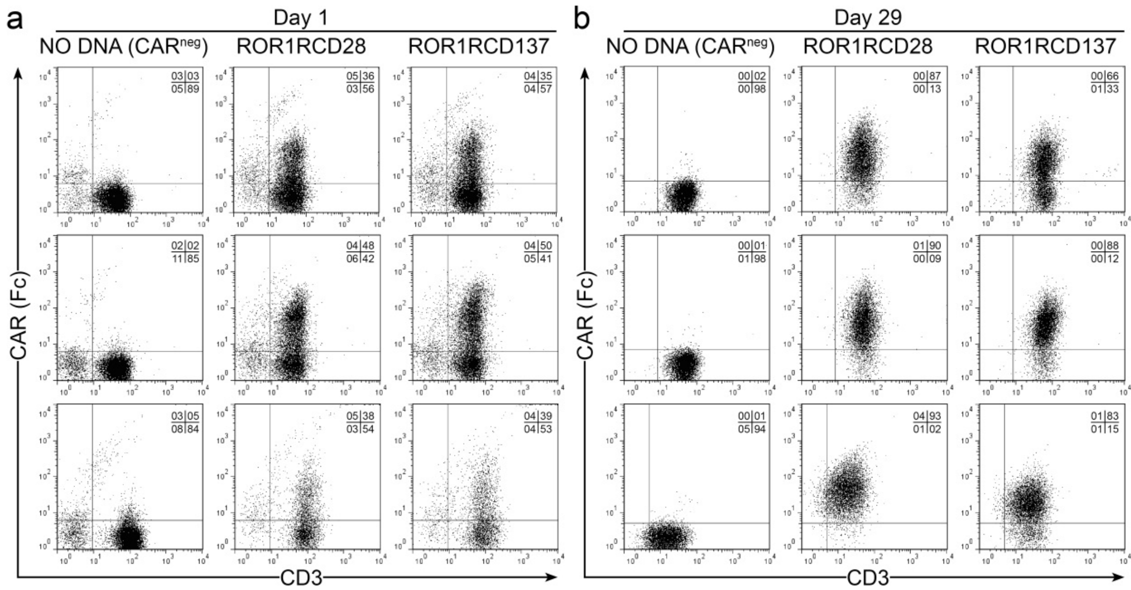

Figure 9. CAR Expression in T cells Before and After Expansion on Clone#1 aAPC. (**a**) Transient expression of ROR1RCD28 (middle) and ROR1RCD137 (right) T cells the day following electroporation where "no DNA" T cells (left) were used as negative controls. (**b**) Stable CAR expression in ROR1RCD28 (middle) and ROR1RCD137 (right) populations. T cells were marked by CD3 staining and CAR^+ cells were detected with anti-Fc antibody. Quadrant frequencies are displayed in upper right corners.

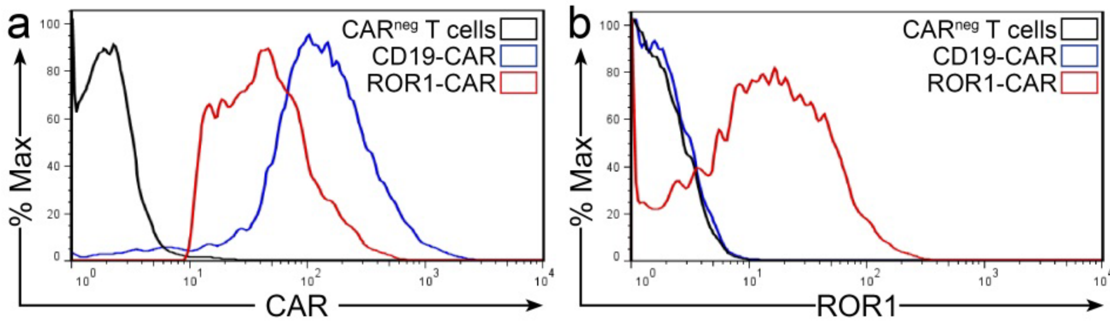

Figure 10. rROR1 Antigen Binding by ROR1-specific T cells. Recombinant ROR1 (rROR1) was purified and conjugated to fluorescent tag for detection of ROR1-specific T cells (ROR1RCD28). CD19-specific CAR^+ T cells (CD19RCD28) and "no DNA" CAR^{neg} T cells were used as negative controls. (**a**) Fc detection of CARs and (**b**) rROR1 binding.

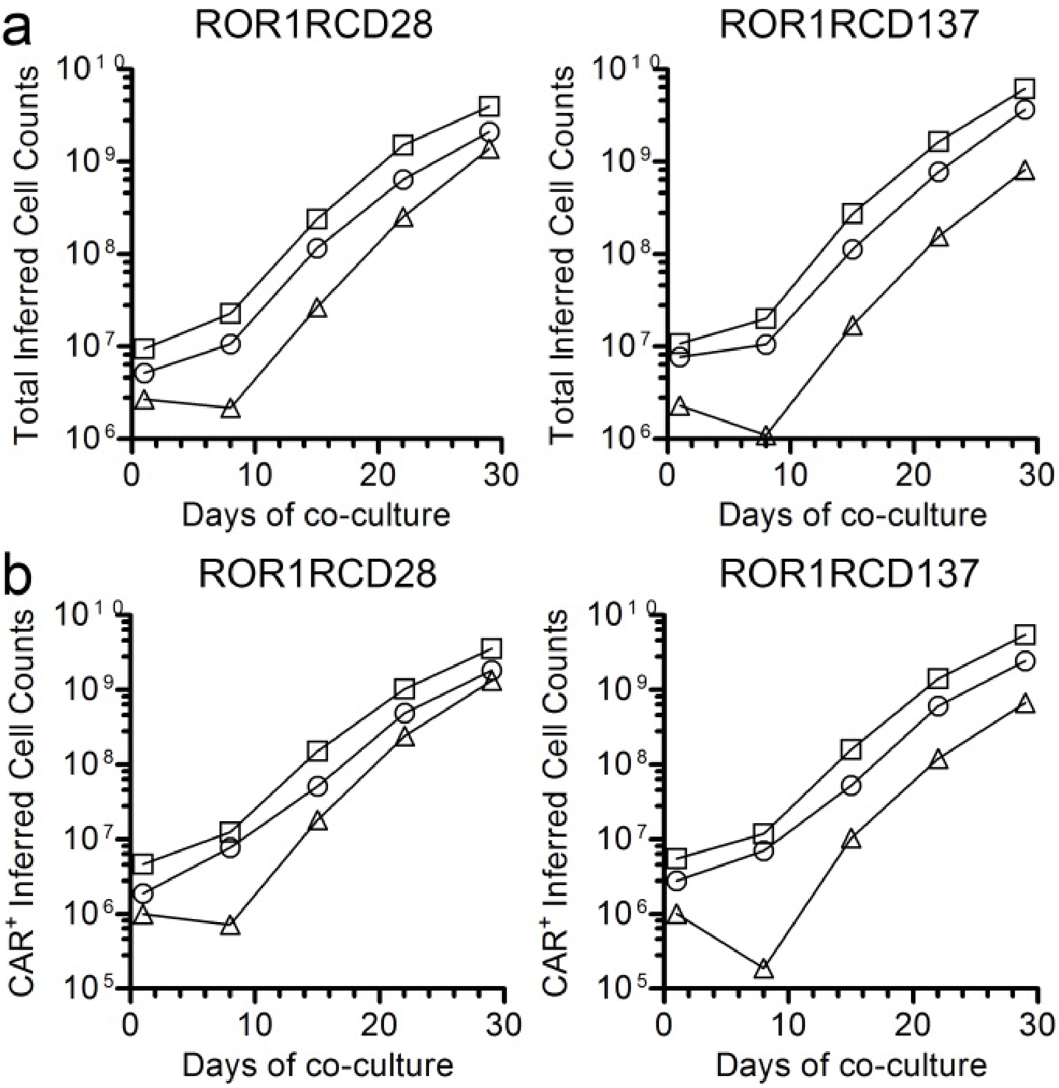

Figure 11. Sustained Proliferation of CAR$^+$ T cells. (**a**) Total cells and (**b**) CAR$^+$ T cell proliferation on clone#1 aAPC. ROR1RCD28 represented on the left and ROR1RCD137 shown on the right. Each symbol represents a different healthy donor.

II.C.5. Immunophenotype of ROR1-specific T cells

II.C.5.a. T cell Immunophenotype of ROR1RCD28 and ROR1RCD137

Following 29 days of expansion on irradiated clone#1 aAPC, ROR1RCD28 and ROR1RCD137 cells were profiled for (i) gene expression using the nCounter gene expression array platform (NanoString) and (ii) T cell surface proteins and memory markers by flow cytometry. A unique panel of lymphocyte genes was assembled for analysis on the nCounter and was termed "Lymphocyte CodeSet Array" or LCA (**Appendix A**). As expected, both δ and ε isoforms of CD3 (CD3D and CD3E, respectively) were highly expressed by both CAR^+ T cell populations, and there was higher expression of both CD3D and CD3E in ROR1RCD28 cells (**Figure 12a**). Expression of CD3ζ was not evaluated at the mRNA level because it could not be distinguished from CD3ζ on CAR intracellular domains. Nonetheless, >97% of CAR^+ T cells were $CD3^+$ on the cell surface (**Figure 12b**). There was also a trend of decreased expression of CD4 and CD8A transcripts in ROR1RCD137 cells relative to ROR1RCD28 and there was ~100 times more CD8A transcript than CD4 (**Figure 12a middle panels**). The same was observed at the protein level where both CARs preferentially expanded $CD8^+$ T cells over $CD4^+$ T cells and on average there were fewer CD4 and CD8 T cells in the ROR1RCD137 culture (**Figure 12b top panels and 12c**). This phenomenon of fewer $CD4^+$ and $CD8^+$ T cells is most likely attributed to small frequencies of γδ T cells (identified by $CD3^+TCR\gamma\delta^+$) that were present in the ROR1RCD137 cultures and not in the ROR1RCD28 cultures (**Figure 12b bottom panels**), because γδ T cells are commonly negative for both CD4 and CD8 but express

CD3.(286) Indeed, γδ T cells can proliferate on aAPC (**Chapters III and IV**), which suggests that they may compete for clone#1 for proliferative signal and diminish ROR1RCD137 cells from reaching >90% CAR^+ T cells. NK cells were present in cultures at Day 15 and were depleted with CD56 microbeads from all cultures, so negligible quantities of $CD3^{neg}CD56^+$ NK cells were detected at the end of the co-culture period two weeks later (**Figure 12b, middle panels**). CD56 was also expressed by T cells at the end of the co-culture period and is associated with MHC-unrestricted cytolysis (**Figure 12a and 12b**).(287) Significant differences between T cell surface protein expression were not observed ($p = 0.322$) between the two CARs in respect of CD3, CD4, CD8, CD56, NK cells, or γδ T cells (**Figure 12c**). These results suggest that CAR^+ T cells have canonical T cell phenotype features and on the basis of these evaluated markers were highly similar.

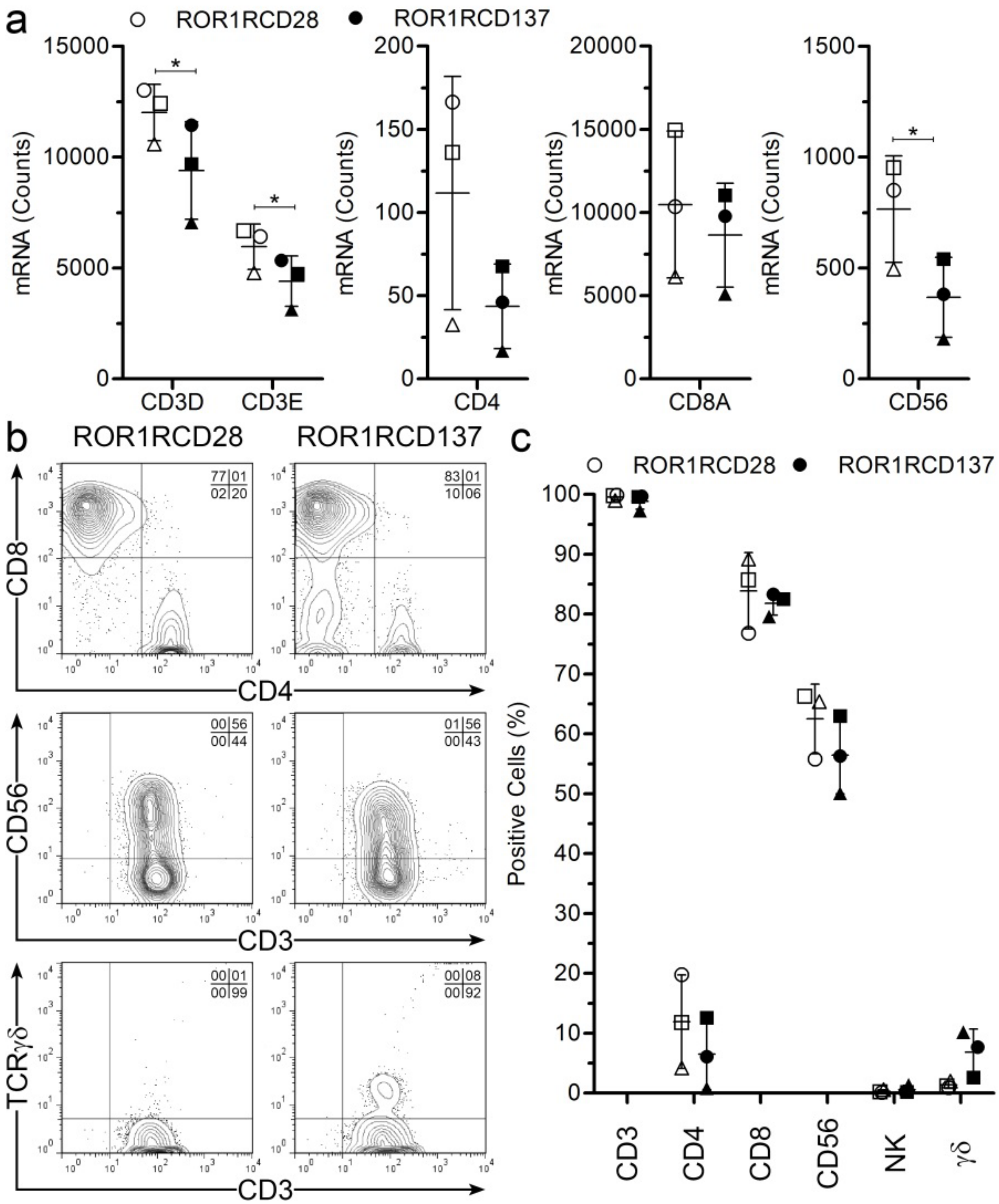

Figure 12. Basic Immunophenotype of CAR$^+$ T cells. After 29 days of expansion on clone#1 aAPC, ROR1RCD28 and ROR1RCD137 cells were (i) lysed for mRNA expression analysis or (ii) phenotyped for T cell surface markers by flow cytometry. (**a**) RNA lysates were interrogated on nCounter gene expression array with "lymphocyte CodeSet array" (LCA) and normalized CD3 (far left), CD4 (middle left), CD8A (middle right), and CD56 (far right). mRNA expression are displayed for ROR1RCD28 (open shapes) and ROR1RCD137 (closed shapes). Student's paired, 2-tailed t-test was used

for statistical analysis (n = 3). *$p<0.05$ (**b**) CD4 (x-axes) and CD8 (y-axes) expression (top panels), CD3 (x-axes) and CD56 (y-axes) expression (middle panels), and CD3 (x-axes) and TCRγδ (y-axes) expression (bottom panels) of one of 3 representative donors. Gate frequencies are in the upper right corners and correspond to gate quadrants. (**c**) Frequencies of cells staining positive for each lymphocyte marker where each shape represents an individual donor, ROR1RCD28 are in open shapes and ROR1RCD137 are in closed shapes, NK cells were defined as $CD3^{neg}CD56^{+}$, γδ T cells were defined as $CD3^{+}TCR\gamma\delta^{+}$, and data are mean ± SD (n = 3).

II.C.5.b. Memory Phenotype of ROR1-specific T cells

Naïve (T_N) and central memory (T_{CM}) T cells have been associated with long-term CAR^+ T cell therapeutic efficacy due to their ability to achieve persistence *in vivo*.(131) Both ROR1RCD28 and ROR1RCD137 cells predominantly expressed memory markers associated with T_N and T_{CM} memory phenotypes at Day 29 of co-culture (**Figure 13**). The mRNA expression of memory-associated genes was first evaluated with LCA, which identified a significant reduction in the inhibitory regulatory gene CTLA4 and an increase in expression of the transcription factor Lef1, which has been described to participate in $CD8^+$ T cell memory formation, in ROR1RCD137 cells relative to ROR1RCD28 cells (**Figure 13a**).(119, 288) As seen with the mRNA gene expression data, surface protein expression of CD28 was significantly ($p = 0.003$; Student's paired, 2-tailed t-test) higher in ROR1RCD137 cells compared to ROR1RCD28, whereas CD27 was highly expressed in both CAR^+ T cell populations suggesting they have not reached terminal differentiation (**Figure 13a, 13b, and 13d**). CAR^+ T cells populations were also similar in their high surface protein expression of lymphoid organ homing and memory markers CD62L and CCR7, suggesting they could home to organs harboring leukemia (**Figure 13a, 13c, and 13d**). A trend of decreased gene expression of SELL (CD62L) gene was observed in ROR1RCD137 cells, whereas CCR7 transcripts were roughly equivalent between the two CAR populations and protein expression was roughly equivalent for both sets as well. There was also a trend of higher expression of the antigen-experienced marker CD45RO over the more naïve-associated marker CD45RA in both populations (**Figure 13d**). Both groups were similar overall ($p = 0.251$; Two-way ANOVA) in expression of CD27, CD28, CD45RA, CD45RO, CD62L, and CCR7. To further analyze memory potential, multi-parameter gating was used to define specific memory populations as naïve (T_N; $CD45RA^+CD27^+CD28^+CCR7^+$), central memory ($T_{CM}$; $CD45RA^{neg}CD27^+CD28^+CCR7^+$), effector memory ($T_{EM}$; $CD45RA^{neg}CD27^+CD28^{neg}CCR7^{neg}$), and effector memory RA (T_{EMRA}; $CD45RA^+CD27^{neg}CD28^{neg}CCR7^{neg}$).(131, 289) Most CAR^+ T cells belonged to T_N and T_{CM} groups with few T_{EM} and T_{EMRA} (**Figure 13e**). ROR1RCD137 had a trend of higher frequencies of cells belonging to T_N and significantly higher T_{CM} groups than ROR1RCD28, and overall the two CAR^+ T cell populations were different ($p = 0.019$; Two-way ANOVA). In aggregate, the surface phenotypes of ROR1-specific CAR T cells suggest their potential for memory and effector functions against $ROR1^+$ malignancies.

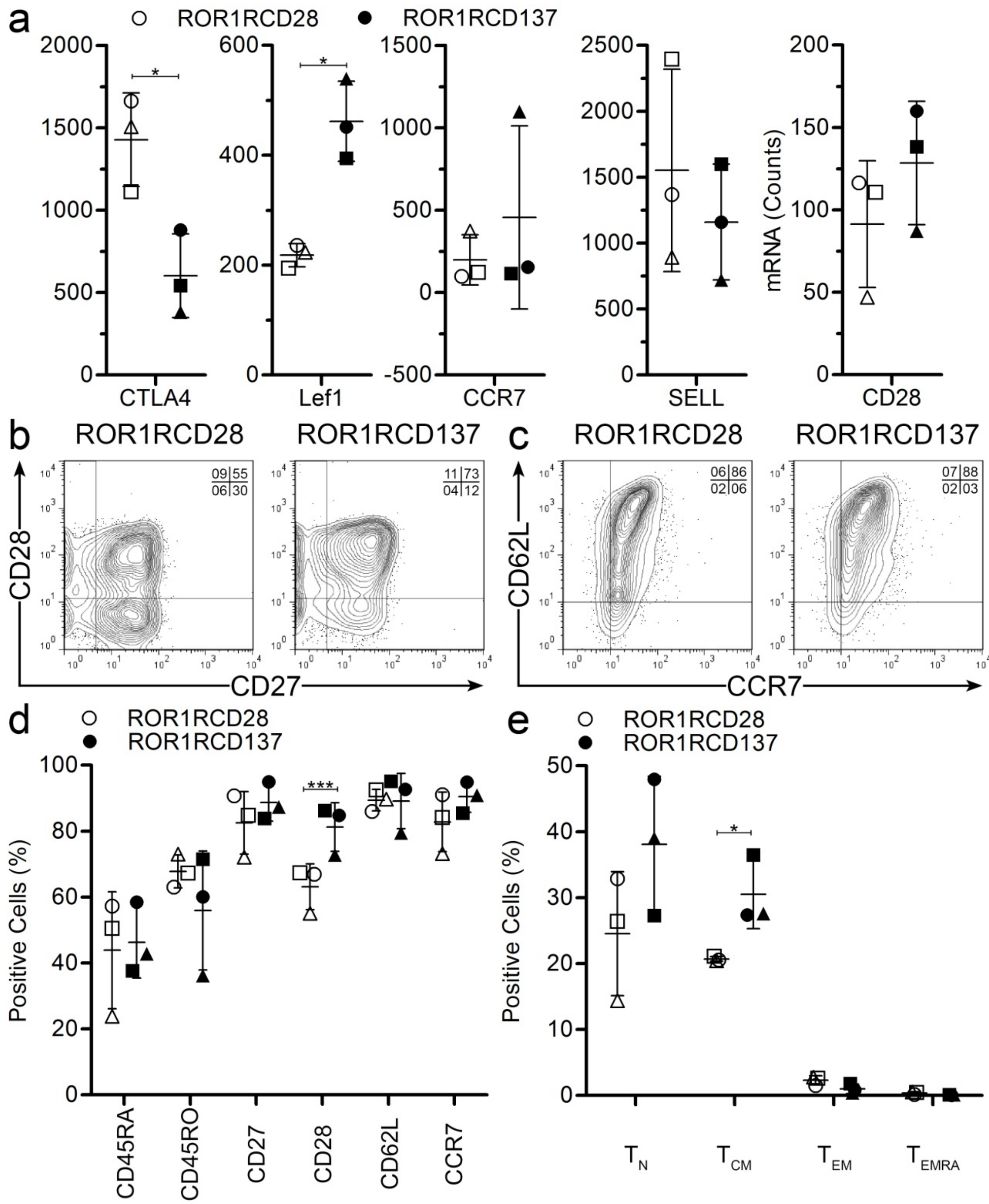

Figure 13. Memory Markers on CAR⁺ T cell Surfaces. After 29 days of expansion on clone#1 aAPC, ROR1RCD28 and ROR1RCD137 cells were (i) lysed for mRNA expression analysis or (ii) phenotyped for T cell surface markers by flow cytometry. (**a**) RNA lysates were run on the nCounter LCA and normalized expression of CTLA4 (far left), Lef1 (middle left), CCR7 (center), SELL (CD62L; middle right), and CD28 (far right) are displayed. Student's paired, 2-tailed t-tests were done for statistical analyses. *p<0.05 (**b**) CD27 (x-axes) and CD28 (y-axes) expression and (**c**) CCR7 (x-axes) and CD62L (y-axes) expression of one of 3 representative donors. (**d**) Frequencies of cells

staining positive for each memory marker. Student's paired, 2-tailed t-tests were done for statistical analyses. ***p<0.001 (**e**) Frequencies of cells staining positive for memory groups (T_N: naïve, T_{CM}: central memory, T_{EM}: effector memory, T_{EMRA}: effector memory RA). Statistical analysis was Student's paired, 1-tailed t-test between CAR groups for each memory group. *p<0.05 For (**a**), (**d**) and (**e**), each shape represents an individual donor, ROR1RCD28 are in open shapes and ROR1RCD137 are in closed shapes, and data are mean ± SD (n = 3).

II.C.6. TCR Repertoire of ROR1-specific T cells

Multiplex gene expression analysis was used to assay differences in TCR genes. Skewing towards a particular TCR clonotype was evaluated between the two CAR populations to assess whether CD28 or CD137 CARs particularly expand a select group of TCRs (**Figure 14**). The "direct TCR expression array" or DTEA was developed to analyze all 45 Vα and 46 Vβ TCR isotypes in a single reaction using the nCounter gene multiplex array platform.(290) After 22 days of expansion on clone#1 aAPC, ROR1RCD28 and ROR1RCD137 were assessed for TCR isotype expression by DTEA (**Figure 14**). Frequencies of TCRα regions were not statistically different between the two CARs ($p = 0.25$; Repeated measures Two-way ANOVA), no obvious trends were observed, and comparisons for each TCRα (Student's paired, two-tailed t-test) resulted in p values >0.05 for all alleles (**Figure 14a**). Similarly, TCRβ isotypes were not significantly different between ROR1RCD28 and ROR1RCD137 when analyzed together ($p = 0.33$) or as individual genes (**Figure 14b**). TCRα and TCRβ were both polyclonal suggesting that skewing to a particular TCR isotype did not occur. Additionally, DTEA measured TCRγ and TCRδ expression where all Vδ counts were 0.9% and 1.9% of the ROR1RCD28 and ROR1RCD137 total TCR frequencies, respectively, and Vγ counts were 6.2% and 8.1% of the ROR1RCD28 and ROR1RCD137 total TCR frequencies, respectively. These results showed that γδ T cells were minor contributors to the total CAR^+ T cell pools, which were mainly αβ T cells as determined by DTEA. Thus, CAR endodomain signaling was not preferential to a particular TCRαβ clonotype but rather generated polyclonal αβ T cells.

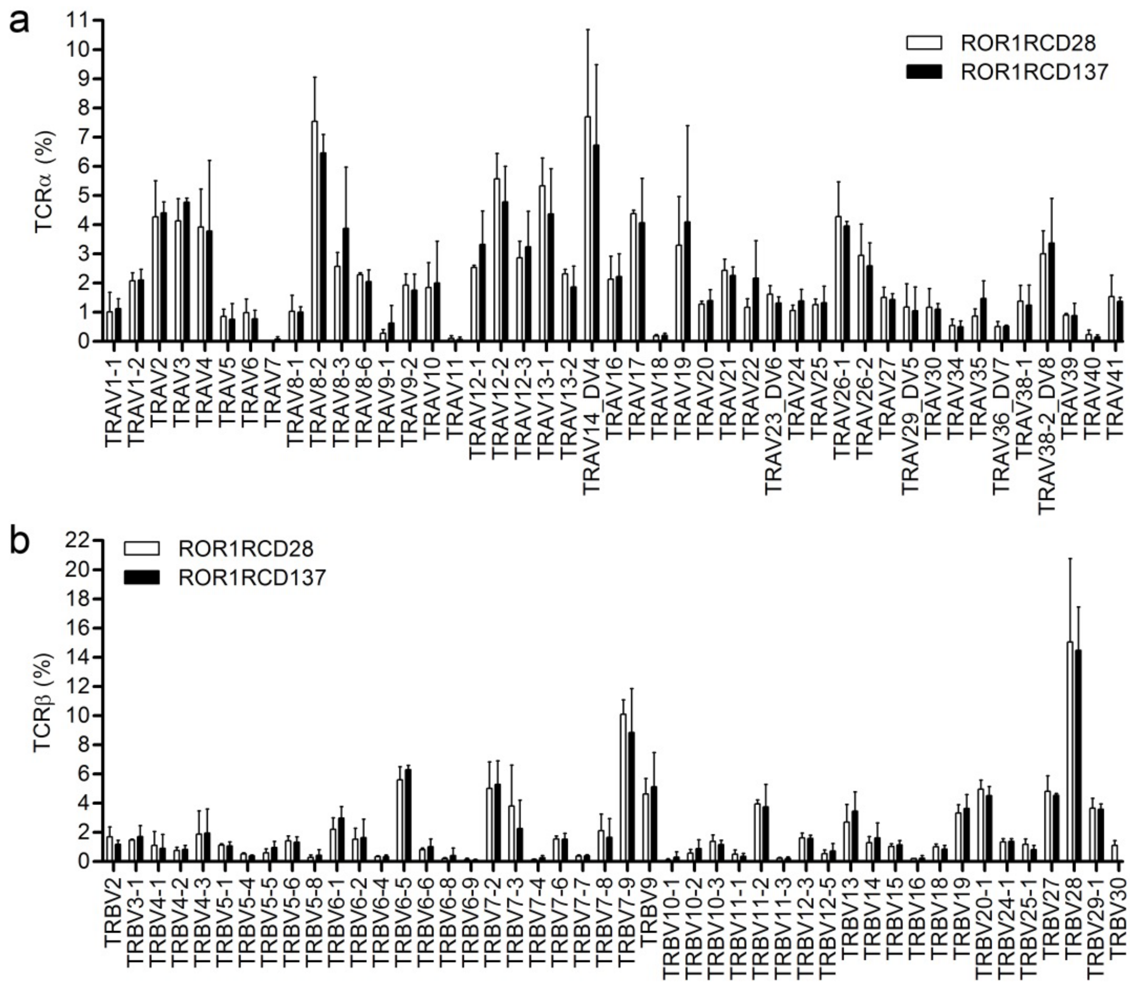

Figure 14. TCRα and TCRβ Expression in ROR1RCD28 and ROR1RCD137 cells. nCounter gene multiplex array was used to interrogate TCR isotype expression with "direct TCR expression array" (DTEA) in CAR-modified T cells after expansion on clone#1 aAPC. Cells were lysed at day 22 of co-culture period. (**a**) TCRα and (**b**) TCRβ expression in ROR1RCD28 (filled bars) and ROR1RCD137 (open bars) T cells.

II.C.7. IFNγ Production by CAR^+ T cells in Response to ROR1

In order to assess whether CAR^+ T cells were functional and specific for $ROR1^+$ tumor cells, IFNγ production was measured by flow cytometry after activation with leukemia cells or TCR agonists. Brefeldin-A was co-cultured with T cells to inhibit IFNγ secretion. Collectively, the data suggest that CAR^+ T cells were specific and functional in responding to $ROR1^+$ tumors.

II.C.7.a. TCR Stimulus with Leukocyte Activation Cocktail

Phorbol myristate acetate (PMA) and Ionomycin were used as leukocyte activation cocktail (LAC) to stimulate the T cells for evaluation of maximal TCR response. LAC mimics TCR activation by activating protein kinase C (PKC) and increasing intracellular Ca^{2+} levels and, therefore, is a measure of non-specific T cell activation.(291, 292) ROR1RCD28 and ROR1RCD137 T cells were mock activated (media only) as a negative control or activated with LAC for 6 hours. Significant expression of IFNγ was measured in response to LAC as seen in example histograms (**Figure 15a**) and average mean fluorescence intensities (MFI) of IFNγ staining (**Figure 15b**). There was a trend of higher production of IFNγ by ROR1RCD28 compared to ROR1RCD137 that was not statistically different ($p = 0.120$). These results established that IFNγ was produced when CAR^+ T cells were activated through canonical TCR signaling pathways and suggested that ROR1RCD28 had higher propensity to express IFNγ relative to ROR1RCD137 cells.

II.C.7.b. Specific IFNγ Production to ROR1$^+$ Leukemia Cells

Both Kasumi2 and NALM6 are B-cell ALL cell lines that express CD19, but only Kasumi2 expresses ROR1 (**Figure 6a**). Thus, they were used to assess responsiveness of CAR$^+$ T cells to human leukemia cells in 6 hours of co-culture. As expected, ROR1RCD28 and ROR1RCD137 T cells produced IFNγ when co-cultured with Kasumi2 cells but not with NALM6 (**Figure 15c**). Similarly to LAC activation, ROR1RCD137 cells produced less IFNγ than ROR1RCD28 cells (**Figure 15d**) in response to the ROR1$^+$ cell line. Nonetheless, ROR1-specific CAR$^+$ T cells responded specifically to ROR1$^+$ leukemia.

II.C.7.c. CAR$^+$ T cells Produce IFNγ in Response to Primary ROR1$^+$ Leukemia Cells but not Healthy ROR1^{neg} B cell LCL

It was important to ensure that ROR1-specific T cells would respond to primary ROR1$^+$ leukemia samples and spare normal B cells. LCL cell lines are immortalized healthy B cells, which served as negative controls in experiments where primary patient samples were used as targets. No IFNγ was produced by CAR$^+$ T cells when co-cultured for 6 hours with allogeneic LCL cell lines (**Figure 15e**). In contrast, significant ($p = 0.004$, Student's paired, 2-tailed t-test) IFNγ was produced by ROR1RCD28 and there was a trend of increased IFNγ production by ROR1RCD137 with CLL but did not reach a measure for statistical significance (**Figure 15e and 15f**). This was the same observation seen in an independent study testing ROR1-specific T cells, albeit with CARs derived from different mAbs specific for ROR1, where less cytokine production was seen with CARs signaling through CD137 relative to those signaling through CD28.(199) Thus, ROR1-specific CAR^+ T cells were functionally responsive to primary $ROR1^+$ leukemia and not to healthy B cells.

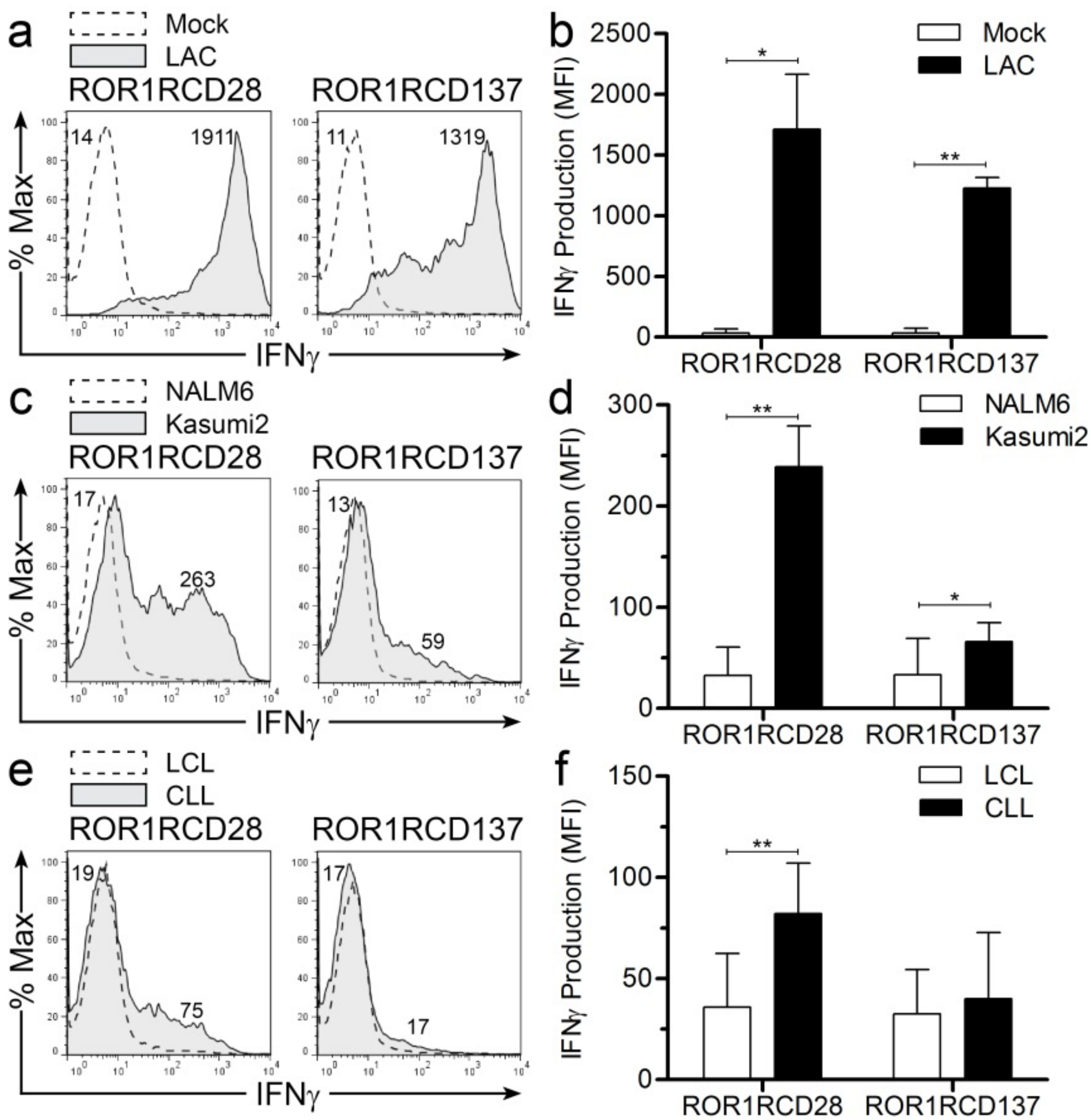

Figure 15. IFNγ Production by ROR1-specific T cells in Response to ROR1^{+} Targets. Brefeldin-A (GolgiPlug) was added to T cells to block IFNγ secretion in order to measure functional responses to agonistic stimulation. At day 29 of co-culture, CAR^{+} T cells were co-cultured for 6 hours at 37^{o}C and cells were gated for CD3^{+}Fc^{+} to assess CAR responses to: (**a**)/(**b**) complete media (Mock) or PMA and Ionomycin (leukocyte activation cocktail; LAC), (**c**)/(**d**) B-ALL cell lines NALM6 (ROR1^{neg}) or Kasumi2 (ROR1^{+}), or (**e**)/(**f**) healthy donor LCL cell line (ROR1^{neg}) or CLL patient sample (ROR1^{+}). Mean fluorescence intensities (MFI) are displayed next to histograms in (**a**), (**c**), and (**e**), which are representative of three CAR^{+} T cell donors. Mean ± SD (n = 3) are displayed in (**b**), (**d**), and (**f**). Student's paired, 1-tailed t-test for statistical analysis. *p<0.05 and **p<0.01

II.C.8. ROR1-specific Cytotoxicity by CAR$^+$ T cells

Cytotoxicity was another important assessment of ROR1-specific CAR$^+$ T cell function. Four-hour chromium release assays (CRA) are the gold-standard technique for *in vitro* killing assays. Thus, CRA was used to test specific lysis of ROR1$^+$ control cells, established tumor cell lines, and primary tumor cells. Significant lysis was only observed against ROR1$^+$ cells suggested that CAR$^+$ T cells were specific in their lytic abilities.

II.C.8.a. CAR$^+$ T cells Lyse Leukemia but not Healthy B cells

The clinical trial based on these data will treat patients with B-cell CLL, so primary B-cell CLL samples were tested as targets by allogeneic ROR1-specific CAR$^+$ T cells. ROR1^{neg} LCLs were used for negative controls for CLL samples (**Figure 6a**). As expected, minimal lysis was observed by ROR1RCD28 and ROR1RCD137 against LCL (**Figure 16a**). In contrast, both ROR1RCD28 and ROR1RCD137 killed patient CLL cells in a dose-dependent manner (**Figure 16b**). More variability was observed in ROR1RCD28 samples in their lysis of CLL compared to ROR1RCD137, which was almost identical amongst donors. These data indicated specific lysis of ROR1$^+$ leukemia by CAR$^+$ T cells while sparing normal B cells.

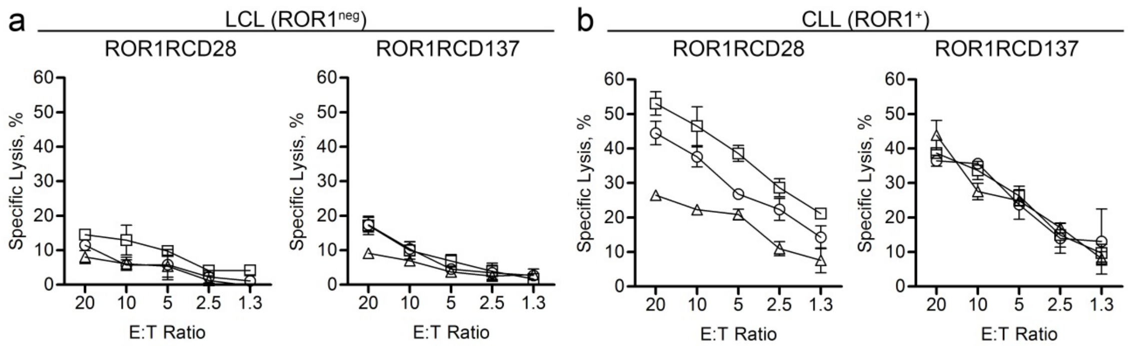

Figure 16. Specific Cytolysis of Primary ROR1^{+} B-cell CLL by CAR^{+} T cells. (**a**) Established ROR1^{neg} B-cell LCL and (**b**) Primary patient ROR1^{+} CLL cells were tested for cytolysis by ROR1-specific CAR^{+} T cells in standard 4-hour CRA. Specific lysis by ROR1RCD28 (left) and ROR1RCD137 (right) at decreasing effector to target (E:T) ratios. Each line and shape represents a different effector donor. Data are mean ± SD of triplicate measurements in CRA.

II.C.8.b. ROR1-restricted Killing of Tumor Cell Lines

A number of established tumor cell lines express ROR1 as an endogenous or introduced protein (**Figure 6**), so they were used for killing assays in parallel to cell lines lacking ROR1 expression. As expected, both ROR1RCD28 and ROR1RCD137 efficiently lysed EL4-ROR1^{+} but showed minimal lysis of EL4-ROR1^{neg} cells (**Figure 17a**). Similar to EL4 data, ROR1^{+} B-ALL cell line Kasumi2 was lysed at significantly higher levels ($p < 0.0001$) compared to ROR1^{neg} B-ALL cell line NALM6 by ROR1RCD28 (**Figure 17b left**). The same was observed for ROR1RCD137 where Kasumi2 was lysed at significantly higher levels ($p < 0.0001$) compared to NALM6 (**Figure 17b right**). In contrast to ROR1-specific CAR^{+} T cells, donor-matched CD19^{+} specific CAR^{+} T cells lysed all three cell lines, which were all CD19^{+} (data not shown), and suggested that ROR1RCD28 and ROR1RCD137 were more discriminant in their killing abilities. Furthermore, ROR1^{+} OvCa cell line EFO27 was lysed at significantly ($p<0.0001$) higher levels than ROR1^{neg} OvCa cell line A2780 by both ROR1RCD28 and ROR1RCD137 (**Figure 17c**). In summary, ROR1-specific CAR^{+} T cells demonstrated effective and specific lysis of ROR1^{+} tumor cells *in vitro*.

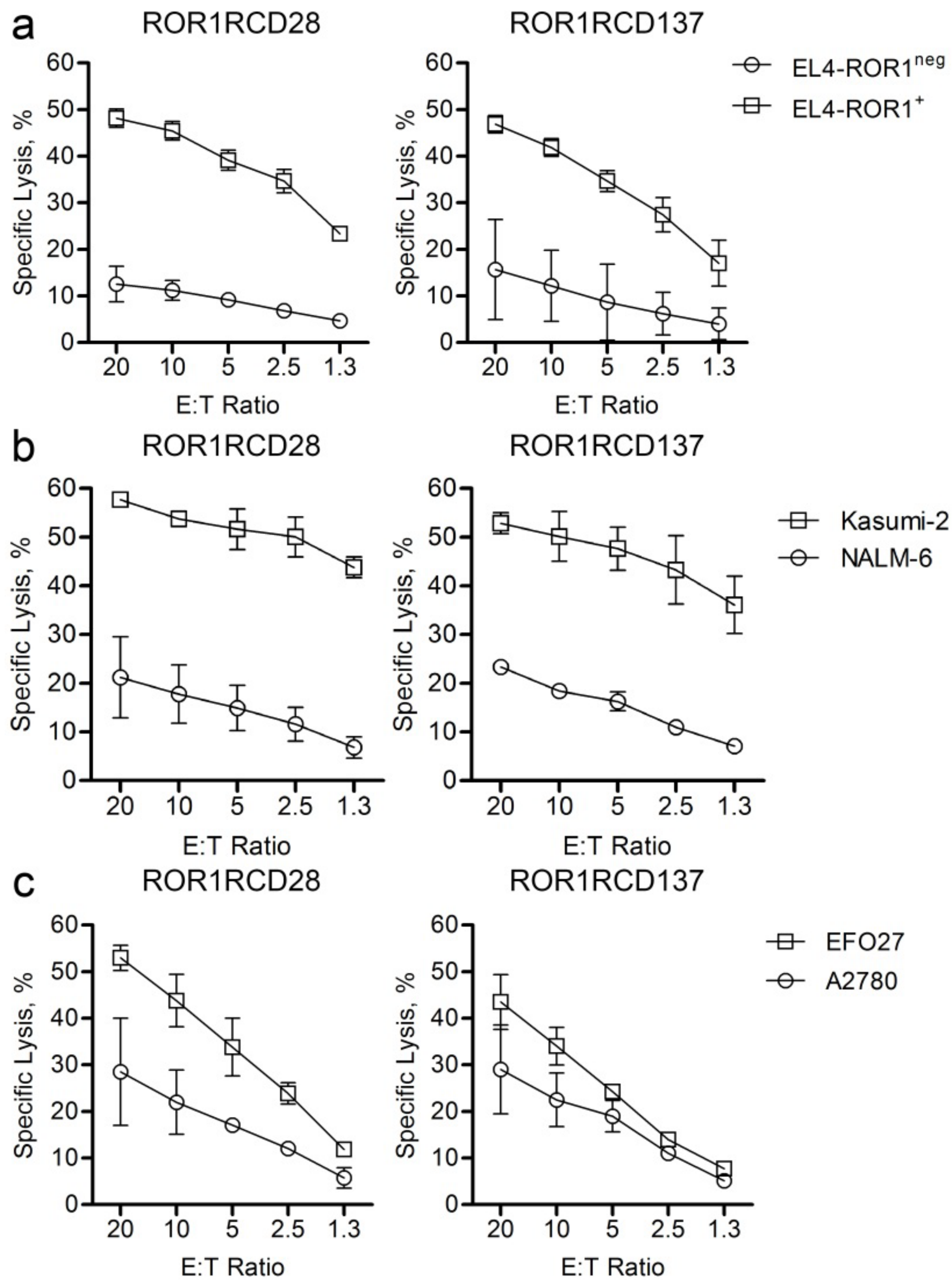

Figure 17. Specific Cytolysis of Established ROR1$^+$ Tumor Cell Lines by CAR$^+$ T cells. Standard 4-hour CRA were used to assess specific lysis of (**a**) EL4-ROR1^{neg} (circles) or EL4-ROR1$^+$ (squares) cells, (**b**) ROR1^{neg} NALM6 (circles) or ROR1$^+$ Kasumi2 (squares) cells, and (**c**) ROR1^{neg} A2780 (circles) or ROR1$^+$ EFO27 (squares) cells by ROR1RCD28 (left) and ROR1RCD137 (right) at decreasing E:T ratios. Each line and shape represents a different target where data are mean ± SD of three donors with triplicate measurements in CRA.

II.C.9. *In Vivo* Leukemia Clearance by ROR1-specific T cells

In order to test the anti-tumor activity of ROR1-specific CAR$^+$ T cells *in vivo*, a mouse model of MRD was implemented for leukemia and ROR1-specific CAR$^+$ T cells were tested as treatment arms. Kasumi2 cells were sensitive to ROR1-specific T cells lysis, so they were genetically modified to express mKate red fluorescence protein to sort transduced cells (**Figure 18a**) and *Firefly Luciferase* (*ffLuc*; bioluminescence reporter) for non-invasive bioluminescence imaging (BLI) of tumor burden *in vivo* (**Figure 18b**). NOD.*scid*.$\gamma_c^{-/-}$ (NSG) mice were used because they lack functional adaptive immune systems and can, therefore, accept human tumor xenografts well. Mice engrafted with Kasumi2-*ffLuc*-mKate had consistent $\log_{10}$-fold increases in bioluminescence flux from their tumors and succumbed to disease after 27 (average) days after engraftment (**Figure 18c circles and 18d top panel**). ROR1RCD28 was able to diminish tumor burden significantly ($p = 0.0004$) above untreated mice as measured by tumor BLI flux (**Figure 18c squares and 18d middle panel**) and was able to increase survival significantly ($p = 0.002$) to an average of 30 days post-engraftment. Furthermore, ROR1RCD137 eliminated tumor burden significantly above both untreated mice ($p = 0.0001$) and ROR1RCD28-treated mice ($p = 0.002$) as measured by tumor BLI flux (**Figure 18c triangles and 18d bottom panels**), and was able to increase survival significantly longer compared to both untreated mice ($p < 0.001$) and ROR1RCD28-treated mice ($p = 0.03$) to 34 days (average) post-engraftment and up to 11 days relative to the first mouse that died in the untreated group and the last mouse that died in the ROR1RCD137 group. ROR1RCD137 cells had consistently lower frequencies of CAR$^+$ T cells (94%, 62%, and 46% at doses 1, 2, and 3, respectively) prior to infusion relative to ROR1RCD28 cells, which expressed CAR at >90% for all three doses. The T cell doses were given based as 10^7 total cells/mouse, so a greater anti-tumor effect was seen with ROR1RCD137 with fewer total CAR^+ T cells, which highlights their ability to outperform ROR1RCD28 in tumor killing *in vivo*. In summary, ROR1-specific CAR^+ T cells can efficiently treat $ROR1^+$ leukemia and, therefore, can now be moved into the clinic for testing in patients with $ROR1^+$ malignancies.

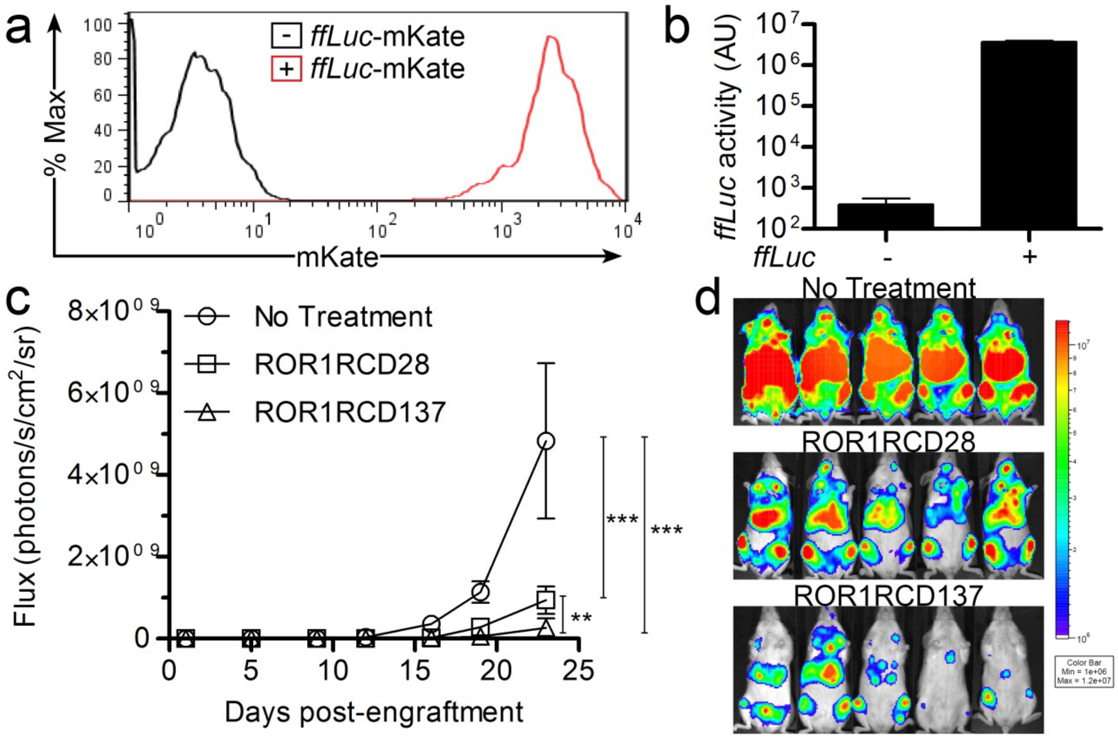

Figure 18. *In vivo* Tumor Clearance by ROR1-specific CAR$^+$ T cells. ROR1$^+$ B-ALL cell line Kasumi2 was transduced with mKate-*ffLuc* lentiviral particles and cells were sorted for uniform mKate expression by FACS. (**a**) mKate expression in parental cell line (black histogram) or transduced cell line (red histogram). (**b**) *In vitro* luciferase activity of parental Kasumi2 cell line (without *ffLuc*) and transduced Kasumi2-*ffLuc*-mKate cells. NSG mice were engrafted with $4x10^4$ Kasumi2-*ffLuc*-mKate cells intravenously (i.v.) and were treated with three doses of 10^7 T cells i.v. to assess the ability of ROR1-specific T cells to manage MRD. High dose (60 kIU) IL2 was given intraperitoneally (i.p.) the day of T cell dosing and the following day. (**c**) Non-invasive bioluminescence imaging (BLI) flux kinetics during experiment where untreated mice are in circles, ROR1RCD28-treated mice are in squares, and ROR1RCD137-treated mice are in triangles. Two-way ANOVA was used for statistical analysis. **p<0.01 and ***p<0.001 (**d**) Representative BLI images at day +23 post-engraftment.

II.D. Discussion

II.D.1. Importance of Developing ROR1-specific T cells for Leukemia Patients

This work aimed to develop pre-clinical data to support a "first-in-man" Phase I clinical trial of ROR1-specific T cell treatments for $ROR1^+$ malignancies. The major advantage of this therapy over the current anti-CD19 cellular therapies is that normal B cells would be spared when targeting ROR1 as CD19 is uniformly expressed on most B cells and is required for B cell function.(56, 57) B cells are the primary arm of the humoral response and are critical for the adaptive immune response in clearance of microbial pathogens.(89) However, people can survive without B cells, albeit under threat of novel pathogens, if they receive serum immunoglobulin replacement therapy.(63) Thus, quality of life would be certainly improved if CAR^+ T cell therapy patients had a normal repertoire of healthy B cells as would be achieved by targeting ROR1 instead of CD19.

II.D.2. ROR1 as a Tumor Target and Safety Concerns in Immunotherapy

ROR1 was originally identified on the surface of CLL cells with absent expression on normal tissues, including cells in the hematopoietic compartment.(66, 75) Subsequently, ROR1 has been described on t(1;19) B-ALL and a number of solid tumors, e.g. breast, ovarian, and pancreatic cancers.(13, 67, 79) Some expression of ROR1 mRNA species was identified in normal lung, pancreas, and adipose tissue, by qPCR of healthy donor tissue panels, and protein expression was later corroborated on the cell surface in adipocytes and in the cytoplasm in pancreatic islet cells and alveolar macrophages by immunohistochemical staining with the 2A2 ROR1-specific antibody.(77, 81) However, this antibody also displayed cytosolic staining of a number of tissues that do not express ROR1 mRNA transcripts, e.g. adrenal glands, cardiac muscle, neurons, colon, endometrium, hypophysis, larynx, liver, ovary, salivary, small intestine, skin, stomach, and thymus, which means that (i) the mRNA expression data is inaccurate or (ii) the 2A2 antibody is not completely specific for ROR1. Testing of the R12 goat antibody specific for ROR1 binding to normal tissues has not yet been reported.(293) In contrast to the 2A2 data, RNAseq analysis did not corroborate ROR1 mRNA presence in normal healthy tissues (Kipps TJ, UCSD, unpublished observations). Moreover, the 4A5 ROR1-specific mAb from which the CAR was developed in this study did not detect ROR1 in healthy tissues by both Western blot and immunohistochemistry.(67, 75) The only reported staining of ROR1 with the 4A5 mAb outside of malignancies was described on hematogones, which are B-cell precursors, and loss of hematogones would impact B cell differentiation but not the mature B cell pool.(66) There is always the risk of potential "on-target/off-target" toxicity of proper antigen recognition by CAR^+ T cells on undesired tissues expressing low levels of antigen, but we are confident that our approach is safe because (i) 4A5 did not stain normal tissue and the CAR was derived from this Ab, (ii) homing to pancreas and or adipose tissue is unlikely given the homing repertoire expressed by CAR^+ T cells which predicted for homing to lymphoid organs (CCR7 and CD62L), and (iii) high tumor burden in many CLL patients will likely be seen first and occupy the T cells from other organs. As a control for adverse events, suicide genes, e.g. inducible Caspase9, can be co-expressed with CAR in order to eliminate T cells *in vivo* with drugs specific for the suicide gene of choice.(294) In the end, these questions will only be answered once clinical trials test these hypotheses in humans.

II.D.3. CD28 versus CD137 in CAR Design

A common debate in CAR immunotherapy at present is whether to use CD28 endodomain, as most investigators have done, or CD137 endodomain, both of which have led to objective clinical responses.(4-7, 32) A direct comparison of CD28 versus CD137 signaling in CD19-specific CARs developed at MDACC (and analogous to the ROR1-specific CARs in design) resulted in almost indistinguishable characteristics *in vitro* but CD137 was superior *in vivo* in leukemia clearance compared to CD28 (Singh H, unpublished observations). In this study, the most notable differences between the two ROR1-specific CARs were in (i) memory phenotype, (ii) *in vitro* IFNγ production, and (iii) *in vivo* tumor clearance. In regards to surface phenotype, both ROR1RCD28 and ROR1RCD137 T cells were almost completely naïve (T_N) and central memory T cells (T_{CM}) after *ex vivo* expansion, and there were more of both T_N and T_{CM} populations in ROR1RCD137 cells (**Figure 13**). Indeed, both of these populations have been correlated to limited effector functions including reduced cytokine production and cytolysis.(132, 135) It is consistent then that ROR1RCD137 cells produced less IFNγ when challenged with $ROR1^+$ targets (**Figures 15**), and fewer cytokine mRNA transcripts were produced by ROR1RCD137 relative to ROR1RCD28 as evaluated by nCounter LCA (data not shown). Indeed, the ability to produce cytokines was inversely correlated with $CD8^+$ T cell efficacy in other T cell immunotherapies.(295) Again, reduced cytokine production was also observed with ROR1-specific CARs signaling through CD137 that were derived from the 2A2 mAb and its higher affinity counterpart R12 mAb.(199) Similar killing was detected by both CAR populations against $ROR1^+$ targets, with a minor exception of primary cell lines where ROR1RCD28 was highly variable in cytolysis between donors and exceeded ROR1RCD137 in killing for 2 out of 3 donors (**Figure 16b**). In contrast to the *in vitro* results, ROR1RCD137 was significantly ($p = 0.0001$) better at eliminating $ROR1^+$ leukemia compared to ROR1RCD28, which was significantly better ($p < 0.0001$) than no treatment (**Figure 18**). Furthermore, these results were achieved with fewer total CAR^+ T cells infused into each mouse, because the same total number was injected but CAR percentage was lower in ROR1RCD137 relative to ROR1RCD28. Possible explanations of the differences are (i) higher frequencies of T_N and T_{CM} memory cells that are correlated with highest CAR^+ T cell responses relative to other classification,(131) (ii) lower expression of inhibitory molecules like CTLA4 (**Figure 13**), (iii) production of other inflammatory molecules other than IFNγ such as IL17, and/or (iv) longer persistence in the mice which has been correlated to memory formation and increased anti-tumor activity.(6, 189, 215, 237) The NSG mice used for *in vivo* studies lack human homeostatic cytokines, e.g. IL7 and IL15, that can improve persistence in patients treated with ROR1-specific T cells and therefore increase the potential of the anti-tumor effects observed in the mouse studies. A side-by-side comparison of the two CARs in clinical trials will be the ultimate test of which CAR is better for cancer treatment.

II.D.4. Immediate Plans for ROR1-specific T cells in Leukemia Treatment

A Phase I clinical trial has been approved by the NIH RAC and is in process for MD Anderson IRB approval. The trial design is to co-infuse ROR1RCD28 and ROR1RCD137 cells in a competitive repopulation experiment to maximize potential therapeutic efficacy and determine which CAR will persist longer in the patients. PCR will be used as a highly-sensitive means to detect persistence of one population over another based on unique oligonucleotides present in the two CAR transposons (SIM for CD28 and FRA for CD137). As this will be the first time ROR1-specific T cells are infused into humans, it is the primary endpoint to determine toxicity and maximum tolerated doses. There is strong evidence that this will work as means to eliminate leukemia while maintaining normal B cells, and will be the first time that ROR1 has been a target of immunotherapy for cancer treatment.

CHAPTER III

Bi-specific T cells Expressing Polyclonal Repertoire of Endogenous γδ T-cell Receptors and Introduced CD19-specific Chimeric Antigen Receptor

III.A. Hypothesis and Rationale

The *hypothesis* of this chapter is that enforced CAR expression on γδ T cells will stimulate them independent of their TCRγδ, thus leading to expansion of γδ T cells with polyclonal TCRγδ repertoire, and would amplify the anti-tumor effects from TCRγδ towards TAA^{+} malignancies through the CAR. The *rationale* for this specific aim is that (i) γδ T cells have inherent anti-tumor immunity through a number of combinations of TCRγ and TCRδ pairings, (ii) the use of γδ T cells in the clinic is currently restricted to Vγ9Vδ2 even though other γδ T cell lineages have anti-tumor reactivity, (iii) CARs stimulate T cells independent of their TCR, (iv) electroporation of SB transposons containing the CAR can be achieved in quiescent PBMC with a polyclonal repertoire of γδ T cells, and (v) CD19-specific CAR transposon plasmids and $CD19^{+}$ aAPC are currently in clinical trials at MD Anderson and these reagents can be used to quickly translate findings from this chapter into clinical trials. Therefore, using a polyclonal set of γδ T cells for CAR-based immunotherapy would allow for targeting the tumor through both CAR and multiple TCRγδ pairings to maximize anti-tumor immunity through bi-specific T cells.

III.B. Introduction

“Chimeric antigen receptors (CARs) re-direct T-cell specificity to tumor-associated antigens (TAAs), such as CD19, independent of major histocompatibility complex (MHC).(57, 186, 189, 272, 296) This genetic modification of T cells has clinical applications as adoptive transfer of CAR^+ T cells with specificity for CD19 can lead to anti-tumor responses in patients with refractory B-cell malignancies.(6, 7, 32, 56) Current trials administer CAR^+ T cells co-expressing αβ T-cell receptor (TCRαβ) derived from a population that represents 95% of the peripheral T-cell pool. However, the remaining 1-5% of circulating T cells expressing TCRγδ (γδ T cells) have clinical appeal based on their endogenous cytotoxicity towards tumor cells as well as their ability to present TAA and elicit an anti-tumor response.(177, 297, 298) This population of T cells directly recognizes TAA, *e.g.*, heat shock proteins, MHC class I chain-related gene A/B (MICA/B), F1-ATPase, and intermediates in cholesterol metabolism (phosphoantigens), in humans.(299) Therefore, broad recognition of tumor cells and anti-tumor activity is achieved by these T cells expressing a diverse TCRγδ repertoire (combination of Vδ1, Vδ2, or Vδ3 with one of fourteen Vγ chains).(300)

More specifically, T cells expressing Vδ1 and Vδ2 have been associated with anti-tumor immunity, but current adoptive immunotherapy approaches are limited to the Vδ2 sub-population due to limited expansion methods of Vδ1 to clinically-sufficient numbers of cells for human applications. For the most part, γδ T cells have been numerically expanded *in vivo* and *ex vivo* using Zoledronic acid (Zol),(301) an aminobisphosphonate that results in selective proliferation of T cells expressing Vγ9Vδ2 TCR.(175, 177, 297) This treatment modality has resulted in objective clinical responses against both solid and hematologic tumors, but has not been curative as a monotherapy. Vδ1 γδ T cells have not yet been infused, but their presence has correlated with complete responses observed in patients with B-cell acute lymphoblastic leukemia (B-ALL) after undergoing αβ T cell-depleted allogeneic hematopoietic stem-cell transplantation (HSCT).(302-305) As both of these sub-populations of γδ T cells are associated with anti-tumor activity, but have not been combined for cell therapy, we sought a clinically-appealing approach to propagate T cells that maintain a polyclonal TCRγδ repertoire.

Recognizing that a CD19-specific CAR can sustain the proliferation of αβ T cells on artificial antigen presenting cells (aAPC) independent of TCRαβ usage,(280) we hypothesized that CAR^+ γδ T cells would expand on aAPC independent of TCRγδ. Our approach was further stimulated by the observation that K562, the cell line from which the aAPC are derived, are a natural target for γδ T cells.(303) We report that CAR^+ γδ T cells can be propagated to clinically-relevant numbers on designer aAPC while maintaining a polyclonal population of TCRγδ as assessed by our "direct TCR expression assay" (DTEA), a novel digital multiplexed gene expression analysis that we adapted to interrogate all TCRγδ isotypes.(290) These CAR^+ γδ T cells displayed enhanced killing of CD19^{+} tumor cell lines *in vitro* compared to polyclonal γδ T cells not expressing CAR. Leukemia xenografts in immunocompromised mice were significantly reduced when treated with CAR^{+} γδ T cells compared to control mice. This study highlights the ability of aAPC to numerically expand bi-specific T cells that exhibit introduced specificity for CD19 and retain endogenous polyclonal TCRγδ repertoire.

III.C. Results

III.C.1. CAR^{+} γδ T cells Numerically Expand on aAPC

To date, it has been problematic to synchronously manipulate and expand multiple γδ T-cell subpopulations for application in humans. Viral-mediated gene transfer typically requires cell division to achieve stable gene transfer and CARs have been introduced into transduced T cells expressing just Vδ2 TCR following the use of aminobisphosphonates to drive proliferation.(306) In contrast, non-viral gene transfer with *Sleeping Beauty* (SB) transposition can be achieved in quiescent peripheral blood mononuclear cells (PBMC) with the full complement of peripheral γδ T cells initially present. Thus, stable expression of CAR can be achieved without prior T-cell propagation, enabling us to investigate if a population of T cells expressing polyclonal TCRγδ chains could then be numerically expanded in a CAR-dependent manner on designer artificial antigen presenting cells (aAPC). PBMC were electroporated (Day 0) with SB transposon/transposase system to enforce expression of a second generation

CD19-specific CAR (CD19RCD28)(57) that signals through chimeric CD28 and CD3ζ. Electroporated cells were sorted using paramagnetic beads to separate the 4.0% ± 1.5% (mean ± standard deviation (SD); n = 4) CAR^{+} γδ T cells from the majority of CAR^{+} αβ T cells. The CAR^{+} γδ T cells were selectively propagated by the recursive additions of γ-irradiated K562-derived aAPC (clone #4, genetically modified to co-express CD19, CD64, CD86, CD137L, and membrane bound IL15)(57) with soluble IL2 and IL21. IL21 is included in the manufacture of our CAR^{+} αβ T cells so it was used to propagate CAR^{+} γδ T cells.(57) Prior experiments predicted that IL2 and IL15 enhance the proliferative potential of γδ T cells, and synergy between IL2 and IL21 has led to improved anti-tumor activity compared with γδ T cells grown with either IL2 or IL21 alone.(174, 178, 307-309) Sham electroporations were undertaken to provide staining control T cells that were propagated by cross-linking CD3 using aAPC loaded with OKT3 to numerically expand CAR^{neg} αβ T cells.(310) As expected, CAR was expressed on the day following electroporation (Day 1) in most of the T cells, including γδ T cells, which comprised up to 10% of the mononuclear cells (**Figure 19a, left**). After 36 days of co-culture on aAPC, the majority of cells co-expressed CD3 and TCRγδ with 30.7% ± 23.3% (n = 4) CAR expression (**Figure 19a, right**). The absolute CAR proportions at Day 36 varied in frequency depending on the donor, but increased compared to the initial populations of CAR^{+} γδ T cells at Day 1 (**Figure 19b**). As we have demonstrated, our aAPC co-culture system enforces CAR expression in αβ T cells (>90% CAR^{+} T cells by 28 days of co-culture),(57) but the apparent lack of the same degree of selective pressure when combined with γδ T cells was attributed to an inherent ability of CAR^{neg} γδ T cells to sustain proliferation on aAPC derived from K562. Continuous proliferation of both CAR^{neg} and CAR^{+} γδ T cells was observed over the tissue culture period. Even so, we could generate up to $1.5x10^{9} \pm 1.2x10^{9}$ (n = 3) CAR^{+} γδ T cells from the $2.8x10^{5} \pm 1.5x10^{5}$ (n = 3) CAR^{+} γδ T cells at the start of the culture (**Figure 19c**). Most of the propagated cells co-expressed CD3 and TCRγδ, but did not express TCRαβ (**Figure 19d**). These data demonstrate that aAPC could be used to sustain proliferation of CAR^{+} T cells co-expressing TCRγδ.

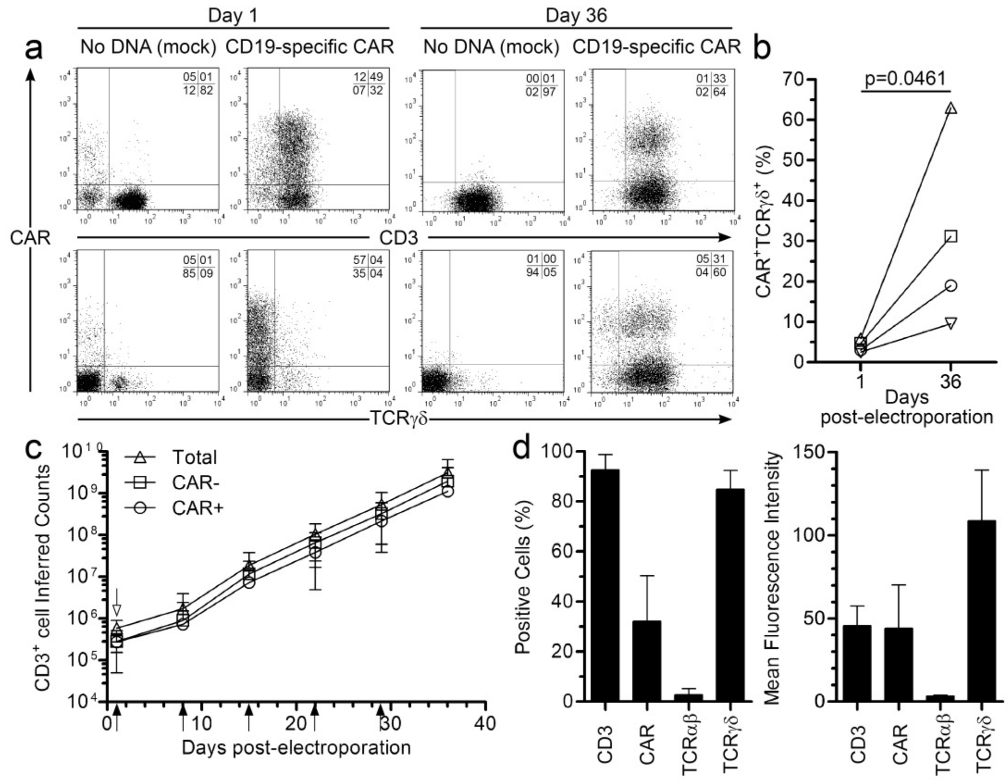

Figure 19. CAR+ γδ T cells Propagate on Designer aAPC. (**a**) Transient (Day 1) and stable (Day 36) expression of CAR in T cells (top) and γδ T cells (bottom) in mock electroporated ("no DNA") or CD19-specific CAR electroporated cells (CD19RCD28). (**b**) Percentage of CAR+ γδ T cells in the culture as transient (Day 1) and stable (Day 36) expression where each shape represents an individual donor. (**c**) Rate of expansion of total γδ T cells (triangles), CAR^neg γδ T cells (squares), and CAR+ γδ T cells (circles) over tissue culture period following paramagnetic bead sorting (open arrow) and recursive stimulation (closed arrows) with aAPC and exogenous IL2 and IL21 administration. (**d**) Percentage-positive cells and mean fluorescence intensity of CD3, CAR, TCRαβ, and TCRγδ at day 36. Data are mean ± SD (n = 4) and quadrant percentages of flow plots are in upper right corner. **This work was originally published in *Molecular Therapy*.** Deniger, D. C., K. Switzer, T. Mi, S. Maiti, L. Hurton, H. Singh, H. Huls, S. Olivares, D. A. Lee, R. E. Champlin, and L. J. Cooper. 2013. Bispecific T-cells Expressing Polyclonal Repertoire of Endogenous gammadelta T-cell Receptors and Introduced CD19-specific Chimeric Antigen Receptor. Mol Ther. 21(3): 638-647.(311) © **Nature Publishing Group**

III.C.2. Immunophenotype of Numerically Expanded CAR$^+$ $\gamma\delta$ T cells

Multi-parameter flow cytometry was used to gate on CAR$^+$ T cells and analyze their expression of cell surface markers (**Figure 20**). TCR$\gamma\delta$ was expressed at high and low densities (**Figure 20a, top**). CD56, a marker of MHC-unrestricted lytic ability,(287) was also expressed on T cells, but the culture contained <1% CD3^{neg}CD56$^+$ NK cells and <1% CD3$^+$Vα_{25}TCR$^+$ NKT cells (data not shown). In contrast to $\alpha\beta$ T cells, no CAR$^+$ $\gamma\delta$ T cells expressed CD4, some were CD8$^+$, but most were CD4^{neg}CD8^{neg}, which is consistent with what is known for $\gamma\delta$ T cells.(286) The relative frequencies for each donor are shown in **Figure 20b**. Markers associated with memory, *e.g.*, CD27, CD28, CD62L, and CCR7, were expressed by CAR$^+$ $\gamma\delta$ T cells (**Figure 20a, bottom**). Both naïve (CD45RA) and antigen-experienced (CD45RO) cells were present after propagation on aAPC, and the T cells were not exhausted as measured by low expression of CD57 (**Figure 20b**). In aggregate, cultures contained a heterogonous mixture of naïve (CD45RA$^+$CD27$^+$CD28$^+$CCR7$^+$; 26.5% ± 6.2%), central memory (CD45RA^{neg}CD27$^+$CD28$^+$CCR7$^+$; 7.8% ± 3.6%), effector memory (CD45RA^{neg}CD27$^+$CD28^{neg}CCR7^{neg}; 10.1% ± 5.4%), and EMRA (CD45RA$^+$CD27^{neg}CD28^{neg}CCR7^{neg}; 7.6% ± 3.4%) T-cell phenotypes.(131, 289) Co-stimulation by enforced expression of CD86 and CD137L (4-1BBL) on aAPC may be important for CAR$^+$ $\gamma\delta$ T-cell numeric expansion due to expression of their receptors CD28 and CD137 (4-1BB), respectively. Molecules associated with homing to bone marrow (cutaneous lymphocyte antigen (CLA) and CXCR4) and lymph nodes (CD62L and CCR7) were present on CAR^+ γδ T cells suggesting that they could migrate to sites known to harbor leukemia. In sum, propagated CAR^+ γδ T cells expressed T cell-associated surface markers that indicate desired potential for memory and homing.

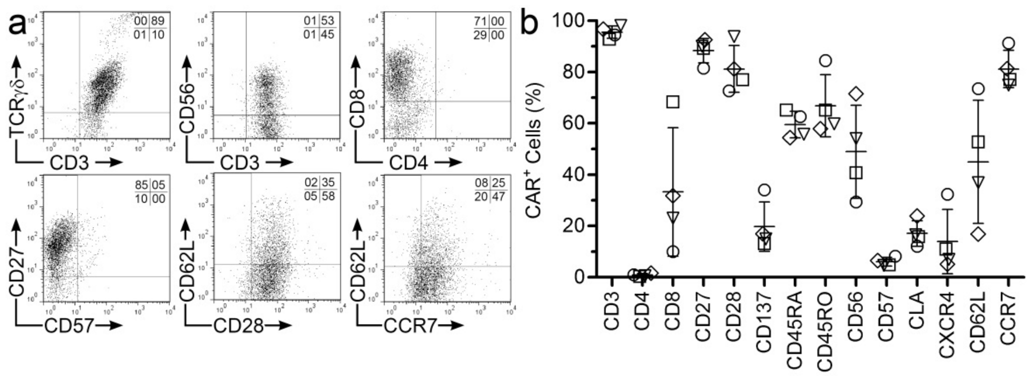

Figure 20. Immunophenotype of Electroporated, Separated, and Propagated CAR$^+$ γδ T cells. (**a**) Expression by flow cytometry of cell-surface markers associated with T cells and memory as gated on CD3$^+$CAR$^+$ cells. (**b**) Percentages of CAR$^+$ T cells expressing T-cell markers where each shape represents a different donor. Data are mean ± SD (n = 4). Quadrant percentages of flow plots are in upper right corner. **This work was originally published in *Molecular Therapy*.** Deniger, D. C., K. Switzer, T. Mi, S. Maiti, L. Hurton, H. Singh, H. Huls, S. Olivares, D. A. Lee, R. E. Champlin, and L. J. Cooper. 2013. Bispecific T-cells Expressing Polyclonal Repertoire of Endogenous gammadelta T-cell Receptors and Introduced CD19-specific Chimeric Antigen Receptor. Mol Ther. 21(3): 638-647.(311) © **Nature Publishing Group**

III.C.3. Direct TCR Expression Assay to Reveal γ and δ TCR Usage in CAR^{+} γδ T cells

We sought to determine that aAPC-propagated CAR^{+} T cells were indeed bi-specific as defined by the presence of a polyclonal population of TCRγδ alleles. Up to now, it has been difficult to determine the pattern of expression of the γ and δ TCR chains. Therefore, we adapted our DTEA to assess the complete TCRγδ transcriptome. This approach takes advantage of the nCounter assay system to measure multiple bar-coded genes in a single reaction with high sensitivity and linearity across a broad range of expression.(312) A multiplexed CodeSet was designed with two sequence-specific probes for each allele to evaluate TCRγδ isotypes. The DTEA was initially validated using Zol to preferentially propagate Vγ9Vδ2 cells from PBMC and, as expected, the resultant TCR usage was dominated by both Vδ2 and Vγ9 at protein (**Figure 21a**) and mRNA levels (**Figure 21b and 21c**). A second validation employed antibodies directed against γδ T-cell subsets (Vδ1 and Vδ2; no commercially available antibodies to Vδ3) to measure their mRNA expression. Vδ1^{neg}Vδ2^{neg}, Vδ1^{+}Vδ2^{neg}, and Vδ1^{neg}Vδ2^{+} cells were sorted from CAR^{neg} T cells (to maximize the number of Vδ2 cells recovered by fluorescence-activated cell sorting, FACS) and subjected to DTEA (**Figure 22a**). As expected, Vδ1^{+}Vδ2^{neg}, Vδ1^{neg}Vδ2^{+}, and Vδ1^{neg}Vδ2^{neg} expressed Vδ1*01, Vδ2*02, and Vδ3*01 mRNA species, respectively (**Figure 22b**). These two strategies supported the validity of the DTEA panel enabling the identity of TCRγδ to be determined in CAR^{+} T cells. Therefore, we measured the mRNA levels for all three Vδ alleles as present in electroporated, separated, and propagated CAR^+ γδ T cells which correlated with multi-parameter flow cytometry on gated CAR^+ T cells to reveal the frequencies of Vδ subsets based on protein expression. The three Vδ populations were present in ascending frequency (Vδ1>Vδ3>>>Vδ2) in the electroporated and propagated T cells (**Figure 22c**). CAR^{neg} γδ T cells displayed similar frequencies of Vδ TCR usage as CAR^+ γδ T cells. DTEA array also assessed Vγ usage, which is of particular utility because only one antibody against Vγ9 is commercially available, thus limiting the tools with which to detect Vγ usage. Of note, Vγ2, Vγ7, Vγ8 (both alleles), Vγ9, and Vγ10 were present in CAR^+ T-cell cultures (**Figure 22d**). A lack of commercially-available antibodies prevented assessment of pairing between individual Vδ and Vγ chains on the T cells. The TCR usage described for γδ T cells was that which was present at the time of functional assays. Our ability to digitally quantify the presence of mRNA species enabled us to determine that the propagated CAR^+ T cells expressed a polyclonal population of TCRγδ chains.

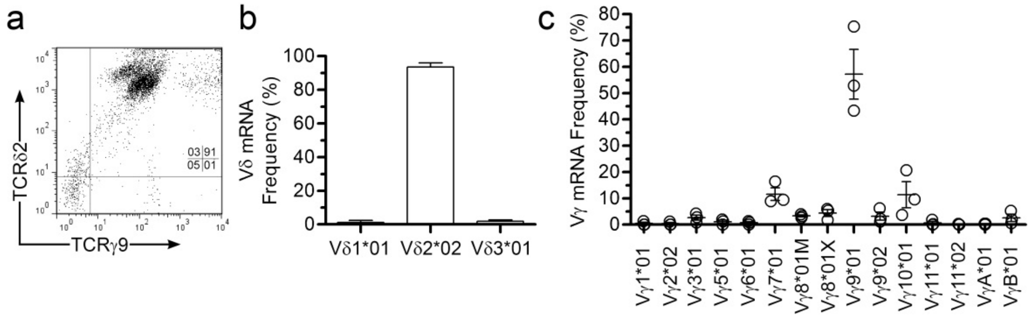

Figure 21. Distribution of Vδ and Vγ in γδ T cells Expanded on Aminobisphosphonate. (**a**) Representative flow cytometry plot from T cells following 36 days of numeric expansion with Zol. (**b**) Vδ and (**c**) Vγ allele mRNA expression in Zol-expanded T cells. Data are mean ± SD (n = 3). Quadrant frequencies of flow plot are displayed. **This work was originally published in *Molecular Therapy*.** Deniger, D. C., K. Switzer, T. Mi, S. Maiti, L. Hurton, H. Singh, H. Huls, S. Olivares, D. A. Lee, R. E. Champlin, and L. J. Cooper. 2013. Bispecific T-cells Expressing Polyclonal Repertoire of Endogenous gammadelta T-cell Receptors and Introduced CD19-specific Chimeric Antigen Receptor. Mol Ther. 21(3): 638-647.(311) © **Nature Publishing Group**

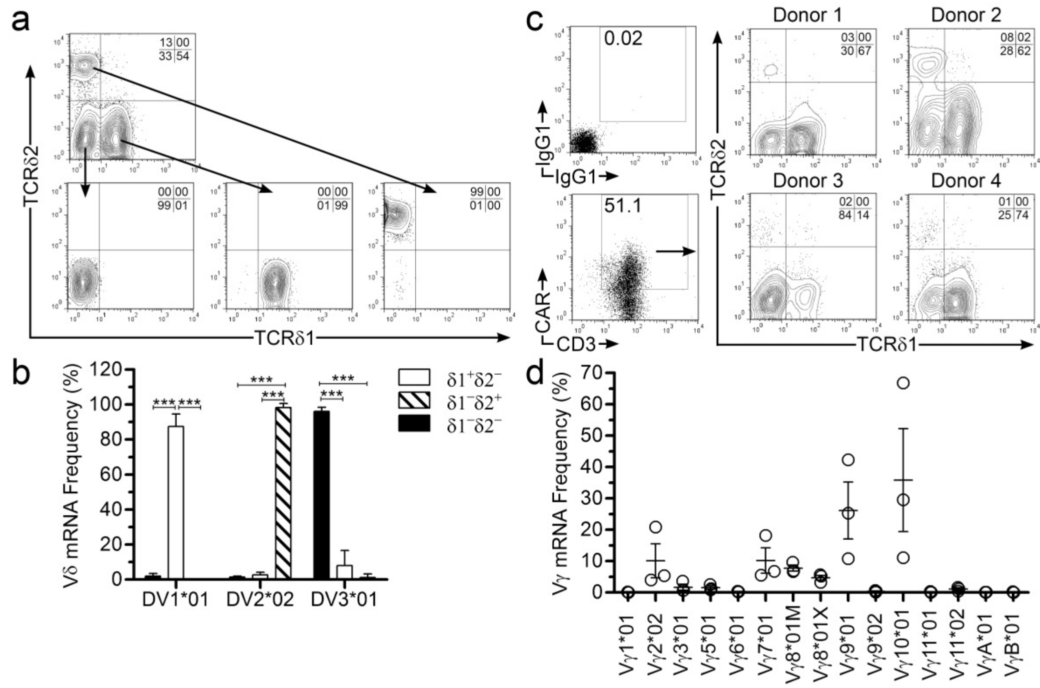

Figure 22. Distribution of Vδ and Vγ in CAR⁺ γδ T cells. (**a**) Representative FACS of Vδ populations (top) into $V\delta1^{neg}V\delta2^{neg}$ (left), $V\delta1^{+}V\delta2^{neg}$ (middle), and $V\delta1^{neg}V\delta2^{+}$ (right) populations and (**b**) Vδ allele mRNA expression in sorted T cells. (**c**) $V\delta1^{neg}V\delta2^{neg}$, $V\delta1^{+}V\delta2^{neg}$, and $V\delta1^{neg}V\delta2^{+}$ frequencies in gated CAR^{+} γδ T-cell populations from four donors. (**d**) Vγ allele mRNA expression in CAR^{+} γδ T cells. Data are mean ± SD (n = 3). Quadrant percentages of flow plots are in upper right corner. **This work was originally published in *Molecular Therapy*** Deniger, D. C., K. Switzer, T. Mi, S. Maiti, L. Hurton, H. Singh, H. Huls, S. Olivares, D. A. Lee, R. E. Champlin, and L. J. Cooper. 2013. Bispecific T-cells Expressing Polyclonal Repertoire of Endogenous gammadelta T-cell Receptors and Introduced CD19-specific Chimeric Antigen Receptor. Mol Ther. 21(3): 638-647.(311) © **Nature Publishing Group**

III.C.4. T cells Produced Pro-inflammatory Cytokines in Response to Stimulation through Endogenous TCRγδ and Introduced CAR

The functional activity of the CAR^+ T cells was assessed by activation with leukocyte activation cocktail (LAC), which was comprised of PMA and Ionomycin. LAC mimics activation through TCR by simulating protein kinase C and increasing intracellular Ca^{2+} to activate phospholipase C (PLC). Measurement of secreted and intracellular cytokines (in the presence of the inhibitor GolgiPlug, which contains Brefeldin A) were performed on genetically modified T cells with and without LAC (**Figure 23a and 23b**). A broad range of cytokines were produced by γδ T cells, with the highest expression of IFNγ, TNFα, and chemokines MIP-1α, MIP-1β, and RANTES (**Figure 23b**). Interleukin-17 (IL17) has been shown to be important for anti-tumor efficacy of γδ T cells and this cytokine was secreted by CAR^+ γδ T cells. These results suggest that TCRγδ can be activated to produce cytokines that could promote inflammation within the tumor. Next, CAR-specific cytokine production was assessed by activation using the murine T-cell lymphoma line EL4 and a genetically modified derivative to enforce expression of human CD19. Both TNFα and IFNγ were produced by CAR^+ γδ T cells in response to CD19 (**Figure 23c**). A less diverse repertoire of cytokines was secreted following CAR stimulation when compared with stimulation of TCRγδ, but IFNγ, TNFα, MIP-1α, MIP-1β, and RANTES were all increased in response to activation through CAR (**Figure 23d**). In aggregate, pro-inflammatory cytokines were upregulated by bi-specific CAR^+ γδ T cells through their TCR and CAR.

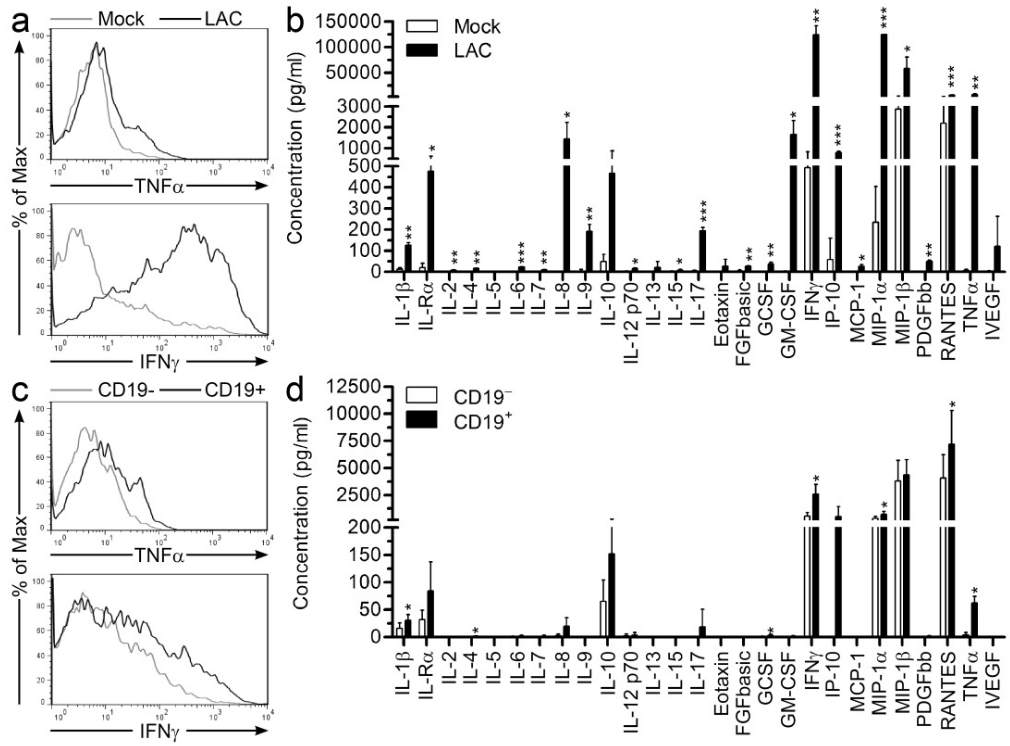

Figure 23. Bi-specific γδ T cells Produce Pro-inflammatory Cytokines when Endogenous TCR and Introduced CAR are Stimulated. (**a**) CAR^+ γδ T cells at Day 35 of co-culture on aAPC were stimulated for 4 hours with a mock cocktail (media alone) or Leukocyte Activation Cocktail (LAC, PMA/Ionomycin) to induce TCR stimulation and then analyzed by flow cytometry. CAR^+ T cells were gated and tumor necrosis factor-α (TNFα, top) and interferon-γ (IFNγ, bottom) production is shown. (**b**) Luminex array (27-Plex) of cytokines secreted by CAR^+ γδ T cells in conditions described in (**a**). (**c**) Similar to (**a**) except that EL4-$CD19^{neg}$ and EL4-$CD19^+$ were used instead of Mock/LAC. (**d**) Same as (**b**) but with EL4-$CD19^{neg}$ and EL4-$CD19^+$ targets. Student's t-test for statistical analysis between (**b**) Mock and LAC and (**d**) EL4-$CD19^{neg}$ and EL4-$CD19^+$ where *$p<0.05$, **$p<0.01$, and ***$p<0.001$. Data are representative of four donors for (**a**) and (**c**) and mean ± SD (n = 3) for (**b**) and (**d**). **This work was originally published in *Molecular Therapy*.** Deniger, D. C., K. Switzer, T. Mi, S. Maiti, L. Hurton, H. Singh, H. Huls, S. Olivares, D. A. Lee, R. E. Champlin, and L. J. Cooper. 2013. Bispecific T-cells Expressing Polyclonal Repertoire of Endogenous gammadelta T-cell Receptors and Introduced CD19-specific Chimeric Antigen Receptor. Mol Ther. 21(3): 638-647.(311) © **Nature Publishing Group**

III.C.5. CAR^{+} γδ T cells Exhibit Enhanced Anti-tumor Effects against CD19^{+} Targets *in vitro*

It was anticipated that γδ T cells would display endogenous cytotoxicity to leukemia cells. Therefore, γδ T cells without CAR were numerically expanded on aAPC in order to test their anti-leukemia activity. Human CD19^{+} B-ALL cell lines (REH, Kasumi2, and Daudi genetically modified to express β2M) were lysed by CAR^{neg} γδ T cells while primary, healthy CD19^{+} B cells were not killed by the same effectors (**Figure 24a**). However, not all B-ALL cell lines were susceptible to efficient lysis by CAR^{neg} γδ T cells. In particular, EL4 and NALM6 cells were largely resistant to cytolysis by γδ T cells. Thus, the ability of the CD19-specific CAR to amplify the inherent anti-tumor activity of γδ T cells was investigated. Enforced expression of CD19 on the surface of EL4 cells improved targeting and killing of this cell line by CAR^{+} γδ T cells at significantly higher ($p = 0.0001$) levels compared with the parental CD19^{neg} EL4 cell line (**Figure 24b**). Similarly, CAR^{+} γδ T cells exhibited improved ability ($p = 0.001$) to kill CD19^{+} NALM6 cells compared with CAR^{neg} γδ T cells (**Figure 24c**). In summary, the introduced CAR enhanced the specific killing capability of genetically modified γδ T cells.

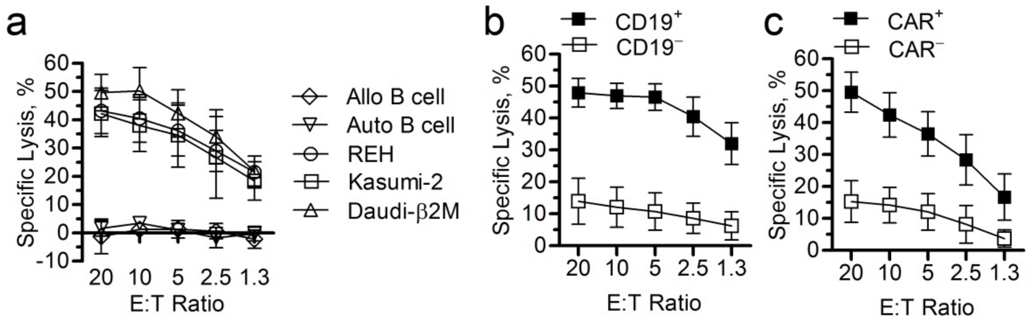

Figure 24. Specific lysis of CD19$^+$ Tumor Cell Lines by CAR$^+$ γδ T cells. (**a**) Standard 4-hour CRA of (**a**) CAR^{neg} γδ T cells against CD19$^+$ B-ALL cell lines (REH, Kasumi2, and Daudi-β2M) or primary CD19$^+$ B cells from autologous (Auto) or allogeneic (Allo) donors, (**b**) CAR$^+$ γδ T cells against EL4-CD19^{neg} (open squares) and EL4-CD19$^+$ (closed squares) tumor cells, and (**c**) CAR^{neg} γδ T cells (open squares) and CAR$^+$ γδ T cells (closed squares) against CD19$^+$ NALM6 tumor cells. Data are mean ± SD from four healthy donors (average of triplicate measurements for each donor) that were pooled from two independent experiments. **This work was originally published in *Molecular Therapy*.** Deniger, D. C., K. Switzer, T. Mi, S. Maiti, L. Hurton, H. Singh, H. Huls, S. Olivares, D. A. Lee, R. E. Champlin, and L. J. Cooper. 2013. Bispecific T-cells Expressing Polyclonal Repertoire of Endogenous gammadelta T-cell Receptors and Introduced CD19-specific Chimeric Antigen Receptor. Mol Ther. 21(3): 638-647.(311) © **Nature Publishing Group**

III.C.6. CAR^+ γδ T cells can Target $CD19^+$ Tumor *in vivo*

The ability of electroporated and propagated γδ T cells to target $CD19^+$ tumor was then investigated *in vivo*. NALM6 is an aggressive $CD19^+$ B-cell leukemia model and immunocompromised mice engrafted with 10^5 NALM6 are moribund in 20 to 25 days when untreated. Control of disseminated NALM6 tumor *in vivo* is dependent on the infused T cells homing to tumor and activating cytolytic machinery in the tumor microenvironment. After adoptive immunotherapy, the burden of tumor was significantly decreased in mice receiving CAR^+ γδ T cells (Donor#4 from **Figure 22c**) compared to untreated mice (**Figure 25**). Mice in treatment group receiving CAR^+ T cells displayed fewer characteristics of the untreated and thus unwell mice, which included lethargy, ruffled coat, temporary hind limb paralysis, and difficulty entering and exiting anesthesia at late stages of the experiment. A uniform date for euthanasia was chosen to measure the anti-tumor effect based on flow cytometry for NALM6 in lymphoid tissue. There was significant anti-tumor activity by the CAR^+ γδ T cell as measured by bioluminescent imaging (BLI) of NALM6-eGFP-*ffLuc* (**Figure 25b**) as exemplified at 22 days after injection of tumor (**Figure 25c**). Non-invasive imaging was corroborated by analysis of presence of tumor cells at necroscopy. Mice that received CAR^+ γδ T cells exhibited significant reductions in tumor burden ($CD19^+eGFP^+$) in the bone marrow, spleen, and peripheral blood (**Figure 25d and 25e**). These data reveal that polyclonal CAR^+ γδ T cells exhibit therapeutic activity *in vivo*.

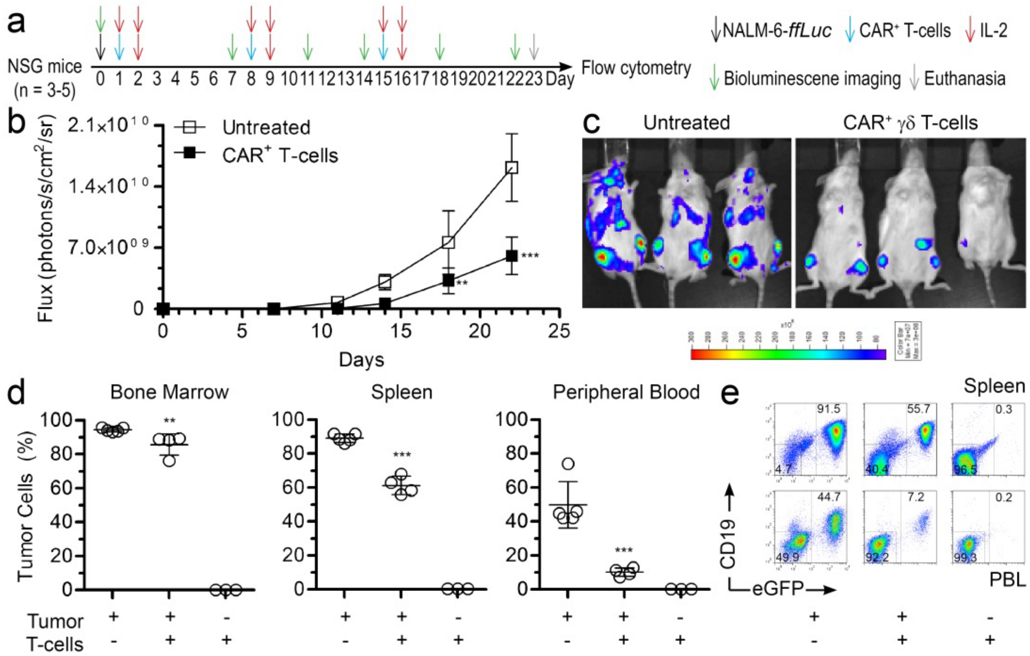

Figure 25. *In vivo* Anti-tumor Activity of CAR^{+} γδ T cells. (**a**) Schematic of experiment. (**b**) BLI derived from eGFP^{+}*ffLuc*$^{+}$CD19^{+} NALM-6 tumor and (**c**) representative images of mice at day 22. (**d**) Post-mortem analysis of tissues and blood where tumor cells (CD19^{+}eGFP^{+}) were detected by flow cytometry. (**e**) Representative flow plots from (**d**). Data are mean ± SD (n = 3 to 5 mice per group, representative of two independent experiments) and gating frequencies in (**e**) are displayed. The percentage of tumor cells is derived from detecting CD19^{+}eGFP^{+} NALM-6 by flow cytometry from post-mortem samples. Statistics performed with (**b**) two-way ANOVA with Bonferroni's post-tests and (**d**) Student's t-test between treated and untreated mice. **p<0.01 and ***p<0.001 **This work was originally published in *Molecular Therapy*.** Deniger, D. C., K. Switzer, T. Mi, S. Maiti, L. Hurton, H. Singh, H. Huls, S. Olivares, D. A. Lee, R. E. Champlin, and L. J. Cooper. 2013. Bispecific T-cells Expressing Polyclonal Repertoire of Endogenous gammadelta T-cell Receptors and Introduced CD19-specific Chimeric Antigen Receptor. Mol Ther. 21(3): 638-647.(311) © **Nature Publishing Group**

III.D. Discussion

III.D.1. Polyclonal Bi-specific T cells for Immunotherapy

We established that introduction of a 2nd generation CAR could (i) drive the numeric expansion of T cells independent of usage of TCRγδ chains and (ii) augment the lytic potential of $CD19^+$ tumors by γδ T cells. Propagating bi-specific CAR^+ T cells with a broad diversity of TCRγδ chains is desirable based on their therapeutic potential. Indeed, γδ T cells other than those expressing Vγ9Vδ2 have been generated from PBMC using TCRγδ-specific and CD3-specific mAbs.(313-315) These prior approaches did not comprehensively measure TCRγδ isotype expression nor did they yield Vδ1 and Vδ3 at frequencies as high as seen in this study. The Vγ2 TCR chain was detected on our T cells, which has been described to pair with Vδ2, and these T cells can have antigen presentation capabilities.(166) Our CAR^+ γδ T cells expressed molecules consistent with antigen presentation, *e.g.*, CD86, CD137L, and HLA-DR (data not shown), and Vγ9Vδ2 cells have served as aAPC for αβ T cells.(298) Future experiments will investigate if our polyclonal CAR^+ γδ T cells also have an ability to serve as aAPC. Also present were T-cell sub-populations expressing Vγ7, and Vγ8, and Vγ10, where the first two chains have been associated with intestinal intraepithelial lymphocytes (iIEL)(316, 317) and the latter chain's functional significance is not yet apparent. In all, our approach is the first to report expansion of CAR^+ T cells that maintained a polyclonal TCRγδ expression.

III.D.2. Changes Observed in Vδ Populations Following Expansion on aAPC

The repertoire of TCRγδ chains employed by CAR[+] T cells was similar to the initial pool of γδ T cells in PBMC with two exceptions. We noted an increase in Vδ3 usage, but this may be advantageous as it is associated with specificity for viruses that could offer enhanced immune responses to viral infections in immunocompromised patients receiving therapy.(165) A decrease in Vγ9Vδ2 usage was also observed compared to the starting frequency of this TCR in PBMC, but this could potentially be increased by priming aAPC with Zol to increase Vγ9Vδ2 ligand expression in the co-culture. Whether this loss of Vγ9Vδ2 TCR expression was due to preferential activation induced cell death or selective out-growth of T cells expressing Vδ1 and Vδ3 TCR is not known. Nonetheless, Vγ9Vδ2 chains were still present in the final T-cell cultures indicating that aminobisphosphonate therapy could drive expansion of this subset of T cells after administration.

III.D.3. Improvements upon CAR Expression on γδ T cells

Recombinant retroviruses have been previously employed to achieve stable expression of CARs in γδ T cells, but this required using an aminobisphosphonate to achieve numeric expansion of T cells before transduction.(175, 318) We now demonstrate propagation of T cells after, rather than before, gene transfer using SB-mediated transposition results in a polyclonal population of bi-specific γδ T cells capable of CAR-mediated (i) production and secretion of pro-inflammatory cytokines in response to CD19, (ii) enhanced lysis of $CD19^+$ tumor targets, and (iii) *in vivo* anti-tumor activity against a $CD19^+$ tumor. The ability of these T cells to exhibit effector functions was not correlated to a particular Vδ or Vγ usage as cells with different Vδ TCR frequencies (**Figure 22c**) produced the same cytokines (**Figure 23**) and displayed similar cytolysis of $CD19^+$ targets (**Figure 24b**). We noted that frequency of CAR expression was more variable on γδ T cells compared with αβ T cells. This was likely due to an endogenous ability of K562 cells to sustain proliferation of γδ T cells independent of CAR. Nevertheless, adoptive transfer of γδ T cells of which 60% expressed CAR could still yield the same *in vitro* lytic ability as 98% CAR^+ γδ T cells (**Figure 26**). This indicated that (i) CAR^+ γδ T cells are potent tumor killers and (ii) >90% CAR expression may not be a critically limiting parameter for predicting therapeutic efficacy. Nonetheless, we are undertaking improvements to increase the expression of CAR on propagated γδ T cells. Furthermore, the chimeric signaling molecules in the CAR endodomain could be specifically designed to enhance triggering of γδ T cells. For example, γδ T cells can be activated through FcγRIIIA (CD16) in the TCR complex,(319) which raises the possibility that signaling through chimeric FcRγ (as compared with CD3ζ in our current design) in a CAR endodomain may improve activation. However, CD16 was not detected on CAR^+ γδ T cells in this study (data not shown). Since clinical responses against $CD19^+$ lymphocytic leukemia have been achieved with T cells expressing a CAR that signaled through 4-1BB (CD137) endodomain,(7, 32) another option is to swap CD28 for CD137 for activation of γδ T cells.

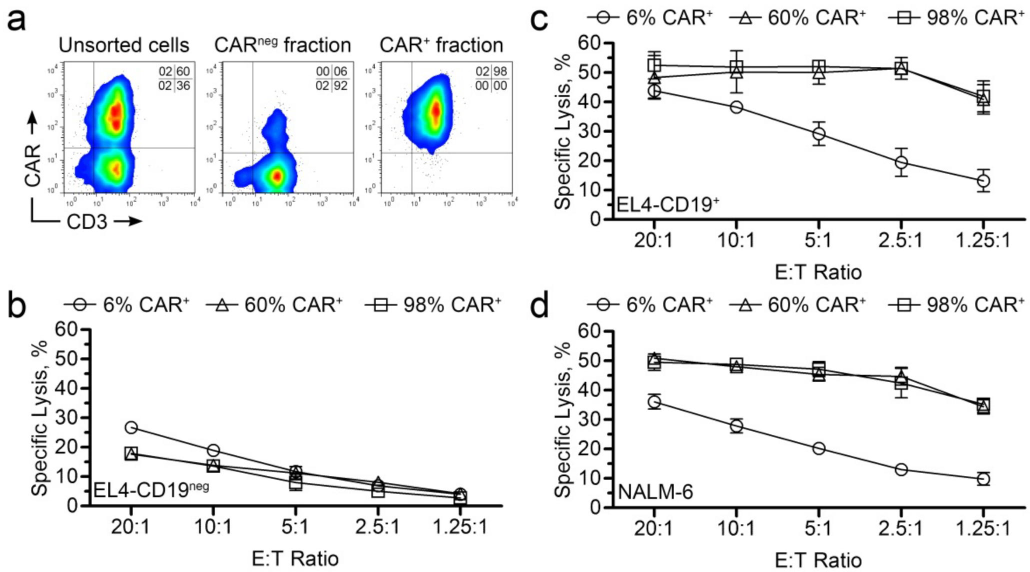

Figure 26. Specific Lysis of CD19^{+} Tumor Cell Lines by CAR^{+}, CAR^{++}, and CAR^{+++} γδ T cells. (**a**) Phenotype of T cells at day 19 of co-culture either unsorted (left) or from CAR sorting at day 15 where CAR^{neg} and CAR^{+} fractions are displayed in the middle and right, respectively. Four-hour CRA (Day 19 of co-culture on aAPC) of γδ T cells genetically modified to enforce expression of CD19-specific CAR with 6% (CAR^{+}, circles), 60% (CAR^{++}, triangles), and 98% (CAR^{+++}, squares) expression of CAR targeting (**b**) EL4-CD19^{neg}, (**c**) EL4-CD19^{+}, and (**d**) CD19^{+} NALM-6 tumor cells. Data are mean ± SD (n = 3). Quadrant frequencies of flow plots are displayed. **This work was originally published in *Molecular Therapy*.** Deniger, D. C., K. Switzer, T. Mi, S. Maiti, L. Hurton, H. Singh, H. Huls, S. Olivares, D. A. Lee, R. E. Champlin, and L. J. Cooper. 2013. Bispecific T-cells Expressing Polyclonal Repertoire of Endogenous gammadelta T-cell Receptors and Introduced CD19-specific Chimeric Antigen Receptor. Mol Ther. 21(3): 638-647.(311) © **Nature Publishing Group**

III.D.4. Improvements on Type of γδ T cell used for CAR Immunotherapy

In addition to improving CAR expression on γδ T cells, the type of γδ T cell arising after electroporation with SB system and propagation on aAPC could be manipulated to further improve anti-tumor activity. For instance, some γδ T cells were observed to secrete IL17, a pro-inflammatory cytokine that has potent, yet context-dependent, anti-tumor effects.(320-324) IL17 producing lineages of T cells can be mutually exclusive from those that secrete IFNγ.(325) Inducible co-stimulator of T cells (ICOS) leads to IL17 polarization in $CD4^+$ T cells and CD28 co-stimulation overcame this effect to dictate that $CD4^+$ T cells now produce IFNγ.(326) CD86 is one of the co-stimulatory molecules on our aAPC and the majority of CAR^+ γδ T cells secrete IFNγ in response to CD19 with diminished production of IL17. Furthermore, the CAR contains a chimeric CD28 endodomain which may contribute to IFNγ polarization in genetically modified T cells. Substitution of chimeric CD28 for ICOS in the CAR and replacement of CD86 on the aAPC with ICOS-ligand (ICOSL) could potentially reverse the polarization to IL17. Given that we can propagate CAR^+ γδ T cells on aAPC we are prepared to design aAPC to evaluate whether we can skew the cytokine profile to reflect the propagation of desired T-cell subsets.

III.D.5. Clinical Significance of Bi-specific T cells

The human application of CAR^+ γδ T cells is appealing given their inherent potential for anti-tumor effects and their apparent lack of alloreactivity.(304) The CAR, SB system, and aAPC are all already in use in our clinical trials. Therefore, we plan to modify our manufacturing scheme in compliance with current good manufacturing practice to generate bi-specific CAR^+ γδ T cells. Our data provides a clinically-appealing approach to numerically expand and manipulate CAR^+ T cells with multiple Vγ and Vδ pairings enabling clinical trials to evaluate their therapeutic potential."

This work was adapted from published work in *Molecular Therapy*. Deniger, D. C., K. Switzer, T. Mi, S. Maiti, L. Hurton, H. Singh, H. Huls, S. Olivares, D. A. Lee, R. E. Champlin, and L. J. Cooper. 2013. Bispecific T-cells Expressing Polyclonal Repertoire of Endogenous gammadelta T-cell Receptors and Introduced CD19-specific Chimeric Antigen Receptor. Mol Ther. 21(3): 638-647.(311) © **Nature Publishing Group**

CHAPTER IV

Artificial Antigen Presenting Cells Propagate Polyclonal Gamma Delta T cells with Broad Anti-tumor Activity

IV.A. Hypothesis and Rationale

The *hypothesis* of this chapter is that aAPC will expand polyclonal γδ T cells that will have broad anti-tumor immunity. The *rationale* for this chapter is that (i) CAR^{neg} polyclonal γδ T cells proliferated in parallel to CAR^{+} γδ T cells described in **Chapter III** on aAPC, (ii) no current expansion protocols exist for polyclonal γδ T cells for the clinic, (iii) aAPC are currently in clinical trials and are available as a master cell bank in the manufacturing facility at MD Anderson, (iv) γδ T cells expressing Vδ1 are correlated with long-term remissions in cancer therapy but have not been directly infused as an adoptive immunotherapy, (v) γδ T cells expressing Vδ2 have shown anti-tumor effects as direct adoptive immunotherapies, (vi) γδ T cells expressing Vδ3 have not been described to have direct anti-tumor immunity leaving a gap in the field of knowledge, and (vii) a polyclonal approach to γδ T cell immunotherapy could target multiple ligands on the tumor through a diverse repertoire of TCRγδ. Therefore, development of an expansion protocol to generate clinically-relevant numbers of polyclonal γδ T cells would have implications as both cancer immunotherapies and for immunologists studying γδ T cells.

IV.B. Introduction

Human γδ T cells exhibit inherent anti-tumor activity and hold promise for immunotherapy of cancer. They are distinguished by the heterodimeric pairing of γ and δ T-cell receptor (TCR) chains from the more prevalent αβ T cell lineage (~95% of circulating T cells), which are defined by TCRα/TCRβ heterodimers.(327) TCRαβ recognizes peptide complexed with MHC but TCRγδ ligands are recognized independent of MHC restriction.(141, 146, 152) Many of these ligands are present on cancer cells, thus raising the possibility that a culturing approach to propagating T cells that maintains a polyclonal repertoire of γδ TCRs may have appeal for human application.

γδ T cells represent 1% to 5% of the T-cell pool in peripheral blood, and many standard T cell expansion protocols are not applicable to γδ T cells.(314, 328) Proliferation of monoclonal γδ T cell populations (Vγ9Vδ2) can be sustained with aminobisphosphonates, e.g. Zol, and clinical trials investigating their anti-tumor efficacy have yielded objective responses treating both solid and hematological cancers.(175, 179, 301) However, this subset of γδ T cells was not curative as a stand-alone therapy.(318) Novel polyclonal γδ T cell expansion protocols are needed to improve upon these findings, but are lacking in clinically-relevant methods to expand multiple γδ T cell subsets in one cellular therapy product.

Since many ligands that signal through γδ TCR are unknown, we hypothesized that a tumor cell line may serve as a cellular substrate for activating these T cells and sustaining their proliferation. aAPC are used to stimulate CAR^+ T cell growth *ex vivo* and are derived from K562 cells, a natural cytolytic target of γδ T cells.(57, 280, 310, 329) As seen in **Chapter III**, CAR-modified γδ T cells expanded on aAPC while expressing multiple TCRγδ alleles and displayed enhanced cytolysis to antigen-positive tumors.(311) Moreover, γδ T cells not expressing CAR were present in CAR^+ γδ T cell cultures in high frequencies (**Figure 19a, bottom right panels**). Therefore, we hypothesized that γδ T cells could expand on aAPC independent of CAR^+ T cells and that these γδ T cells would maintain a polyclonal TCRγδ repertoire. Given that the aAPC are available as a master-cell bank, these data provide a translational pathway for adapting γδ T cells for human application. Thus, this could be the first time that polyclonal γδ T cells could be used for cancer immunotherapy.

IV.C. Results

IV.C.1. Propagation of γδ T cells on aAPC

As seen in **Chapter III**, aAPC clone#4 sustained the proliferation of γδ T cells in cultures containing CD19-specific CAR^+ γδ T cells.(311) To assess whether γδ T cells could numerically expand on aAPC without expression of CAR, quiescent γδ T cells were isolated from peripheral blood and stimulated by recursive additions of γ-irradiated aAPC clone#4 in presence of IL2 and IL21 (**Figure 27a**). It was observed that γδ T cells represented a small fraction of PBMC (3.2% ± 1.2%; mean ± SD; n = 4), but after 22 days of co-culture on aAPC the cultures contained a homogeneous population of γδ T cells (97.9% ± 0.6%) as assessed by co-expression of CD3 and

TCRγδ (**Figure 27b**). Cultures yielded $>10^9$ γδ T cells from $<10^6$ total cells in three weeks of co-culture (**Figure 27c**), which represented a $4.9 \times 10^3 \pm 1.7 \times 10^3$ fold increase over a 22-day culture period. Although γδ T cells were rare in peripheral blood, they were readily sorted then expanded on aAPC to sufficient numbers for experiments and potential clinical application.

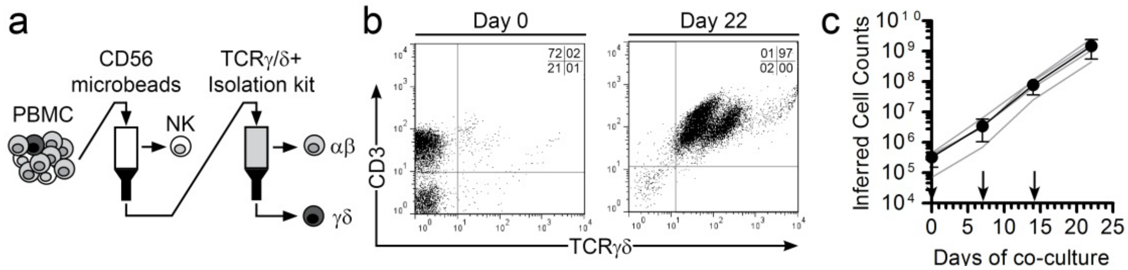

Figure 27. Sustained Proliferation of γδ T cells on aAPC and IL2/21. (**a**) Schematic of experimental design where NK cells are in open shapes, αβ T cells are in light gray shapes, and γδ T cells are in dark gray shapes. Columns represent paramagnetic isolation. (**b**) Expression by flow cytometry of CD3 (y-axis) and TCRγδ (x-axis) in PBMC prior to isolation of γδ T cells isolation (Day 0) and after 22 days of co-culture on aAPC/IL2/IL21. One representative donor is shown and quadrant gate frequencies are displayed in the upper right corners of flow plots. (**c**) Total inferred cell counts of viable cells during co-culture period. Black lines are mean ± *S*D from 4 healthy donors, gray lines are individual donors, and arrows represent addition of γ-irradiated aAPC.

IV.C.2. Roles for Co-stimulation and Cytokine Support in γδ T cell Proliferation on aAPC

The mechanism of γδ T cell proliferation on aAPC was unknown. Addition of cytokines and co-stimulation by aAPC were likely candidates for supporting growth of on aAPC. In order to assess which surface molecules on the clone#4 aAPC (membrane-bound IL15 (mIL15), CD86, and CD137L) were important for γδ T cell expansion with IL2 and IL21, parental K562 cells were genetically modified to express (i) mIL15 (cloneA6), (ii) mIL15 and CD86 (clone A3), or (iii) mIL15 and CD137L (clone D4) and were subcloned for uniform transgene expression (**Figure 28a**). Co-cultures with exogenous IL2 and IL21 were initiated with γδ T cells and γ-irradiated (i) parental K562 cells, (ii) clone A6 aAPC, (iii) clone A3 aAPC, (iv) clone D4 aAPC (**Figure 28b**), or (v) clone#4 aAPC (**Figure 8 middle panels**) in parallel with T cells receiving cytokines only. IL2 and IL21 in combination sustained limited γδ T cell proliferation, which was increased when K562 cells were added to co-cultures. Slightly less expansion was observed when either mIL15 or mIL15 and CD86 were added to K562 cells. However, significantly higher γδ T cell propagation was only observed with mIL15$^+$CD137L$^+$ and mIL15$^+$CD86$^+$CD137L$^+$ aAPC over IL2 and IL21 alone. After establishing that co-stimulation on aAPC was necessary for γδ T cell proliferation, IL2 and IL21 were added separately or in combination to assess their contribution to growth on clone#4 aAPC. No γδ T cell expansion was observed when both IL2 and IL21 were removed from co-cultures, addition of IL2 alone resulted in more proliferation than IL21 alone, and combination of both IL2 and IL21 displayed additive growth of γδ T cells (**Figure 28c**).

This validated our approach to use both IL2 and IL21 for maximum γδ T cell yield following co-culture on clone#4 aAPC and strongly suggested that both aAPC co-stimulation and cytokine support were critical for maximum γδ T cell proliferation *ex vivo*.

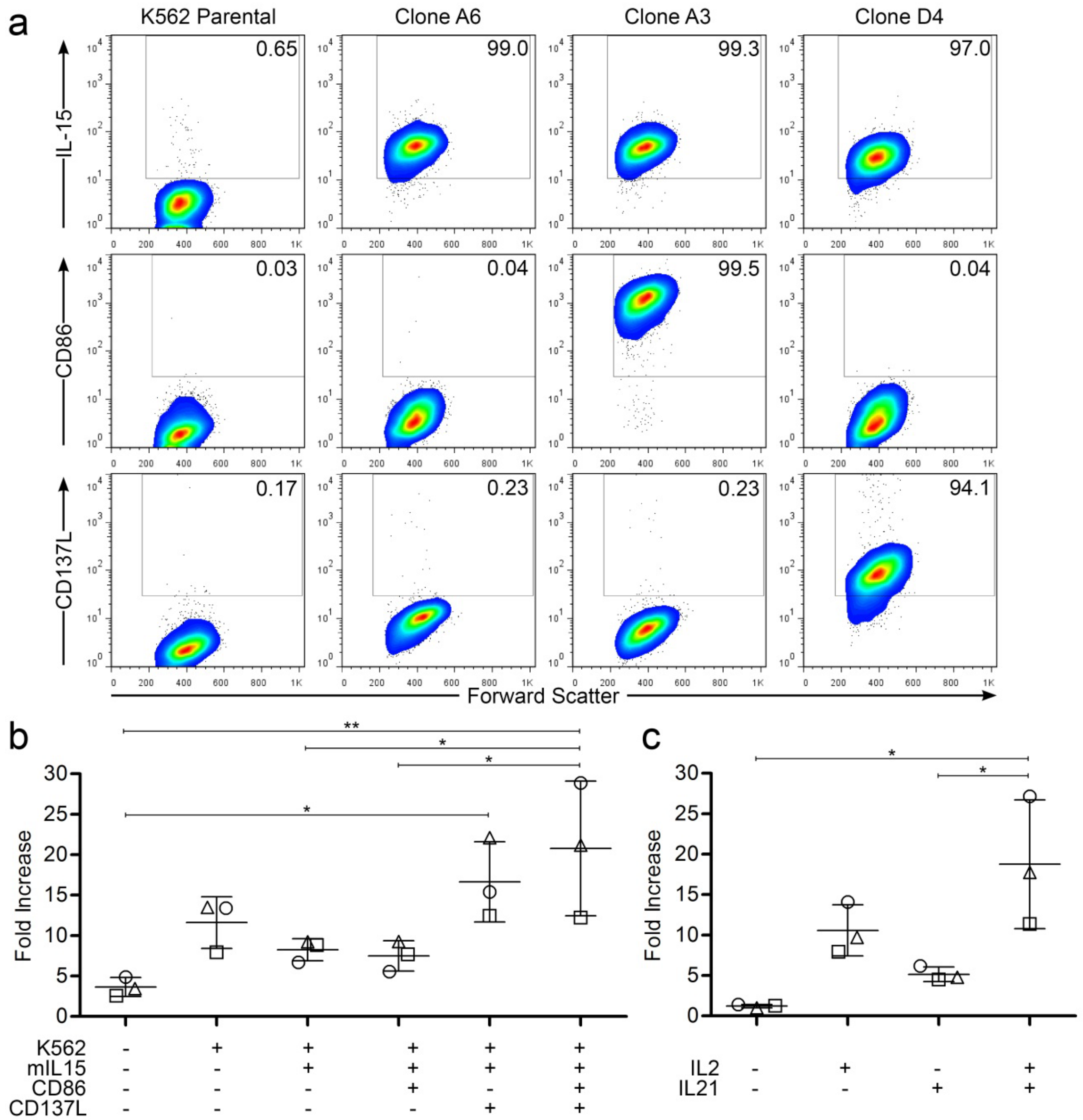

Figure 28. Co-stimulation and Cytokine Requirements for γδ T cell Expansion on aAPC *ex vivo*. (**a**) Surface phenotype of aAPC expressing single co-stimulatory molecules with membrane-bound IL15 (mIL15). (**b**) γδ T cell proliferation was measured after 10 days of growth with IL2 and IL21 on (i) no aAPC, (ii) parental K562 cells, (iii) $mIL15^+$ aAPC (clone A6), (iv) $mIL15^+CD86^+$ aAPC (clone A3), (v) $mIL15^+CD137L^+$ aAPC (clone D4), or (vi) clone#4 aAPC. All aAPC were γ-irradiated prior to co-culture. (**c**) Co-cultures were initiated with clone#4 aAPC and either (i) no cytokines, (ii) 50 U/mL IL2, (iii) 30 ng/mL IL21, or (iv) 50 U/mL IL2 and 30 ng/mL IL21. Fold changes were calculated relative to the input cell numbers. Two-way ANOVA with Bonferroni's post-tests was used for statistical analysis. *$p<0.05$ and **$p<0.01$

IV.C.3. UCB-derived γδ T cells Expansion on aAPC

Umbilical cord blood (UCB) is a source of γδ T cells with unique use for immunotherapy because they have limited immunological education and thus potential utility in allogeneic settings. Moreover, UCB-derived γδ T cells should have a younger phenotype and could (theoretically) have a longer range of responsiveness before anergizing or undergoing senescence. However, UCB has limited volumes and γδ T cells are a small fraction of an already limited resource. Fluorescence activated cell sorting (FACS) was used to isolate γδ T cells in order to maximize yields and purity of this valuable resource. Indeed, clone#4 aAPC induced substantial proliferation of γδ T cells derived from UCB (**Figure 29a**). After 35 days of co-culture on clone#4 with IL2 and IL21, there was a 10 million-fold increase in cell number as an average of 10^{11} UCB-derived γδ T cells (Range: $6x10^9$ – $3x10^{11}$; n = 5) were propagated from just 10^4 γδ T cells at the start of the culture. Because few cells were isolated (10^4 per donor), two more stimulations were performed for UCB compared to PBMC to highlight their potential for proliferating to clinically relevant numbers. As expected, γδ T cell cultures were pure as assessed by uniform expression of CD3 (**Figure 29b**) and TCRγδ (**Figure 29c**) without expression of TCRαβ (**Figure 29d**) or presence of $CD3^{neg}CD56^{+}$ NK cells (**Figure 29b**). Collectively, these data demonstrate that aAPC clone#4 when used with IL2 and IL21 could sustain the proliferation of γδ T cells *ex vivo* from limited starting populations.

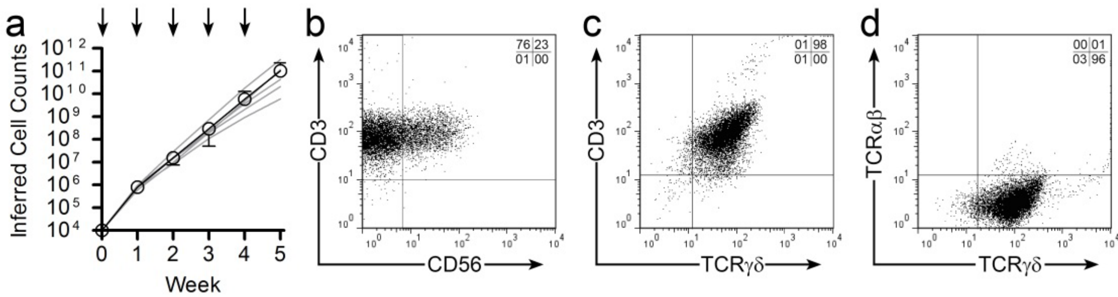

Figure 29. Expansion of UCB-derived γδ T cells on aAPC. γδ T cells were sorted by FACS following staining with CD3 and TCRγδ and were stimulated weekly with clone#4 aAPC, IL2, and IL21 (**a**) Total inferred cell numbers from co-cultures where black line represents mean ± SD (n = 5) and gray lines are individual donors. Arrows represent stimulations with aAPC. Expression of (**b**) CD3 (y-axis) and CD56 (x-axis), (**c**) CD3 (y-axis) and TCRγδ (x-axis), and (**d**) TCRαβ (y-axis) and TCRγδ (x-axis) of one representative donor by flow cytometry after 5 weeks of expansion on aAPC with IL2 and IL21. Quadrant frequencies are displayed in upper right corners.

IV.C.4. Frequency of γ and δ TCR Usage in aAPC-propagated γδ T cells

Previously, CAR$^+$ γδ T cells expanded on clone#4 aAPC maintained polyclonal repertoire of TCRγ and TCRδ chains, and γδ T cells proliferating in parallel to CAR$^+$ γδ T cells also maintained polyclonal TCRγδ distribution (**Chapter III**).(311) Whether the aAPC-expanded γδ T cells would do the same was of great interest, because if so then this would represent the first ever clinically-viable approach to expand multiple γδ T cells subsets in one cellular product for cancer therapy.

IV.C.4.a. Vδ and Vγ mRNA Expression

Now that it is established that γδ T cells can expand on aAPC independently of CAR$^+$ T cells (**Figures 27, 28, and 29**), the TCR isotype variable (V) region repertoire was evaluated at the mRNA level by DTEA. As anticipated, mRNA species for all three Vδ alleles were identified (**Figure 30a**) and Vγ2, Vγ5, Vγ7, Vγ8 (two alleles), Vγ9, Vγ10, and Vγ11 mRNA species were co-expressed in the aAPC-expanded γδ T cells from PBMC (**Figure 30b**). Similar polyclonal TCR expression of Vδ (**Figure 30c**) and Vγ (**Figure 30d**) was observed in γδ T cells expanded from UCB with fewer Vδ2 cells, more Vγ2 and Vγ5 cells, and presence of Vγ3 cells not seen in PBMC. Thus, aAPC are able to repeatedly expand γδ T cells with polyclonal TCR repertoire from both PBMC and UCB.

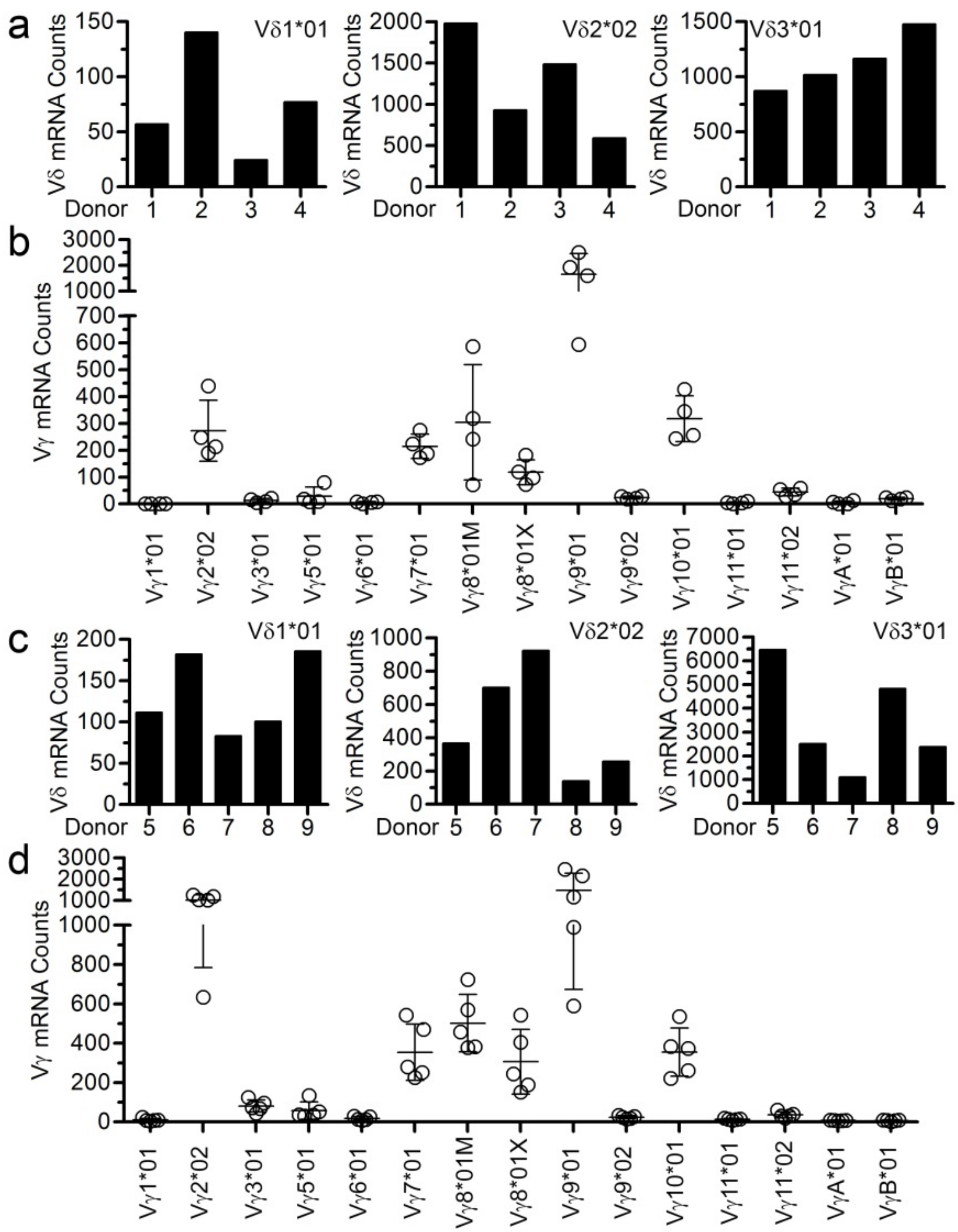

Figure 30. Pattern of Vδ and Vγ mRNA Usage on aAPC-expanded γδ T cells. Quantification of mRNA species coding for (**a**) Vδ1*01, Vδ2*02, and Vδ3*01 alleles from left to right, respectively, and (**b**) Vγ alleles in PBMC-derived γδ T cells by DTEA at day 22 of co-culture on aAPC/IL2/IL21. Each circle represents an individual donor's γδ T cells and lines show mean (horizontal) ± SD (vertical). Quantification of mRNA species coding for (**c**) Vδ and (**d**) Vγ alleles in UCB-derived γδ T cells by DTEA at day 34-35 of co-culture on aAPC/IL2/IL21 as described for PBMC. Numbers correlate with identification of PBMC (1-4) and UCB (5-9) donors described further in **Figure 31**.

IV.C.4.b. TCRγδ Surface Protein Expression

After establishing Vγ and Vδ mRNA expression from a number of different isotypes, surface expression of TCRγ and TCRδ was investigated. However, there are only 3 commercially available antibodies specific for individual TCRγδ isotypes, which are specific for TCRδ1, TCRδ2, and TCRγ9. As was seen in CAR^{+} γδ T cells, aAPC-expanded γδ T cells from PBMC stained for all three Vδ populations ($TCR\delta1^{+}TCR\delta2^{neg}$, $TCR\delta1^{neg}TCR\delta2^{+}$, and $TCR\delta1^{neg}TCR\delta2^{neg}$), corroborating DTEA detection of Vδ1, Vδ2, and Vδ3 populations of γδ T cells, respectively (**Figure 31a**). Moreover, TCRδ expression frequencies followed the trend of TCRδ1>TCRδ3>TCRδ2, and most TCRδ2 chains paired with TCRγ9 (**Figure 31b**). Fewer TCRδ2 cells were seen in UCB-derived γδ T cells (**Figure 31c**) compared to PBMC-derived γδ T cells (**Figure 31a**), but UCB-derived γδ T cells followed the same TCRδ1>TCRδ3>TCRδ2, trend and most Vδ2 paired with Vγ9 as expected (**Figure 31d**). Analysis of other Vγ pairings with Vδ could not be performed because there are no other Vγ-specific commercially antibodies available. Thus, aAPC-expanded γδ T cells were polyclonal at both mRNA and protein levels, and this protocol therefore represents the first clinically-relevant expansion approach of polyclonal γδ T cells.

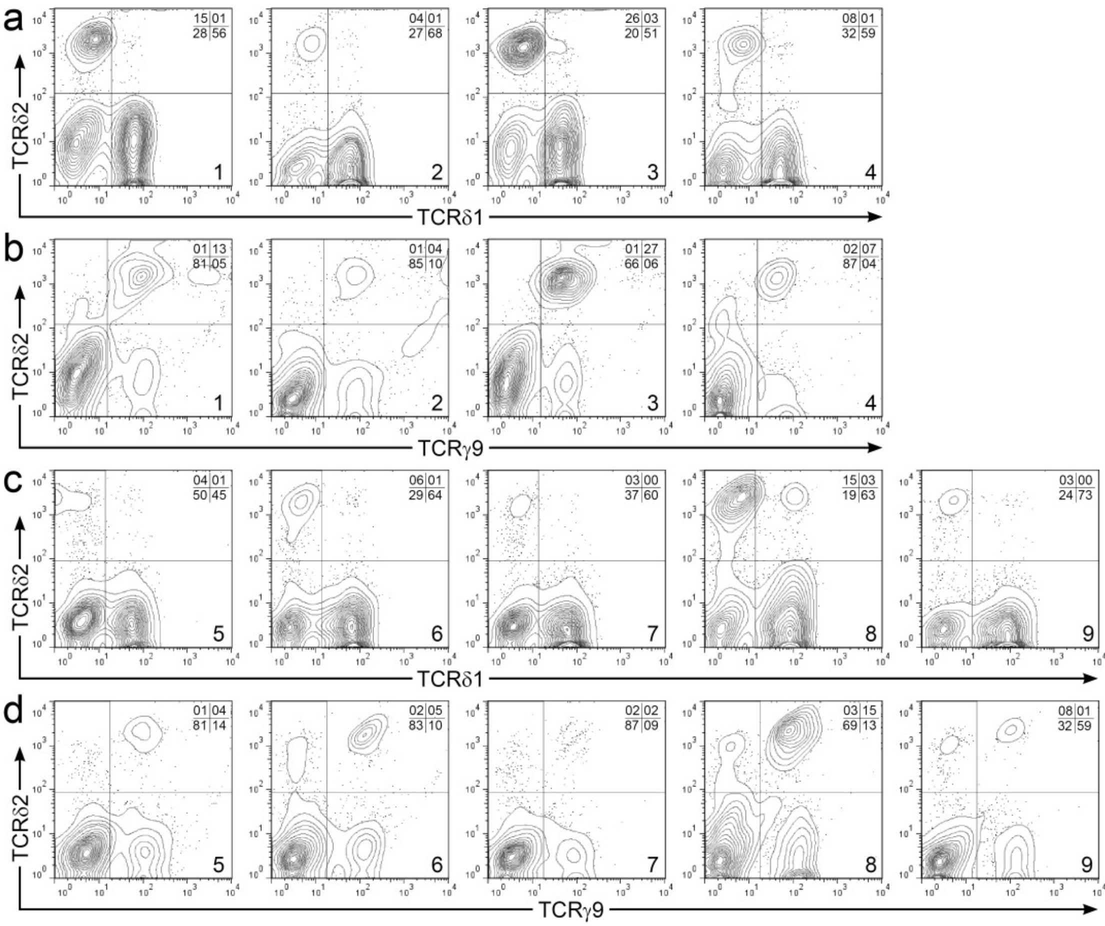

Figure 31. TCRδ and TCRγ Isotype Surface Expression on aAPC-expanded γδ T cells. Expression by flow cytometry of (**a**) TCRδ2 (y-axes) and TCRδ1 (x-axes) and (**b**) TCRδ2 (y-axes) and TCRγ9 (x-axes) in PBMC-derived γδ T cells at day 22 of co-culture on aAPC/IL2/IL21. Expression by flow cytometry of (**c**) TCRδ2 (y-axes) and TCRδ1 (x-axes) and (**d**) TCRδ2 (y-axes) and TCRγ9 (x-axes) in UCB-derived γδ T cells at day 35 of co-culture on aAPC/IL2/IL21. Numbers in lower right corners correlate with identification of PBMC (1-4) and UCB (5-9) donors also shown in **Figure 30** and quadrant frequencies are displayed in upper right corners.

IV.C.4.c. Validation of Vδ3 Subset and Vδ Lineage Propagation

Little is known about the Vδ3 lineage of γδ T cells and no reports have been made to date about their role in anti-tumor immunity. Because this study has implications for showing the first ever evidence that this subset can mediate anti-tumor effects, further validation that the TCRδ1^{neg}TCRδ2^{neg} cells were, in fact, Vδ3 cells was warranted. Complicating this matter is the fact that no commercially available antibodies for TCRδ3. However, an indirect means was successfully used by combining FACS and DTEA. As there are only three Vδ populations in humans and there are antibodies to two of the isoforms, a combination of DTEA and FACS was used to in two ways to confirm the various populations. First, γδ T cells expanded in the presence of CAR^{+} T cells (**Chapter III**) were sorted for TCRδ1^{+}TCRδ2^{neg}, TCRδ1^{neg}TCRδ2^{+}, and TCRδ1^{neg}TCRδ2^{neg} γδ T cells populations by FACS and they expressed only Vδ1*01, Vδ2*02, and Vδ3*01 mRNA, respectively (**Figure 22a and 22b**).(311) The second approach directly applied the same techniques to γδ T cells expanded on aAPC as described in **Chapter IV** without CAR^{+} T cells. Again, TCRδ1^{+}TCRδ2^{neg}, TCRδ1^{neg}TCRδ2^{+}, and TCRδ1^{neg}TCRδ2^{neg} populations isolated by FACS consisted primarily of Vδ1*01, Vδ2*02, and Vδ3*01 mRNA, respectively, and were therefore denoted Vδ1, Vδ2, and Vδ3, respectively (**Figure 32a**). It is important to note that Vδ1*01 only resulted in ~150 mRNA counts whereas Vδ2*02 and Vδ3*01 ranged in the ~1000-2000 mRNA count range (**Figure 30**), so the purity as measured by mRNA counts appeared to have contaminating Vδ1 cells in Vδ2 and Vδ3 populations but these populations were minor contributors in the Vδ1 population as measured by flow cytometry (**Figures 32d and 32e**). Furthermore, the FACS sorted Vδ1, Vδ2, and Vδ3 populations were expanded on clone#4 aAPC in the presence of exogenous IL2 and IL21 as separate co-cultures populations and even after 15 days of isolated growth the same Vδ mRNA signatures were observed suggesting the cells remained pure during propagation (data not shown). As expected, all three Vδ populations proliferated well on aAPC as separate populations (**Figure 32b**), where fold increase capability was ranked as Vδ1>Vδ3=Vδ2 although there were no statistically different differences (**Figure 32c**). Indeed, there are more Vδ1 cells in polyclonal populations (**Figures 30 and 31**), which may be due to a slight increase in their ability to proliferate on aAPC. Importantly, populations expressed the appropriate TCR alleles on the γδ T cell surface where Vδ1, Vδ2, and Vδ3 subsets were pure for $TCR\delta1^{+}TCR\delta2^{neg}$, $TCR\delta1^{neg}TCR\delta2^{+}$, and $TCR\delta1^{neg}TCR\delta2^{neg}$, respectively, after 15 days of isolated expansion on aAPC (**Figure 32d and 32e**). All separated Vδ subsets co-expressed CD3 and TCRγδ verifying that they were, in fact, γδ T cells (**Figures 34a and 34b**). Collectively, these results showed that (i) $TCR\delta1^{neg}TCR\delta2^{neg}$ γδ T cells contained the Vδ3 lineage, (ii) DTEA accurately measured Vδ mRNA, (iii) Vδ1, Vδ2, and Vδ3 lineages are stimulated by aAPC leading to their proliferation, and (iv) aAPC-expanded γδ T cells are truly polyclonal.

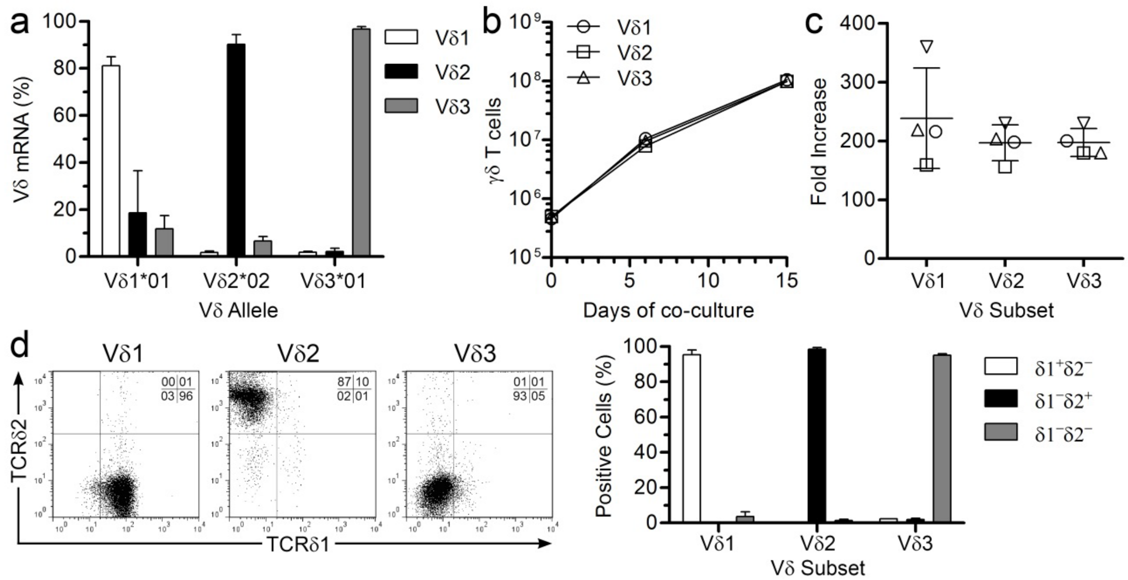

Figure 32. Vδ Subset Separation, Propagation, and Resultant TCR Expression on Sorted T cells. PBMC were sorted for γδ T cells with paramagnetic beads and were expanded for 2 weeks on aAPC/IL2/IL21. They were then sorted into three populations (Vδ1, Vδ2, and Vδ3) by FACS. Separated populations were stimulated for 2 weeks on aAPC/IL2/IL21. (**a**) DTEA detection of Vδ1*01, Vδ2*02, and Vδ3*01 mRNA species in Vδ1, Vδ2, and Vδ3 subsets following FACS purification. (**b**) Proliferation of Vδ lineages on aAPC as separated populations. (**c**) Fold increases of each Vδ population where each shape represents a different donor. (**d**) Representative flow cytometry plots of TCRδ1 (x-axes) and TCRδ2 (y-axes) expression in Vδ1, Vδ2, and Vδ3 subsets (from left to right). Quadrant frequencies are displayed in upper right corner. (**e**) Frequencies of TCRδ1^{+}TCRδ2^{neg}, TCRδ1^{neg}TCRδ2^{+}, and TCRδ1^{neg}TCRδ2^{neg} cells in Vδ1, Vδ2, and Vδ3 subsets. Data are mean ± SD (n = 3-4).

IV.C.5. Immunophenotype of γδ T cells Expanded on aAPC

Functional outcomes, e.g. memory formation, homing to tissues, and effector mechanism, can be predicted by the expression of lymphocyte-specific proteins on the T cell surface. Thus, a panel of markers was used to identify the immunophenotype of γδ T cells cultures first as a polyclonal population to be used as therapy and then as sorted Vδ populations to gain insight into lineage differences.

IV.C.5.a. Immunophenotype of Polyclonal γδ T cell Population

The ultimate goal for the clinic is to use a polyclonal population of T cells for immunotherapy in order to have a multivariate approach to cancer immunotherapy, so extensive phenotyping of the γδ T cell surfaces was performed as a mixed Vδ population. After 22 days of co-culture on aAPC, few αβ T cells (TCRαβ) and NK cells ($CD3^{neg}CD56^{+}$) were detected in the cultures where strong staining for γδ T cells (TCRγδ) was observed (**Figure 33a**). Most γδ T cells were $CD4^{neg}CD8^{neg}$, as expected,(286) but some CD8 and CD4 expression was observed (**Figure 33b**). These T cells were highly activated as measured by expression of CD38 and CD95. IL2 receptors (CD25; IL2Rα and CD122; IL2Rβ) were detected, but limited surface expression of IL7Rα (CD127) was identified. γδ T cells were not exhausted as evidenced by the absence of CD57 and PD1. Most cells expressed CD27 and CD28 co-stimulatory ligands and had a preference towards antigen-experienced (CD45RO) over naïve (CD45RA) characteristics. Homing to the skin, lymph nodes, and bone marrow has potential as evidenced by CCR4, CXCR4/CLA, and CCR7/CD62L expression, respectively. In aggregate, the surface phenotypes of γδ T cells indicated that they were highly activated and antigen experienced with potential for memory formation and homing to tissues.

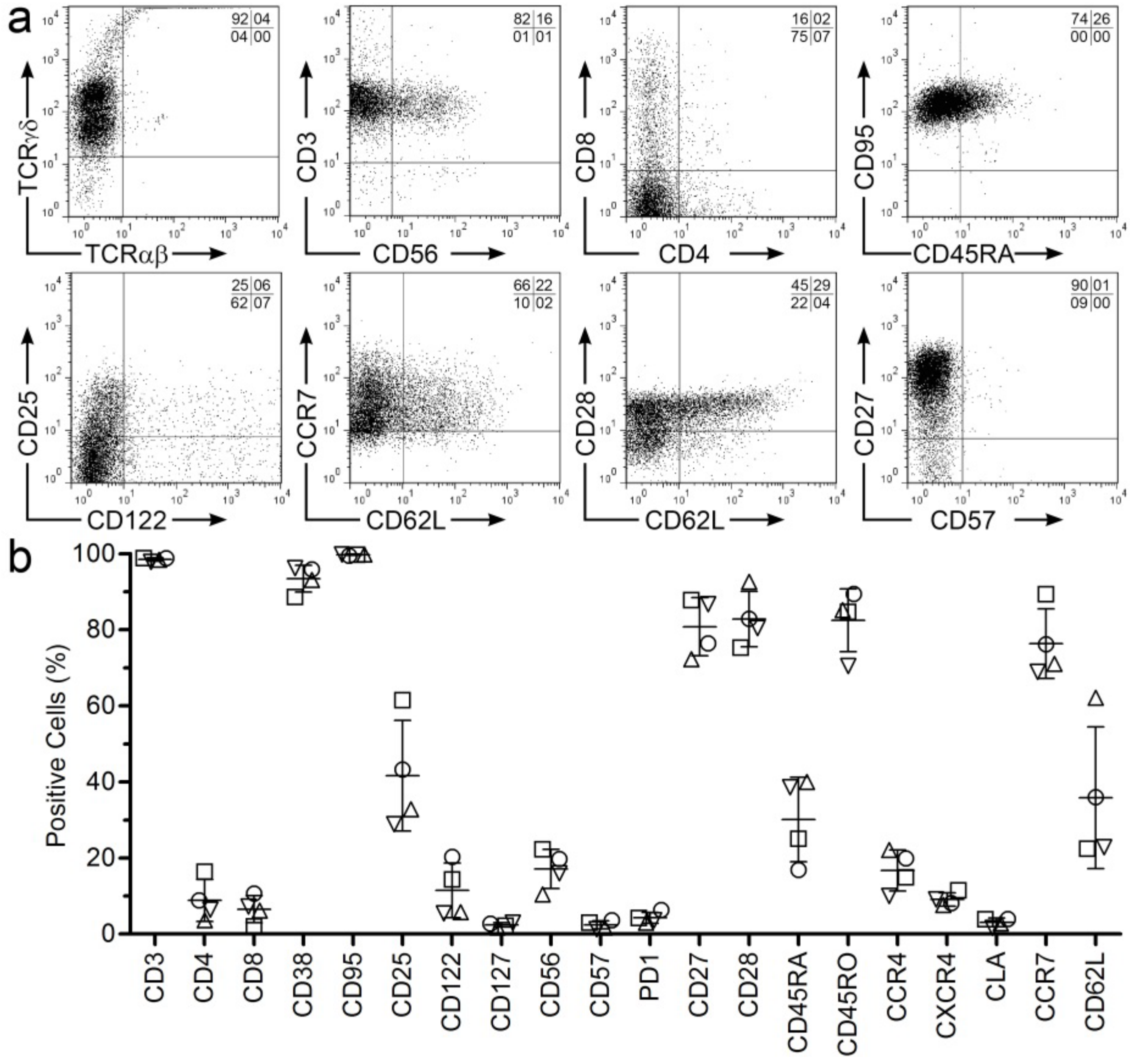

Figure 33. Immunophenotype of Polyclonal γδ T cells Propagated on aAPC, IL2, and IL21. (**a**) Gating (one representative of four donors is shown) and (**b**) frequency of T cell surface makers by flow cytometry of T cells at Day 22 of culture. Lines show mean (horizontal) ± SD (vertical) and symbols represent individual donors.

IV.C.5.b. Immunophenotype of Vδ1, Vδ2, and Vδ3 Subsets

It is of interest to identify differences amongst Vδ1, Vδ2, and Vδ3 lineages that could enable us to predict functional responses and therapeutic efficacy. In particular, distinct differences were observed in TCRγδ cell surface density and memory-associated markers. TCRγδ often stained as a two populations with distinct MFI when co-stained with CD3 (**Figure 27b**). Separation of Vδ1, Vδ2, and Vδ3 subsets clearly identified Vδ2 as the low (43 ± 9; mean ± SD; n = 4), Vδ3 as the medium (168 ± 40), and Vδ1 as the high (236 ± 56) MFI populations in TCRγδ staining (**Figure 34a**). CD4 and CD8 are not commonly expressed on γδ T cells, but there were differences detected in limited surface expression of both CD4 and CD8 between the separated subsets (**Figure 34b**). Vδ1 and Vδ3 cells consistently expressed more CD4 and CD8 than did Vδ2 cells ($p = 0.001$; Two-way ANOVA), and there were significantly more $CD4^+$ Vδ1 and $CD8^+$ Vδ3 cells than $CD4^+$ Vδ2 and $CD8^+$ Vδ2 cells, respectively (**Figure 34c and 34d**). CCR7 and CD62L mediate homing to the lymph nodes and other secondary lymphoid organs. $CD8^+$ T cells expressing CCR7 and/or CD62L were described as T_{CM} cells but $CCR7^{neg}CD62L^{neg}$ were defined as T_{EM} cells.(330, 331) Almost all Vδ1 and Vδ3 cells were $CCR7^+CD62L^{neg}$, but larger proportions of Vδ2 cells were $CCR7^{neg}CD62L^{neg}$ with roughly equal remaining proportions staining as single or double positive for CCR7 and CD62L, suggesting Vδ1 and Vδ3 were T_{CM} and Vδ2 cells were mostly T_{EM} (**Figure 34e**). CD27 and CD28 are both memory markers for $CD8^+$ T cells, especially in the absence of CD45RA, and have important roles as co-stimulatory molecules for T cell activation.(332) CD27 expression followed the order of Vδ1>Vδ3>Vδ2 but all were

>80% $CD27^+$ (**Figure 34f y-axes**). In contrast, there was almost no difference between the three Vδ populations in CD28 expression (**Figure 34f x-axes**). Human γδ T cell memory has been most extensively reported as combinations of CD27 and CD45RA expression where $CD27^+CD45RA^+$, $CD27^+CD45RA^{neg}$, $CD27^{neg}CD45RA^{neg}$, and $CD27^{neg}CD45RA^+$ correspond to T_N, T_{CM}, T_{EM}, and T_{EMRA}, respectively (**Figure 34g**).(151, 333) Indeed, these were the markers that showed the most convincing differences between the Vδ populations although all subsets contained at least some of each population. More specifically, the most T_N cells were Vδ1, the most T_{CM} were Vδ3, the most T_{EM} cells were Vδ2, and virtually no T_{EMRA} were detected (**Figure 34h**). Given these differences in surface memory phenotype, different functional abilities were expected from the γδ T cell subsets and a polyclonal approach to adoptive T cell therapy could utilize these different attributes as needed.

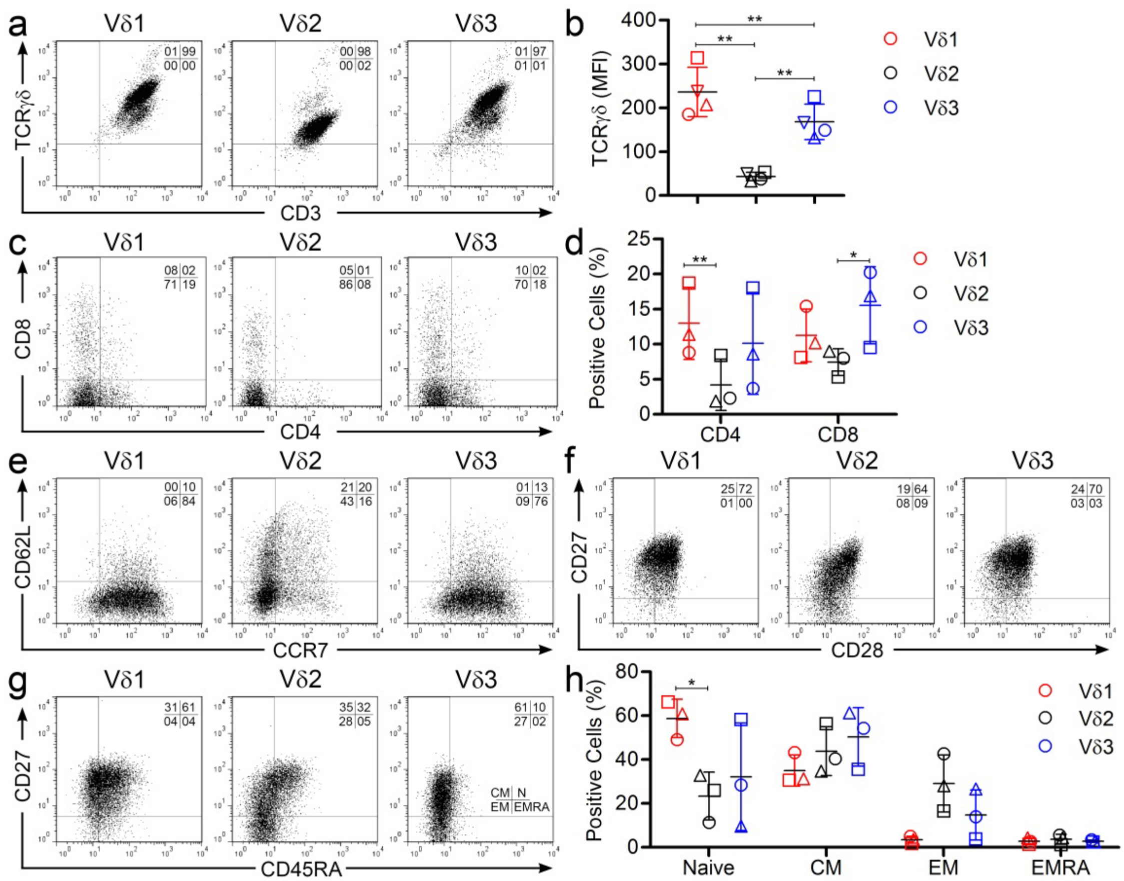

Figure 34. Immunophenotype of Vδ Lineages Propagated on aAPC, IL2, and IL21. After 15 days of proliferation as separated populations, Vδ1, Vδ2, and Vδ3 subsets were stained for lymphocyte markers. (**a**) Representative flow cytometry plots of CD3 (x-axes) and TCRγδ (y-axes) expression in Vδ1, Vδ2, and Vδ3 subsets (from left to right). (**b**) Mean fluorescence intensities (MFI) of TCRγδ staining in Vδ1 (red), Vδ2 (black), and Vδ3 (blue) subsets where each shape represents a different donor and data are mean ± SD (n = 4). (**c**) Representative flow cytometry plots of CD4 (x-axes) and CD8 (y-axes) expression in Vδ1, Vδ2, and Vδ3 subsets (from left to right) and (**d**) summary of frequencies in Vδ1 (red), Vδ2 (black), and Vδ3 (blue) cells where data are mean ± SD (n = 3) and each shape represents a different donor. Representative flow cytometry plots of (**e**) CCR7 (x-axes) and CD62L (y-axes), (**f**) CD28 (x-axes) and CD27 (y-axes), and (**g**) CD45RA (x-axes) and CD27 (y-axes) expression in Vδ1, Vδ2, and Vδ3 subsets (from left to right). Plots are representative of three normal donors. (**h**) Memory phenotypes based on CD27 and CD45RA displayed in lower right corner of Vδ3 in (**g**) where each shape represents a different donor and data are mean ± SD (n = 3).

IV.C.6. Polyclonal γδ T cells Secrete Pro-inflammatory Cytokines and Chemokines

To determine whether γδ T cells would foster an inflammatory environment during therapy, a multiplex analysis (27-Plex Luminex) of cytokines and chemokines was performed on polyclonal γδ T cells following culture on aAPC. LAC and mock activation was used as described in **Chapters II and III**. There was no significant production of anti-inflammatory T_H2 cytokines IL4, IL5, and IL13, and there was a small increase in IL10 production from baseline (**Figure 35a**). In contrast, IL1Ra, IL6, and IL17 were significantly secreted by γδ T cells and have roles together for T_H17 inflammatory responses (**Figure 35b**). Moreover, pro-inflammatory T_H1 cytokines IL2, IL12 (p70), IFNγ, and TNFα were all significantly produced by γδ T cells when TCR was stimulated compared to mock stimulated controls (**Figure 35c**). High expression of chemokines CCL3 (macrophage inflammatory protein-1α; MIP1α), CCL4 (MIP1β), and CCL5 (regulated and normal T cell expressed and secreted; RANTES) were also detected (**Figure 35d**). CCR5 binds to all three of these chemokines,(334) but only 6% ± 2% (mean ± SD; n = 4) of γδ T cells expressed this receptor. Nonetheless, recruitment of other immune cells expressing CCR5 is possible based on γδ T cell's production of CCL3, CCL4, and CCL5. In aggregate, TCR stimulation in γδ T cells led to a largely pro-inflammatory response desired for cell-based cancer therapies.

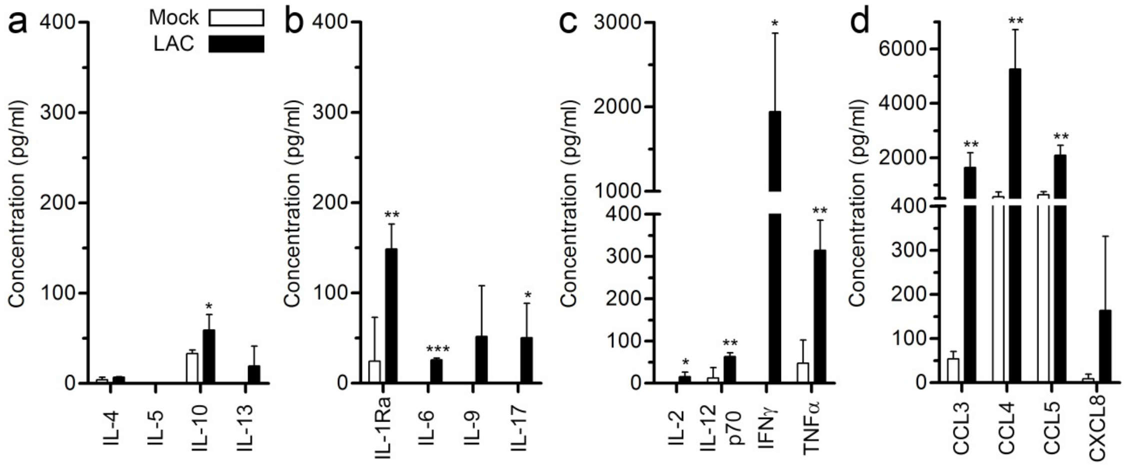

Figure 35. Cytokines and Chemokines Secreted by Polyclonal γδ T cells. At Day 22 of culture on aAPC/IL2/21, T cells were incubated with complete media (mock) or leukocyte activation cocktail (LAC; PMA/Ionomycin) for 6 hours at 37^{o}C. Conditioned media was interrogated on 27-Plex Luminex array to detect cytokines and chemokines. (**a**) T_H2 cytokines, (**b**) T_H17 cytokines, (**c**) T_H1 cytokines, and (**d**) Chemokines. Data are mean ± SD from 4 healthy donors. Student's t-test performed for statistical analysis between mock and LAC groups for each molecule. *p<0.05, **p<0.01, and ***p<0.001

IV.C.7 TCRγδ Involvement in Vδ1, Vδ2, and Vδ3 Production of IFNγ

After establishing that polyclonal γδ T cells produced pro-inflammatory cytokines upon non-specific TCR stimulation, it was of interest to evaluate whether they would respond to tumor cells through their TCRγδ. IFNγ was produced most highly of all the cytokines interrogated by Luminex (**Figure 35c**), so it was chosen as a marker for γδ T cell response to OvCa in a classical intracellular cytokine staining (ICS) assay. Co-cultures with polyclonal γδ T cells and two different OvCa cell lines were incubated at 37^{o}C for 6 hours in the presence of the secretory pathway inhibitor Brefeldin-A (GolgiPlug) in order to trap IFNγ within the T cells. Parallel co-cultures were set up with (i) normal mouse serum (NMS) for negative control or (ii) neutralizing TCRγδ antibody (clone IMMU510) for 1 hour prior to co-culture and during the duration of co-culture. Surfaces of T cells were stained for CD3, TCRδ1, and TCRδ2 in order to separate Vδ1, Vδ2, and Vδ3 populations from tumor cells (**Figure 36a, 36b, and 36c**). Tumor cells alone and T cells without tumor cells served as negative staining controls. As anticipated, each Vδ subset produced IFNγ in response to OvCa in the NMS (negative blocking control) treated cells (**Figure 36d**). Furthermore, the amount of IFNγ produced followed the order Vδ2>Vδ1>Vδ3 as evidenced by IFNγ MFI of 855 ± 475, 242 ± 178, and 194 ± 182 (mean ± SD; n = 4), respectively. Addition of antibody neutralizing TCRγδ significantly inhibited IFNγ production by all three Vδ subsets where Vδ2 was most affected (**Figure 36d, 36e, and 36f**). Therefore, polyclonal γδ T cells responded to tumor cells indicating that they have specific anti-tumor effects through their TCRγδ.

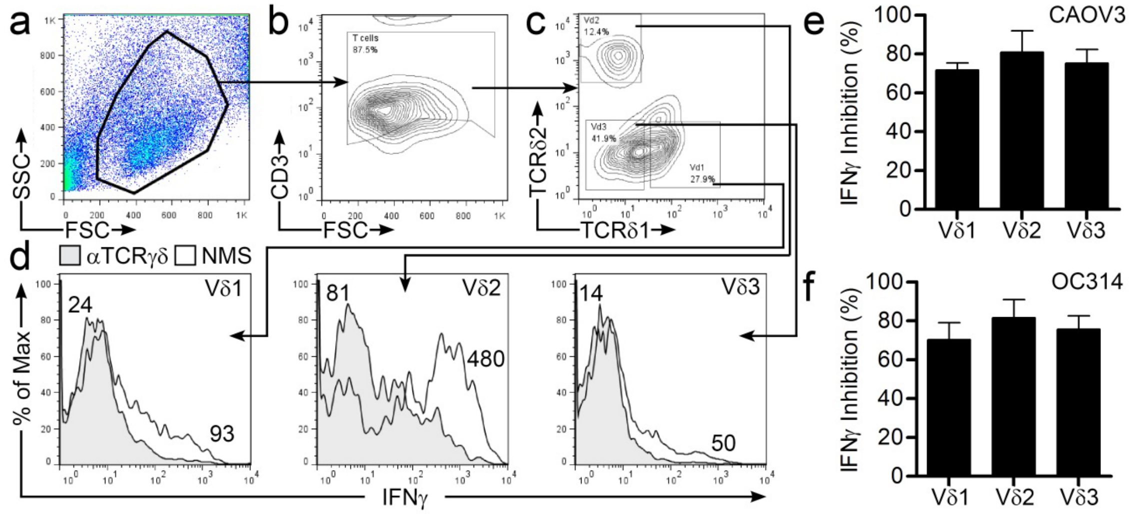

Figure 36. TCRγδ-specific IFNγ Production by Vδ1, Vδ2, and Vδ3 Subsets. Polyclonal γδ T cells were incubated for 1 hour prior to co-culture and during co-cultures with normal mouse serum (NMS; negative control) or neutralizing TCRγδ antibody (αTCRγδ; clone IMMU510). Co-cultures were initiated in the presence of the secretory inhibitor BrefeldinA (GolgiPlug) where polyclonal γδ T cells and one of two OvCa cell lines (CAOV3 or OC314) and were incubated at 37^{o}C for 6 hours. Cells were stained for TCRδ1, TCRδ2, CD3, and IFNγ in order to gate each T cell subset and assess IFNγ production. The gating strategy was (**a**) separation of forward and side scatter (FSC and SSC, respectively) in activated T cell gate, (**b**) isolation of CD3^{+} T cells from contaminating tumor cells in T cell gate, and (**c**) separation into Vδ1, Vδ2, and Vδ3 based on TCRδ1^{+}TCRδ2^{neg}, TCRδ1^{neg}TCRδ2^{+}, and TCRδ1^{neg}TCRδ2^{neg}, respectively. (**d**) Histogram comparisons of Vδ1, Vδ2, and Vδ3 gates (from left to right) co-cultured with CAOV3 and treated with NMS (open) or αTCRγδ (shaded). Numbers next to histograms are MFI. Flow plots are representative of 1 of 3 normal donors and of co-cultures with OC314 cells. Percent inhibition for each Vδ subset was calculated by the following equation: Inhibition (%) = 100 – 100 x $[(MFI_{TUMOR + T\ CELL} - MFI_{T\ CELL\ ONLY})_{\alpha TCR\gamma\delta} / (MFI_{TUMOR + T\ CELL} - MFI_{T\ CELL\ ONLY})_{NMS}]$. Data are mean ± SD (n = 3).

IV.C.8. Broad Anti-tumor Cytolysis by Polyclonal γδ T cells

After establishing that γδ T cells were functional in producing pro-inflammatory molecules, their ability to lyse a broad range of tumor cell lines was investigated against healthy donor cells and established hematological and solid tumor cell lines.

IV.C.8.a. Polyclonal γδ T cells Lyse Hematological Tumors

We previously established that γδ T cells could lyse B-ALL cell lines (Daudi-β2M, Kasumi2, and REH) but not healthy autologous or allogeneic B cells.(311) This observation was confirmed again with healthy autologous and allogeneic B cells, which were not lysed by polyclonal γδ T cells (**Figure 37a**). However, the same effectors were able to kill allogeneic B-ALL cell lines cALL2 and RCH-ACV (**Figure 37b**). T-ALL cell lines (Kasumi3 and Jurkat) were also sensitive to γδ T cell killing suggesting that γδ T cells could be used to kill T cell malignancies (**Figure 37c**). CML cell line K562 was also killed by γδ T cells and has been a well-known target for γδ T cell cytolysis.(303) Moreover, K562-derived clone#4 aAPC were lysed by γδ T cells, as expected (**Figure 37d**). Thus, polyclonal γδ T cells propagated on aAPC have anti-tumor immunity towards hematological malignancies.

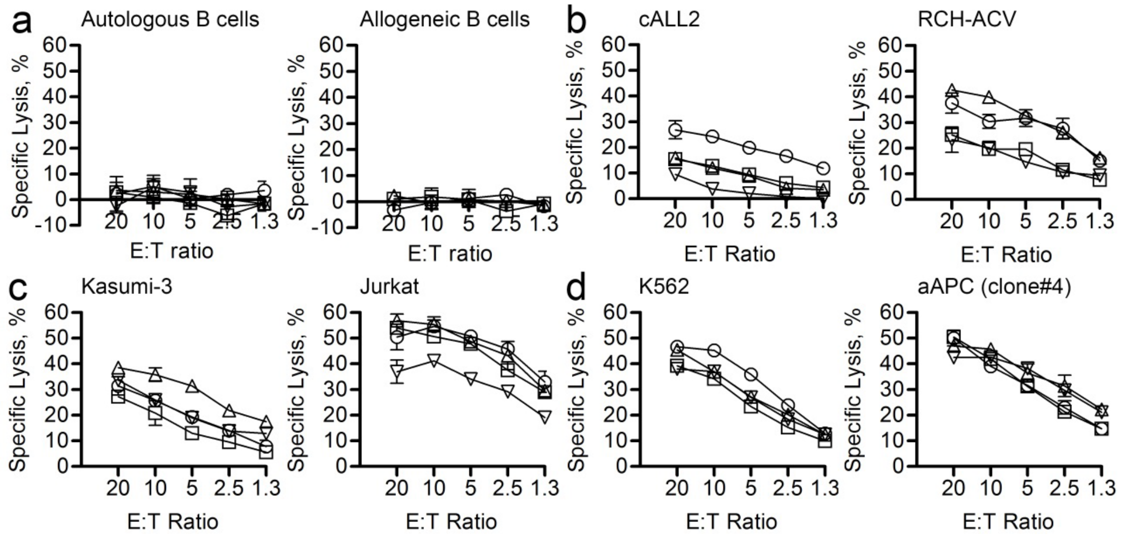

Figure 37. ***In vitro*** **Cytolysis of Hematological Tumor Cells by γδ T cells.** Standard 4-hour CRA were performed with increasing effector (γδ T cells) to target (E:T) ratios against (**a**) B cells from autologous donors or from an allogeneic donor (one of four representative donors), (**b**) B-ALL cell lines cALL2 and RCH-ACV, (**c**) T-ALL cell lines Kasumi3 and Jurkat, and (**d**) CML cell line K562 and its derivative clone#4 aAPC. Each line represents an individual effector where data are mean ± SD (n = 3 wells per assay).

IV.C.8.b. Polyclonal γδ T cells Lyse Solid Tumors

After establishing that polyclonal γδ T cells could lyse hematological tumor cells, solid tumor cell lines were evaluated for killing using standard 4-hour CRA. Established PaCa and OvCa cell lines were tested because of their high likelihood for sensitivity to anti-tumor immunity with a lack of current cellular therapies. Several PaCa cell lines (CaPan2, MiaPaCa2, Su8686, and BxPc3) cell lines were lysed by γδ T cells in a dose-dependent manner where BxPc3 cells were killed most efficiently (**Figure 38a**). Next, eight OvCa cell lines were lysed by polyclonal γδ T cells in the following order: CAOV3 > EFO21 > UPN251 > IGROV1 > OC314 > Hey > A2780 > OVCAR3 (**Figure 38b**). Moreover, there was an average of >60% maximum cytolysis observed against CAOV3 in one donor after 4 hours at an effector to target (E:T) ratio of 20:1. Therefore, polyclonal γδ T cells were able to kill solid tumors *in vitro* and other solid tumor cell lines may also be sensitive to cytolysis by γδ T cells.

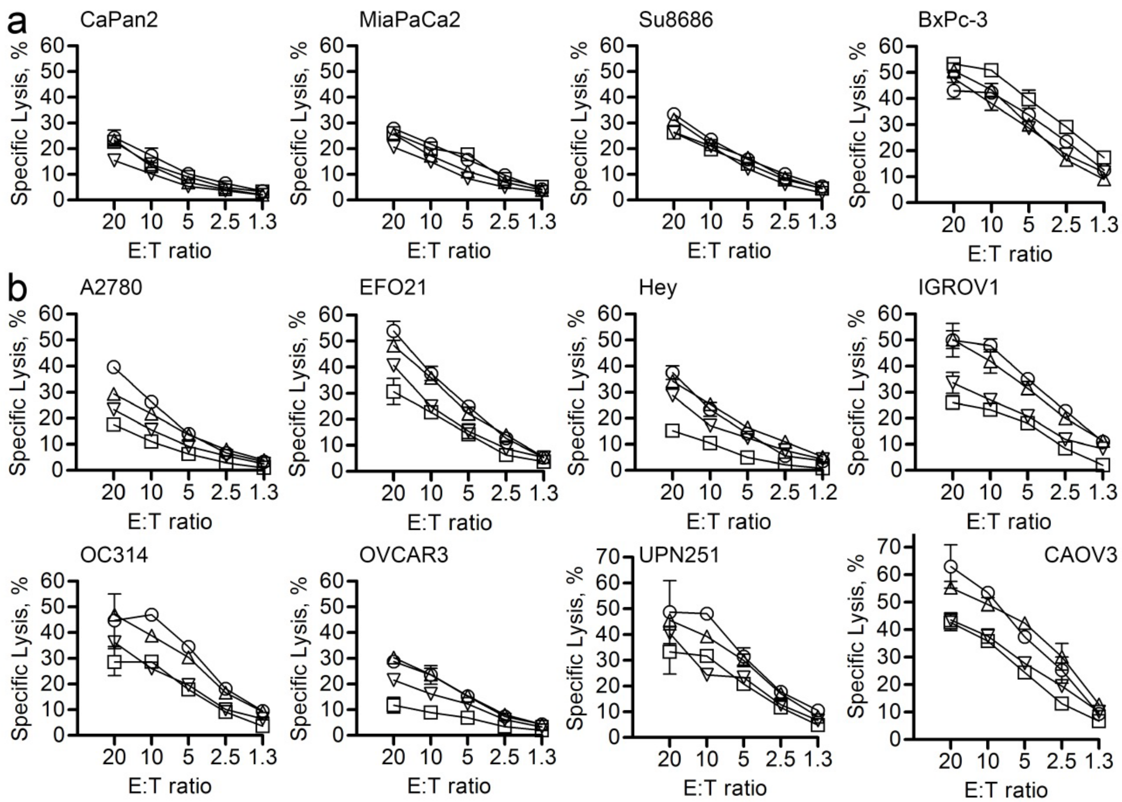

Figure 38. *In vitro* Cytolysis of Solid Tumor Cells by γδ T cells. Standard 4-hour CRA were performed with increasing effector (γδ T cells) to target (E:T) ratios against (**a**) PaCa cell lines CaPan2, MiaPaCa2, Su8686, and BxPc3 and (**b**) OvCa cell lines A2780, EFO21, Hey, IGROV1, OC314, OVCAR3, UPN251, and CAOV3. Each line represents an individual effector where data are mean ± SD (n = 3 wells per assay).

IV.C.8.c. Mechanism of Tumor Cytolysis by γδ T cells was Multi-factorial

We sought to determine if polyclonal γδ T cell cytolysis was directly dependent upon the TCRγδ by neutralizing killing with antibodies. Confounding these assays was the observation that γδ T cells displayed high levels of DNAM1 and NKG2D (data not shown), which can mediate cytolysis by both T cells and NK cells.(335, 336) Moreover, there was not a clear-cut choice for TCRγδ neutralizing antibody since the company information for TCRγδ-specific antibodies did not report on neutralization. In the end, the TCRγδ-specific antibody used for staining in this study (clone B1, BD Biosciences) and clone IMMU510 TCRγδ-specific antibody (IM) from Thermo Fisher were used for neutralization studies. Also, because there were many activating receptors (TCRγδ, DNAM1, NKG2D) on the γδ T cell surface, a pool of all antibodies was used for maximum inhibition and to assess if there was additivity or synergy between the receptors in killing. Hematological tumor cell line (Jurkat) and solid tumor cell line (OC314) were chosen as targets because of their reported expression of DNAM1 and NKG2D ligands and their sensitivity to cytolysis by polyclonal γδ T cells (**Figures 37c and 38b**).(337-339) An E:T ratio of 12:1 was chosen where effectors were pre-incubated with the antibodies and antibodies were present during the 4-hour CRA. NMS was used as a negative control and parallel wells were initiated without antibodies to determine maximum cytolysis for normalization purposes. Antibodies targeting NKG2D, DNAM1, and TCRγδ (clone B1) had minimal effect on reducing cytolysis against Jurkat (**Figure 39a**) and OC314 (**Figure 39b**) relative to NMS. However, there was a statistically significant increase in killing against Jurkat with DNAM1 antibody and significant decrease in killing against OC314 with NKG2D antibody. In contrast, TCRγδ (IM) antibody significantly neutralized killing of both Jurkat and OC314 cells compared to NMS and reduced the killing by an average of 40% in both cell lines (**Figures 39a and 39c second bars from right**). Furthermore, a pool of all four antibodies (NKG2D, DNAM1, TCRγδ (B1), and TCRγδ (IM)) resulted in synergistic inhibition of γδ T cell cytolysis of Jurkat (65% ± 8%) and OC314 (71% ± 10%) cells (**Figures 39a and 39c bars to far right**). Moreover, dose-dependent inhibition was observed by both TCRγδ (IM) and pooled antibodies when concentrations were diluted from 3.0 μg/mL (shown in **Figures 39a and 39c**) to 1.0 μg/mL and 0.3 μg/mL against Jurkat (**Figure 39b**) and OC314 (**Figure 39d**). Similar results were seen with targeting IGROV1 (data not shown), which is also known to express DNAM1 and NKG2D ligands and was sensitive to polyclonal γδ T cell killing (**Figure 38b**).(337, 339, 340). In sum, these results suggested that killing by γδ T cells is multi-factorial with an emphasis on the TCRγδ to mediate cytolysis.

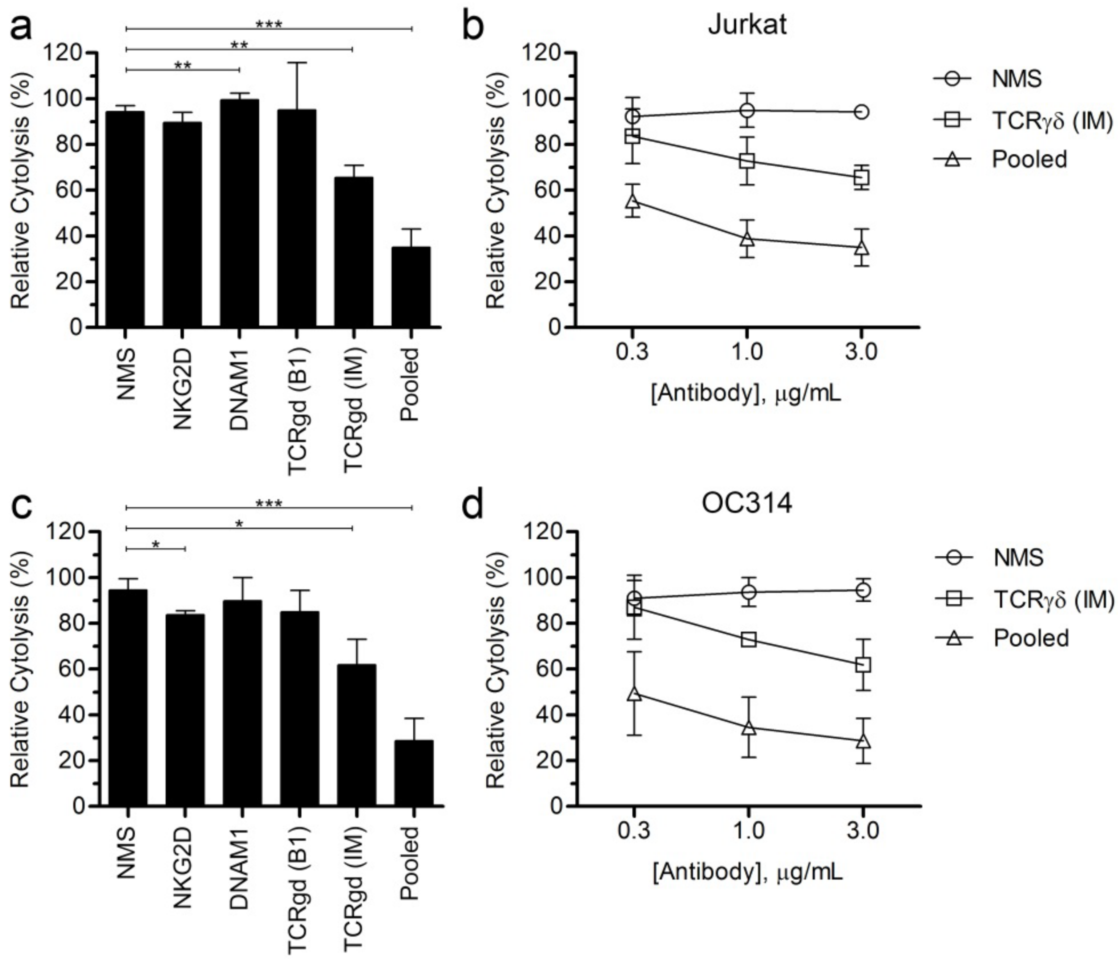

Figure 39. Neutralization of Polyclonal γδ T cell Cytolysis. Neutralizing antibodies to NKG2D, DNAM1, TCRγδ (B1), TCRγδ (IM) were used to block killing of Jurkat or OC314 tumor targets at an E:T ratio of 12:1 in standard 4-hour CRA. Antibodies were pre-incubated with T cells for 1 hour and kept in the CRA at 0.3, 1.0, or 3.0 μg/mL. NMS was used for antibody controls and specific lysis was normalized to wells without antibody to yield relative cytolysis as defined by: Relative cytolysis (%) = $(\text{Specific Lysis})_{\text{With Antibody}}$ / $(\text{Specific Lysis})_{\text{Without Antibody}}$ x 100. Relative cytolysis of Jurkat cells by (**a**) all antibodies at 3.0 μg/mL and (**b**) NMS, TCRγδ (IM), and pooled antibodies at tested concentrations. Relative cytolysis of OC314 cells by (**c**) all antibodies at 3.0 μg/mL and (**d**) NMS, TCRγδ (IM), and pooled antibodies at tested concentrations. Data are mean ± SD (n = 4). Two-way ANOVA with Bonferroni's post-tests were used for statistical analysis.

IV.C.8.d. Importance for TCRδ in γδ T cell Cytolysis

Because the separated Vδ subsets displayed differences in memory phenotype and cytokine production, it was of interest to evaluate their ability to directly lyse solid and hematological tumors. Acute killing was evaluated with standard 4-hour CRA against Daudi-β2M, Jurkat, K562, clone#4 aAPC, and OvCa cell lines (CAOV3, IGROV1, OC314, and UPN251) all of which displayed high levels of susceptibility to lysis by polyclonal γδ T cells (**Figures 37 and 38**). All eight tumor cell lines were lysed by the separated Vδ lineages, but a distinct order of lysis was observed against all targets where Vδ2>>Vδ3>Vδ1 in killing capabilities (**Figure 40**). As the phenotype indicated that Vδ1 cells were mainly naïve, it was expected that they would have the most limited cytolytic ability, which is what was observed. Likewise, T_{CM} have less immediate effector function relative to T_{EM} cells, and these memory populations were dominated by Vδ3 and Vδ1, respectively. Importantly, this was the first report of anti-tumor activity by Vδ3 cells. It was interesting that all three Vδ lineages lysed clone#4 aAPC roughly equally which supports their similar proliferation (**Figure 32**). Long-term killing assays were then set up to assess whether equivalent killing could be achieved during 48 hours of co-culture between Vδ subsets and OvCa cell lines CAOV3, OC314, and UPN251 (**Figure 41**). Indeed, >95% of CAOV3 and UPN251 cells were eliminated by all three subsets in two days. Likewise, 96% ± 4% of OC314 cells were killed by Vδ2 cells, and Vδ1 and Vδ3 achieved 76% ± 5% and 89% ± 5% (mean ± SD; n = 3) killing, respectively, in 48 hours of culture. Collectively, the Vδ subset lineage was important for cytolysis in both acute and long-term conditions, and established that each Vδ lineage propagated on aAPC was capable of tumor killing.

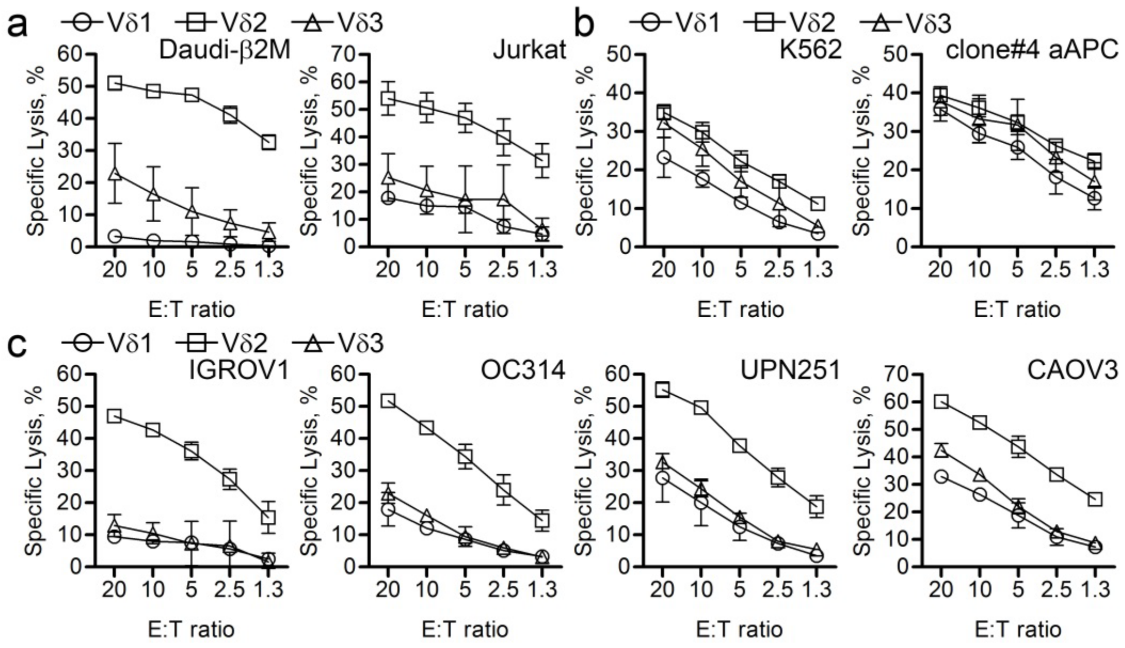

Figure 40. γδ T cell Subset Acute Cytolysis. Vδ subsets were tested in 4-hour CRA against (**a**) ALL cell lines Daudi-β2M and Jurkat, (**b**) CML cell line K562 and its derivative clone#4 aAPC, and (**c**) OvCa cell lines IGROV1, OC314, UPN251, and CAOV3. Vδ1 (circles), Vδ2 (squares), and Vδ3 (triangles) are displayed as mean ± SD from averaged triplicate measurements from four normal donors.

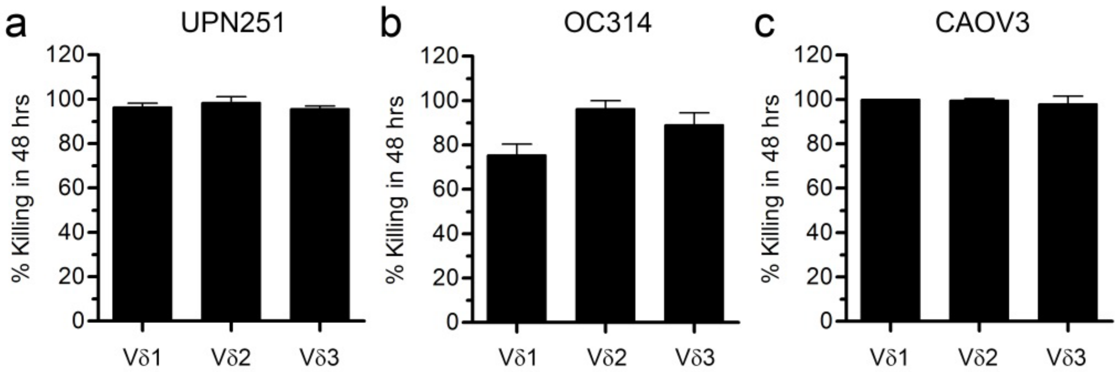

Figure 41. γδ T cell Subset Long-term Killing. CAOV3, OC314, and UPN251 cells were seeded in wells of 6-well plates and incubated overnight so that they would adhere to the wells. T cells from Vδ1, Vδ2, or Vδ3 subsets were then added and co-cultured in the wells with tumor cells for 2 days. Remaining adherent cells were enzymatically removed from the wells and counted for viable cells. Tumor cells without T cells were positive control and T cells without tumor cells was the negative control. Killing (%) = $(\text{Viable cells})_{\text{Co-culture}}$ / $(\text{Viable cells})_{\text{Tumor only}}$ x 100. Data are mean ± SD (n = 3).

IV.C.9. Clearance of Established Tumor Xenografts by Polyclonal γδ T cells

As polyclonal γδ T cells are being proposed as a therapy for cancer patients, a model to test their efficacy *in vivo* was evaluated. NSG mice were used for their ability to accept human tumor xenografts well and were injected with CAOV3-*ffLuc*-mKate tumor cells intraperitoneally (i.p.) then randomized into treatment groups to establish a model of high tumor burden. This was a model for advanced OvCa disease as many women with OvCa do not usually develop metastases outside of the peritoneal cavity but local tumor growth and ascites result in disease pathology.(37) After 8 days of engraftment (denoted Day 0) either PBS (negative control) or γδ T cells (escalating doses) were administered i.p. to the mice (**Figure 42**). Tumor burden was monitored during the experiment with non-invasive BLI following D-luciferin administration. Established tumors were clearly visible by BLI after 8 days of engraftment at Day 0 (**Figure 42a top panels**), which continued to grow in mock (PBS) treated mice (**Figure 42a bottom left panels**) but were eliminated in mice treated with polyclonal γδ T cells (**Figure 42a bottom right panels**) at 72 days post-treatment initiation. All mice treated with PBS had increased BLI flux measurements ($p = 0.018$) whereas polyclonal γδ T cell-treated mice had significantly decreased ($p = 0.004$) BLI flux (**Figure 42b**). Moreover, treatment with γδ T cells improved overall survival ($p = 0.0001$) compared to mock-treated mice where 90% of mice survived OvCa and hazard ratio for mice without treatment was 20.4 (**Figure 42c**). In sum, polyclonal γδ T cells were effective in treating cancer *in vivo* and represent an attractive approach to cell-based cancer treatment.

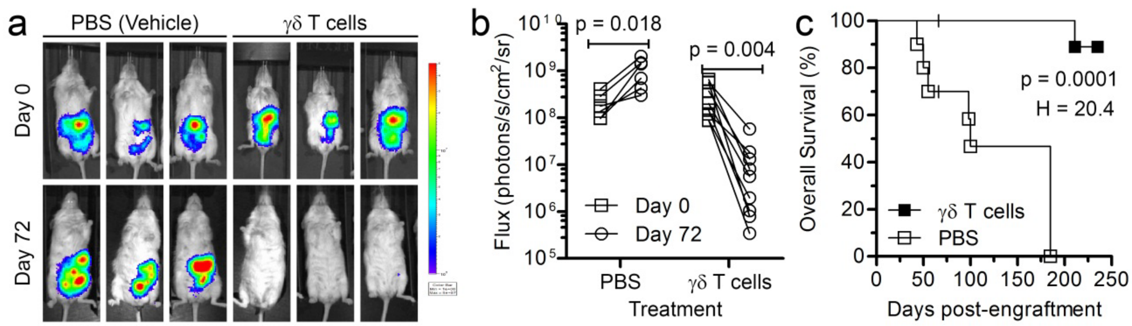

Figure 42. *In vivo* Tumor Clearance by Polyclonal γδ T cells. CAOV3-*ffLuc*-mKate tumor cells ($3x10^6$) were injected i.p. into NSG mice at Day -8 and were allowed to engraft until Day 0 when treatment was started with either PBS (vehicle/mock) or polyclonal γδ T cells. Four doses were given with $3x10^6$, $6x10^6$, $10x10^6$, and $15x10^6$ on days 0, 7, 14, and 21, respectively, to create a dose escalation scheme. (**a**) BLI flux images at Day 0 (top panels) or Day 72 (bottom panels) in PBS-treated (left 3 panels) or polyclonal γδ T cell-treated (right 3 panels) mice. Mice displayed are representative of 10 total mice. (**b**) BLI flux measurements of mice at Day 0 (squares) and Day 72 (circles) where lines are drawn between the same mouse. Student's paired, 2-tailed t-tests were used for statistical analysis and p values are displayed above treatment groups. (**c**) Overall survival of mice treated with PBS (open squares) or polyclonal γδ T cells (closed squares). Gehan-Breslow-Wilcoxon Test was used to calculate p value. H = hazard ratio.

IV.D. Discussion

IV.D.1. Importance of Polyclonal γδ T cells for Immunotherapy

This study establishes clone#4 aAPC as a cellular platform for the sustained proliferation of populations of γδ T cells that exhibit broad reactivity against hematologic malignancies and solid tumors. T cells expressing certain Vδ TCR usage have been associated with clinical responses against cancer. For example, the Vδ1 TCR subset correlated with complete responses observed in patients with ALL and AML who underwent αβ T cell-depleted allogeneic HSCT.(302, 304, 305) However, Vδ1 cells have not been directly infused for therapy. This chapter established direct evidence that Vδ1 cells could mediate anti-tumor immunity and strengthens support for their use in adoptive T cell cancer treatments. In contrast to Vδ1 and Vδ3 cells, T cells expressing Vδ2 TCR have been directly infused and generated responses against solid and hematological tumors, but complete responses were unpredictable and sometimes not directly correlated to Vδ2 therapy (175, 318). Similarly, Vδ2 cells expanded in this chapter had the most immediate anti-tumor cytotoxicity and cytokine production, and aAPC-based expansions could build upon these early successes of Vδ2 T cell infusions. A role for T cells expressing Vδ3 TCR in targeting tumors is unknown, but these lymphocytes have been correlated with immunity to HIV and CMV.(165, 183) Thus infusion of this T-cell subset could be beneficial to immunocompromised patients. Importantly, these results are the first to directly show that Vδ3 cells have anti-tumor activity and this study could, therefore, represent a significant contribution to both translational research strategies and to immunologists studying γδ T cell function. In aggregate, the data herein lend impetus to adoptive transfer of γδ T cells that maintain expression of all Vδ TCR types as investigational treatment for tumors and opportunistic viral infections.

IV.D.2. Potential Ligands for TCRγδ on aAPC

The molecules on aAPC that stimulate TCRγδ for their numeric expansion are not known. K562-derived aAPC express endogenous MICA and MICB molecules (329) which are ligands for both Vδ1 and NKG2D.(152) NKG2D was expressed (40% ± 16%; mean ± SD, n = 4) on aAPC-expanded γδ T cells that were also predominantly Vδ1 cells (**Figure 31**). Polyclonal γδ T cells also demonstrate expression (26% ± 7%) for other activating NK receptors (NKp30, NKp44, and NKp46), which may contribute to γδ T cell function. Two ligands described for Vδ2 TCR are surface mitochonrial F_1-ATPase and phosphoantigens, both of which are described in K562 cells.(171, 172, 297, 299) Indeed, enhanced responses of T cells expressing Vγ9Vδ2 were observed when K562 cells were treated with aminobisphosphonates,(172) and a similar strategy could be employed upon co-culture with clone #4 to increase the frequency of Vδ2 TCR usage.(173) Otherwise, patients receiving polyclonal γδ T cells could be primed to expand Vδ2 cells *in vivo* through administration of aminobisphosphonates. Now that aAPC have been established as a means to propagate polyclonal γδ T cells, these molecular questions can be answered and used for future therapies.

IV.D.3. Co-stimulation in Polyclonal γδ T cell Expansion

We introduced co-stimulatory molecules to improve the ability of aAPC to propagate γδ T cells. CD28 and CD137 (4-1BB) expressed on γδ T cells bind CD86 and CD137L, respectively, expressed on aAPC. The absence of both CD86 and CD137L abrogated γδ T-cell proliferation and expression of single co-stimulatory molecules only partially restored the ability of γδ T cells to proliferate (**Figure 28b**). The benefit of using other molecules' involvement in co-stimulation has not been evaluated to date. CD70 is expressed on γδ T cells (36% ± 15%) concurrently with its receptor CD27 (**Figure 33**), which may allow for *trans*- or *cis*- stimulation independent of the aAPC that does not express CD70. CD27 has been described as a marker for γδ T cells that produce IFNγ, and $CD27^{neg}$ γδ T cells commonly secrete IL17, a potent cytokine that has powerful, yet context-dependent anti-tumor activities.(127, 333, 341) Current studies are investigating whether other co-stimulation combinations, i.e. ICOS without CD86, can improve the propagation and/or change the phenotype of γδ T cells – especially in regards to improving production of IL17 that can have potent anti-tumor effects. It may be that a cocktail of cytokines and neutralizing antibodies is required to propagate IL17-producing γδ T cells, which was required for expansion of $CD4^+$ T_H17 cells *ex vivo* on stimulating beads.(326) Indeed, the addition of IL2 and IL21 was also crucial for the numeric expansion of γδ T cells so the strategy will likely need addition of these two exogenous cytokines (**Figure 28c**). In the end, the aAPC co-culture system provides a clinically relevant methodology to tailor the type of therapeutic γδ T cell produced for adoptive T cell therapy.

IV.D.4. Polyclonal γδ T cells Apparently Lack Allogeneic Responses to Healthy Tissue

An attractive therapeutic strategy is to employ third party allogeneic γδ T cells as an "off-the-shelf" therapy. This may be feasible, as γδ T cells have reduced potential to cause graft-versus-host disease (GvHD) resulting from inappropriate TCR-mediated recognition of normal host tissue (305). Unlike TCRαβ that recognizes peptides in the context of MHC, TCRγδ is not known to be subject to MHC restriction.(141, 298, 299) Thus, matching recipient and donor T cell MHC may not be needed, raising the possibility that propagated γδ T cells from one donor can be infused into multiple recipients. Autologous T cells expressing Vγ9Vδ2 TCR have been adoptively transferred and intravenous administration of aminobisphosphonates was used for *in vivo* numeric expansion of this T-cell subset.(175, 179, 318) To date, the infusion of allogeneic γδ T cell has not been reported. We have evaluated aAPC-expanded γδ T cells for allogeneic responses and are not able to detect such reactivity. For example, γδ T cells proliferate (**Figure 43a**) and secrete IFNγ (**Figure 43b**) when co-cultured with OKT3-loaded aAPC, but not when co-cultured with autologous or allogeneic B cells. Allogeneic tumor cell lines were lysed by our γδ T cells, but healthy B cell donors were spared (**Figures 24a and 37a**). Further, formation of colonies from hematopoietic stem cells was inhibited by allogeneic NK cells, but not by allogeneic γδ T cells (**Figure 43c**). Autologous EBV-transformed LCL stimulated γδ T cells suggesting they may react with EBV antigens (data not shown) as indicated by previous studies.(342, 343) Bi-specific αβ T cells expressing CARs specific for GD2 or CD19 and grown on LCL have shown excellent anti-tumor immunity and could be applicable for the γδ T cell population using aAPC.(236, 344) The ability to infuse donor-derived γδ T cells when needed, rather than wait the availability of an autologous product raises the therapeutic potential of this T-cell subset. This adds to our development of “off-the-shelf” cells as we previously reported that zinc finger nucleases can be used to eliminate expression of TCRαβ to help generate “universal” CAR^{+} T cells.(345)

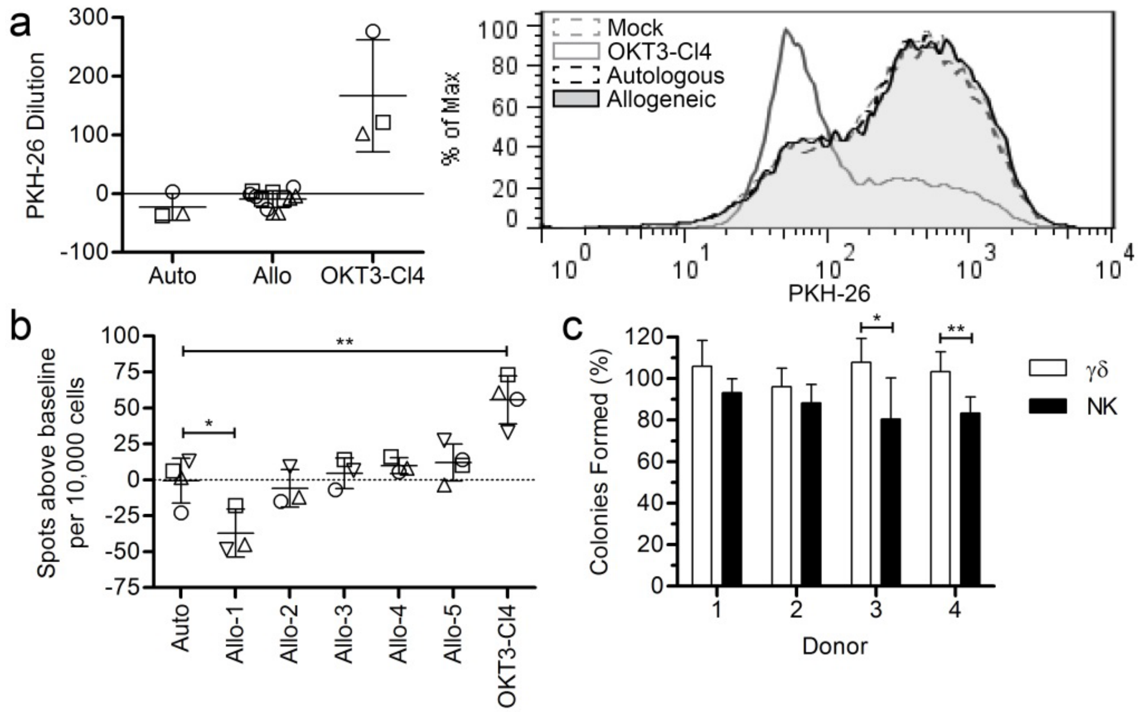

Figure 43. Absence of Allogeneic Responses by Polyclonal γδ T cells to Partially Mis-matched Donors' Healthy Cells. (**a**) Polyclonal γδ T cells were labeled with red fluorescent dye (PKH-26) and co-cultured with (i) media only (mock), (ii) autologous B cells, (iii) allogeneic B cells from normal donors (n = 5), or (iv) OKT3-loaded clone#4 aAPC (positive control) for 3 days at 37^{o}C without exogenous cytokines. Proliferation was measured by dilution of PKH-26 dye MFI and each group was normalized to mock treated T cells after gating for $CD3^{+}TCR\gamma\delta^{+}$ cells. Each shape represents a polyclonal γδ T cell effector (n = 3). Representative flow cytometry plot is displayed to the right. (**b**) The same co-cultures set up in (**a**) were initiated overnight in an IFNγ ELISpot assay plate, except that cells were not labeled with PKH-26. Spots were enumerated and normalized to mock-treated cells for each donor, which is represented by an individual shape. (**c**) Hematopoietic stem cell (HSC) colony forming unit assays were set up with co-cultures of donor-matched NK cells or γδ T cells and PBMC containing a fixed number of HSC and co-cultures were added to semi-solid media supplemented with cytokines for colony formation. HSC cultures without co-cultured lymphocytes were used as negative controls for inhibition of colony formation and to normalize co-culture colony formation. Student's paired, 1-tailed t-tests were used for statistical analysis. *p<0.05 and **p<0.001.

IV.D.5. Application of Polyclonal γδ T cells for Immunotherapy

These data demonstrate that our aAPC can be used to generate large numbers of γδ T cells that maintain polyclonal TCR repertoire and have an ability to kill tumor cells. Clone#4 has been produced as a master cell bank and thus there is a clear path to generating clinical-grade γδ T cells for human application. A polyclonal approach to γδ T cell immunotherapy is supported by the ability to of aAPC generate T_N, T_{CM}, and T_{EM} γδ T cells from Vδ1, Vδ2, and Vδ3 lineages (**Figure 34**) that could then produce a range of effector functions including production of pro-inflammatory cytokines (**Figures 35 and 36**), exerting direct cytotoxicity against tumors (**Figures 37, 38, 39, 40 and 41**), and eliminating solid tumor xenografts (**Figure 42**). Thus, immediate tumor cytotoxicity can be achieved mainly through effector and T_{EM} cells and long-lived anti-tumor immunity could be repopulated in patients with T_N and T_{CM} γδ T cells. Clinical trials can now, for the first time, test the efficacy of polyclonal γδ T cell transfers in cancer treatments of both solid and hematological tumors.

CHAPTER V

General Discussion and Future Directions

V.A. Dissertation Summary

The central aim of this dissertation was to develop and test novel cellular immunotherapies for cancer treatment. This was tested in three independent specific aims. First, ROR1-specific CARs were able to re-direct αβ T cells towards leukemia without affecting normal B cells, and this represented an improvement from current CD19-specific CAR strategies that result in normal B cell aplasia (**Chapter II**). Current CD19-specific CAR and $CD19^{+}$ aAPC are currently in clinical trials at MD Anderson and were the fastest way to translate a strategy to use CAR^{+} γδ T cells for immunotherapy. Therefore, the second approach used polyclonal γδ T cells expressing TCRγδ with anti-tumor reactivity as sentinels of CD19-specific CAR anti-tumor immunity. These CAR^{+} γδ T cells may have clinical bi-specific anti-leukemia efficacy due to targeting the tumor through both TCR and CAR (**Chapter III**). The last aim evaluated the broad anti-tumor activity of polyclonal γδ T cells expanded on aAPC, and established that they can be an effective option for leukemia, PaCa, and OvCa (**Chapter IV**). The translation of these pre-clinical methods into the clinical trials will give people facing cancer treatment new, safe, and effective options.

V.B. Combinational Cellular Immunotherapies

Using more than one cell immunotherapy product in therapy may lead to therapeutic additivity, or better yet, synergy. Indeed, clinical trials have already combined HSCT with CD19-specific CARs to target B-cell leukemia.(263, 346) The trials are still in the enrolling stages, so it will take time to determine whether they are better than historical controls. Similar to HSCT and CD19-specific CAR$^+$ T cells, CARs can be paired to other cellular products to increase anti-tumor efficacy. For instance, polyclonal γδ T cells had inherent anti-tumor immunity towards ovarian and pancreatic cancers (**Chapter V**) and ROR1 is a TAA expressed on both PaCa and OvCa where ROR1$^+$ OvCa cells were lysed by ROR1-specific CAR$^+$ T cells (**Figure 16c**),(67) so a combinational immunotherapy of ROR1-specific αβ T cells and polyclonal γδ T cells could be used. In fact, the 4A5 mAb specific for ROR1 and from which the CAR was derived detected ROR1 at some level in 11 of 12 OvCa cell lines (**Figure 6c and data not shown**). Given the potent anti-tumor activity of polyclonal γδ T cells towards OvCa (**Figure 38b**), the two approaches could be done together to increase tumor clearance. Moreover, patients with low ROR1 antigen expression and resistance to γδ T cell-mediated cytolysis may be sensitive to synergistic killing by ROR1-specific CAR$^+$ αβ T cells and polyclonal γδ T cells. Also, γδ T cells are unlikely to participate in GvHD in allogeneic transplantation, so a universal bank of polyclonal γδ T cells could be established that was known to have high anti-tumor immunity or containing a particular set frequency of Vδ1, Vδ2, and Vδ3 populations with maximum efficacy.(305) Polyclonal γδ T cells could also be used as front-line therapy before addition of HSCT, CAR$^+$ T cells, TILs, etc. in order to prime the tumor microenvironment for adaptive immune cells with broader tumor specificity or to reveal neo-tumor antigens. Furthermore, the bystander effects of $\gamma\delta$ T cells in the microenvironment are largely unknown, and tumor lysis could lead to other resident cell types, e.g. NK cells, macrophages, DCs, etc. to have renewed reactivity to the tumor.(347) Indeed, B-ALL cell lines coated with mAb were lysed by $CD16^+$ $V\gamma9V\delta2$ cells via antibody-dependent cell-mediated cytotoxicity (ADCC), and subsequently the $V\gamma9V\delta2$ had APC function to generate antigen-specific $CD8^+$ $\alpha\beta$ T cell responses to known B-ALL peptides, e.g. PAX5.(348, 349) The advantage of polyclonal $\gamma\delta$ T cells expanded on aAPC is that there may be sufficient direct tumor lysis that ADCC would not be necessary. However, the APC function of aAPC-expanded polyclonal $\gamma\delta$ T cells has not yet been studied. Lastly, melanoma may be an ideal target for combinational cellular immunotherapy because it is one of the most responsive tumors to immunotherapy and many T cells specific to melanoma peptides, e.g. MART1 and gp100, have been well characterized for rapid detection of antigen-specific responses. As aAPC have already been adapted for melanoma TIL studies (Forget MA, unpublished observation),(274) it is a logical next step to evaluate whether polyclonal $\gamma\delta$ T cells can induce antigen-specific $CD8^+$ T cell responses to melanoma. If successful, this approach could impact the TIL expansion protocols to adapt them to a wider range of patients. In aggregate, there are many combinatory approaches that can be taken to increase the therapeutic payload to cellular immunotherapy.

V.C. Generation of IL17-producing T cells for Adoptive Immunotherapy

IL17 has been shown to have potent anti-tumor effects when used in the tumor microenvironment, and therefore secretion of IL17 by transferred T cells homing to the tumor may have potent anti-tumor immunity.(321, 323, 350) T cells that produce IL17 can be mutually exclusive from those who produce IFNγ. Indeed, most of the T cells expanded on aAPC in this dissertation, with or without CARs, produced IFNγ, and the expanded γδ T cells secreted IL17 in diminished quantities compared to IFNγ (**Figures 23 and 35**). CD27 has been a marker for these cytokines in γδ T cells where $CD27^{neg}$ and $CD27^{+}$ are associated with IL17 and IFNγ, respectively.(333, 351, 352) It holds then that ~80% of polyclonal γδ T cells stain positive for CD27 (**Figures 33 and 34**). CD28 co-stimulation was shown to inhibit T_H17 polarization in $CD4^+$ T cells through ICOS co-stimulation,(326) and so it may be that CD86 co-stimulation by aAPC and/or CD28 endodomains in the CAR lead to polarization towards IFNγ in polyclonal γδ T cells, CAR^+ γδ T cells, and CAR^+ αβ T cells. Replacement of CD28 for ICOS in the CAR(s) and CD86 for ICOSL in the aAPC can be tested to see if these can generate T cells that secrete IL17. Another strategy comes out of the observation that ROR1-specific CAR^+ T cells signaling through CD137 produce less IFNγ than do those signaling through CD28 (**Figure 15**). This may be due to (i) CD137 signaling yielding less inflammatory cells or (ii) CAR-CD137 cells expressed other cytokines that have yet to be detected. Clinical trials out of The University of Pennsylvania (PI: June, CH) using CD19-specific CAR^+ T cells for ALL and CLL treatment have shown that responders had high serum IL6.(4, 7) This cytokine has importance for macrophages, inflammatory response (of particular interest in his trials as patients underwent massive fevers from T cells attacking their leukemia), and polarization of $CD4^+$ T cells from T_{REG} to T_H17.(127) In regards to the latter, release of immunosuppression by T_{REG} and production of IL17 could explain these impressive complete responses. **Chapter II** did not directly evaluate the influence of T_{REG} cells on CAR^+ T cell function or IL6 and IL17 production, but experiments using intracellular cytokine staining or multiplex arrays can be used to pursue this line of questioning. Development of an aAPC-based expansion of IL17 secreting T cells would allow for direct testing of their benefit relative to IFNγ-producing cells, and may lead to rationales to use one or both of them in the clinic for cancer therapies.

V.D. Importance of Polyclonal γδ T cells to Immunology

One of the major accomplishments of this dissertation was creating a method for expanding polyclonal γδ T cells (**Chapter IV**), which has broader applications outside of immunotherapy to the immunology and cancer biology fields. For example, few mAb exist that are specific for TCRγδ isotypes, which limits their detection in correlative studies and other assays.(165) Given the ability of aAPC to expand large numbers of polyclonal γδ T cells, mice can be immunized to generate mAb specific for desired TCRγδ isotypes, e.g. Vδ3 and Vγ isotypes outside of Vγ9. Commercial and academic use of these detection antibodies have tangible outcomes, including diagnostic and/or prognostic profiling of γδ T cell TIL within tumors. Other major unknowns are the ligands for many TCRγδ heterodimers. Generation of γδ T cell clones could be used to determine the specific ligands of Vδ/Vγ combinations and therefore lead to future studies on γδ T cell affinity towards a particular disease. Moreover, the ligands on the K562-derived aAPC that TCRγδ binds are unknown. Likely candidates include IPP (Vδ2) and MICA/B (Vδ1) but their exact roles have not been determined.(155, 172) Elucidation of these interactions could assist attempts to tailor the aAPC for total γδ T cell expansion, expansion of a particular γδ T cell lineage, or polarization towards a certain γδ T cell phenotype. Thus, aAPC could be an excellent source for the study of fundamental γδ T cell immunobiology and could yield answers not currently accessible because of limited starting cell numbers and ineffective polyclonal expansion protocols.

V.E. Potential Benefits and Issues with Cellular Immunotherapy

Although promising, there may be some limitations to the immunotherapies created in this dissertation. First, patients with advanced B-cell leukemia disease often have few T cells in their peripheral blood, and have even fewer γδ T cells.(6) In some cases, the residual autologous T cells are functionally unresponsive and difficult to expand to clinically-relevant doses.(353) Preliminary studies using CD19-specific CAR have indicated that CAR^+ T cells can be generated from CLL patients with <5% T cells at the start of culture (Huls MH, unpublished observation). Other options would be to use haplo-identical or MHC-matched T cells. However, this is not always feasible, so allogeneic γδ T cells could be an ideal choice because of they are generally thought to recognize antigens outside of MHC-restriction.(304, 342) Of course, if normal hematopoiesis resumes in the patients then the γδ T cell graft may be rejected, but there may still be a therapeutic window. Another unknown is whether γδ T cells will be subjected to the same regulation by T_{REG} cells or other immunosuppressive forces. Some γδ T cells have been reported to have immunosuppressive function, and it would be of interest to identify these cells and eliminate them from the adoptive T cell product prior to infusion.(354) The tumor microenvironment is also of interest because it often contains hypoxic areas containing malignant cells resistant to conventional treatments.(355, 356) In preliminary experiments, the co-culture system was adapted to assess γδ T cell proliferation as a function of oxygen tension. No difference in proliferative capacity ($p = 0.404$) was observed when the cultures were in hypoxia (1% O_2) or normoxia (20% O_2) and stimulated with clone#4 aAPC, IL2, and IL21, indicating that γδ T cells have potential to operate within the bone marrow or hypoxic tumor milieu (**Figure 44**). Thus, administration of graded doses of autologous and allogeneic γδ T cells in humans will test the ability of γδ T cells to home and recycle effector function in the tumor microenvironment. In the end, clinical trials will be the ultimate test of whether these potential pitfalls out weight the anti-tumor benefits to cancer patients.

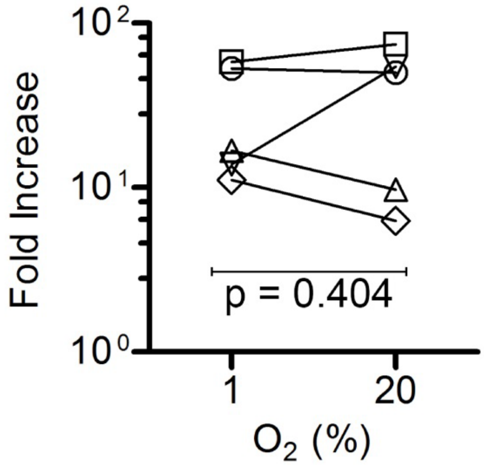

Figure 44. Proliferation of γδ T cells in Hypoxia Compared to Normoxia. Co-cultures were initiated in parallel with γδ T cells and aAPC in the presence of exogenous IL2 and IL21 in incubators set with either 1% O_2 (hypoxia) or 20% O_2 (normoxia) and were normalized to starting quantities 10 days after culture initiation. Student's paired, 2-tailed t-test was used for statistical analysis.

V.F. Clinical Applications of Dissertation Immunotherapies

As of June, 2013 there are immediate plans to use immunotherapies detailed in **Chapters II and IV** in the clinic. A Phase I clinical trial was written to co-administer autologous ROR1RCD28 and ROR1RCD137 T cell populations into CLL patients after lymphodepletive (Cytoxan and Fludarabine) chemotherapy. Proof-of-principle studies have established protocols for expanding CAR^+ T cells from patient samples by using an "electroporation-then-sort" strategy used for growing CAR^+ γδ T cells (**Chapter III**). Patient PBMC will be electroporated with SB transposase and SB transposase plasmids and sorted on paramagnetic beads the following day to deplete $CD19^+$ T cells. Co-culture on aAPC led to CAR^+ T cell growth in the translation research lab (TRL) built to translate lab protocols to the current good manufacturing practices (cGMP) facility. As more data has arrived, the support for utilizing only ROR1RCD137 in the clinical trial has gained momentum and may be the treatment modality tested instead of a competitive re-population experiment of both CAR^+ T cell populations. This investigational new drug (IND) application passed rigorous examination by the National Institutes of Health (NIH) Recombinant DNA Advisory Committee (RAC) with approval in December 2012. Review at the MD Anderson IRB is underway before sending the trial for final IND approval by the Food and Drug Administration (FDA). A second CAR trial has been proposed for treatment of leukemia with both ROR1-specific T cells and the chemotherapy dasatinib, which leads to increased surface expression of ROR1 in t(1;19) B-ALL cells and could minimize the risk for ROR1 antigen escape.(13) In regards to **Chapter IV** translation, a compassionate IND (CIND) has been written to treat a late stage CLL patient with autologous or allogeneic polyclonal γδ T cells in the case that the autologous γδ T cells do not proliferate or respond to the tumor. If allogeneic γδ T cells are infused into this patient, this will represent the first time that purified polyclonal γδ T cells from an allogeneic host were ever infused into a human. There is great optimism that the polyclonal γδ T cells can home to secondary lymphoid tissues harboring CLL and that they can eliminate the leukemia. These two trials are, hopefully, the beginning of the trials to come that will apply ROR1-specific T cells, CAR^+ γδ T cells, and polyclonal γδ T cells for human cancer immunotherapies.

CHAPTER VI

MATERIALS AND METHODS

VI.A. DNA Plasmids and Construct Cloning

All plasmids in this study were propagated in dam$^{-/-}$ bacteria (C2925, Invitrogen, Grand Island, NY) and purified as single cell bacteria clones with EndoFree Plasmid Maxi Kit (Qiagen, Valencia, CA). Plasmids were cleared for transfection when (i) identity was confirmed by analytical digestion, (ii) samples were negative for endotoxin, and (iii) had spectrophotometer readings of $1.80 < A_{260}/A_{280} < 2.00$.

VI.A.1. Tumor Antigens

VI.A.1.a. ROR1

The extracellular and transmembrane domains of ROR1 (Accession: NM_005012), termed dROR1, were cloned into a SB vector (pSBSO). The open reading frame (ORF) was codon optimized for expression in human cells and cloned into a shuttle vector (pMK-RQ) by GeneArt (Invitrogen). Codon-optimized dROR1/pMK-RQ and GlySer-EGFP-mIgG1(CooP)/pSBSO plasmids were digested with *NheI* and *XhoI* restriction enzymes and were purified from pMK-RQ and GlySer-EGFP-mIgG1 fragments, respectively, by gel electrophoresis. Purified dROR1 and pSBSO fragments were ligated with T4 DNA Ligase (Promega, Madison, WI) to create dROR1/pSBSO plasmid, which was then amplified in the presence of kanamycin for large-scale purification. Identity of the purified plasmid was confirmed with digestions of (i) *ClaI*, (ii) *ClaI* and *SmaI*, (iii) *PvuII*, and (iv) *PvuI* and *SmaI* enzymes to distinguish between parental plasmids and dROR1/pSBSO.

VI.A.1.b. CD19

The extracellular and transmembrane domains of human CD19 (Accession: M84371), termed Delta-CD19, were cloned into a pSBSO with linked F2A cleavage site and neomycin resistance (NeoR) for enforced dCD19 expression (performed by Olivares S). As with dROR1, the ORF was codon optimized for expression in human cells and cloned into a shuttle vector by GeneArt. In order to create the final vector, codon-optimized dCD19 from plasmid vector Delta-CD19(CoOp)-F2A-SStomato/pSBSO and Neomycin resistance from plasmid vector Myc-FFLuc(CoOp)-Neo/pSBSO, were digested with *ZraI*/*SpeI* and *EcoRV*/*SpeI* restriction enzymes respectively. The fragments (Neo-insert and Delta-CD19(CoOp)-F2A-X/pSBSO-vector) were purified by gel electrophoresis. Purified fragments were ligated with T4 DNA Ligase to create Delta-CD19(CoOp)-F2A-Neo/pSBSO plasmid, which was then amplified in the presence of kanamycin for large-scale purification. Identity of the purified plasmid was confirmed with digestions of *SacI* restriction enzyme to distinguish between parental plasmids and Delta-CD19-F2A-NeoR/pSBSO.

VI.A.2. Co-stimulatory Molecules

VI.A.2.a. CD86 and CD137L

The entire ORF for CD86 (Accession: EF064748.1) and CD137L (Accession: NM_003811.3) were codon optimized and synthesized by GeneArt and were then cloned into pSBSO (performed by Ang S).

VI.A.2.b. IL15-IL15Rα Fusion Construct

This construct will produce an IL15 that is membrane-bound, but also presented in the context of IL15Rα. A fusion of IL15 (NM_000585.4) to the full length IL15Rα (NM_002189.3) was constructed with a serine-glycine linker and a C-terminal Flag (x3) motif attached to generate membrane bound IL15 (mIL15). The signal peptides for IL15 and IL15Rα were omitted and the IgE signal peptide (gb|AAB59424.1) was used for the mIL15 fusion protein. As with dROR1, mIL15 was codon optimized and synthesized by GeneArt and was then subcloned into GlySer-EGFP-mIgG1(CooP)/pSBSO using *NheI* and *XhoI* restriction sites.

VI.A.3. Chimeric Antigen Receptors

Cloning of second generation CD19-specific CAR signaling through CD28 and CD3ζ (CD19RCD28) has been previously described.(57, 272, 281) The CAR was modified to replace CD28 endodomain for CD137 endodomain as a synthetic cDNA sequence

(GeneArt) that was cloned back into the original plasmid with *SmaI* and *SpeI* restriction endonucleases to create another second generation CD19-specific CAR signaling though CD137 and CD3ζ (CD19RCD137). These plasmids were further manipulated to contain "SIM" and "FRA" oligonucleotides at the 3' end of the CD19RCD28 and CD19RCD137 transposons, respectively, by shuttling the entire CARs into new pSBSO backbones with *NheI* and *XhoI* enzymes (CD19-specific CAR work performed by Olivares S). Heavy and light chain immunoglobulin sequences from the 4A5 mAb hybridoma were provided by Dr. Thomas J Kipps (UCSD) and were used to assemble the following ROR1R sequence *de novo* (GeneArt) from 5' to 3' (i) murine IgGκ signal peptide, (ii) V_L, (iii) Whitlow linker, (iv) V_H, and (v) the first 73 amino acids of the IgG4 stalk, and ROR1R sequence was shipped to MD Anderson as ROR1R(CoOp)/pMK-RQ plasmid. Amplification of ROR1R fragment from ROR1R(CoOp)/pMK-RQ was done by PCR with the following primers: ROR1RCoOpF (GCTAGCCGCCACCATGGGCTGGTCCTGCATC) and ROR1Rrev (GCTCCTCCC GGGGCTTTGTCTTGGC). The PCR product was cloned into pCR4-TOPO with TOPO TA Cloning Kit (Invitrogen) to generate ROR1R(CoOp)/pCR4-TOPO and the sequence was verified with T7 and T13-0 primers by Sanger sequencing (DNA Sequencing Core, MDACC). Then *NheI* and *SmaI* were used to digest ROR1R(CoOp)/pCR4-TOPO and CD19RCD28mZ(CoOp)/pEK plasmids and appropriate bands were purified by gel electrophoresis and ligated with T4 DNA Ligase to generate ROR1RCD28mZ(CoOp)/pEK. The ROR1-specific CAR was then transferred into a SB transposon by digestion of CD19RCD28mZ(CoOp)/pSBSO-MCS and ROR1RCD28mZ(CoOp)/pEK with *NheI* and *SpeI*, removal of phosphates by

Antarctic Phosphatase from pSBSO-MCS digestion, isolation of ROR1RCD28mZ and pSBSO-MCS bands by gel electrophoresis, and ligation with T4 DNA Ligase to generate ROR1RCD28mZ(CoOp)/pSBSO-MCS. The final ROR1RCD28 transposon plasmid was constructed by digesting CD19RCD28mZ(CoOp)/pSBSO-SIM with *NheI*, *XmaI*, and *Antarctic Phosphatase* and ROR1RCD28mZ(CoOp)/pSBSO-MCS with *NheI*, *XmnI*, and *XmaI*, purifying appropriate bands by gel electrophoresis, and ligating them together with T4 DNA Ligase to generate ROR1RCD28CD3z/pSBSO-SIM plasmid. Similarly, the final ROR1RCD137 transposon plasmid was constructed by digesting CD19R-CD28Tm-41BBCyt-Z(CoOp)/pSBSO-FRA with *NheI*, *XmaI*, and *Antarctic Phosphatase* and ROR1RCD28mZ(CoOp)/pSBSO-MCS with *NheI*, *XmnI*, and *XmaI*, purifying appropriate bands by gel electrophoresis, and ligating them together with T4 DNA Ligase to generate ROR1RCD137CD3z/pSBSO-FRA plasmid. Identities of final ROR1R plasmids were distinguished from CD19R plamids by *PmlI* enzyme and pSBSO-SIM and pSBSO-FRA plasmids were distinguished by *BsrGI* enzyme (**Figure 7**).

VI.B. Cell Culture

Three media formulations were used herein for tissue culture. First, RPMI-CM was composed of RPMI (Gibco, Grand Island, NY), 10% heat-inactivated fetal bovine serum (FBS; Hyclone, Logan, UT), and 1% Glutamax-100 (Gibco). Similarly, RPMI-NaPyr-CM was RPMI, 10% FBS, 1% sodium pyruvate solution (Gibco), and 1% Glutamax-100. Last, DMEM-CM was made with DMEM (Sigma, St. Louis, MO), 10%

FBS, 1% sodium pyruvate solution, and 1% Glutamax-100. All tissue culture work was performed with 5% CO_2 at 37^oC in humidified conditions unless otherwise stated.

VI.B.1. Established Tumor Cell Lines

Jurkat, HCT-116, Kasumi3, and K562 cell lines were acquired from American Type Culture Collection (ATCC; Manassas, VA). K562-derived aAPC (clone #4 and clone#9) were acquired as previously described from the University of Pennslyvania courtesy of Dr. Carl June.(57, 275, 278, 279) B-cell acute lymphoblastic leukemia (B-ALL) cell lines cALL2, Kasumi2, REH, and RCH-ACV cell lines were gifts from Dr. Jeff Tyner (OHSU), pancreatic cancer cell lines (BxPC3, CaPan2, MiaPaCa2, and Su8686) were donated by Dr. Viji Ramachandran (MDACC), and ovarian cancer cell lines (A2780, CAOV3, EFO21, Hey, IGROV1, OC314, OVCAR3, and UPN251) were provided by Dr. Robert Bast (MDACC). Cell cultures were maintained in (i) RPMI-CM: K562 parental cells, clone#1 aAPC, clone#4 aAPC, clone A6 aAPC, clone A3 aAPC, clone D4 aAPC, Jurkat, cALL2, Kasumi2, REH, RCH-ACV, and Kasumi3, (ii) RPMI-NaPyr-CM: A2780, EFO21, EFO27, Hey, IGROV1, OC314, OVCAR3, SKOV3, and UPN251, or (iii) DMEM-CM: CAOV3, BxPC3, CaPan2, MiaPaCa2, and Su8686. UPN251 cells were supplemented with insulin-transferrin-selenium solution (Gibco). Identities of all cell lines were confirmed by STR DNA Fingerprinting at MDACC's Cancer Center Support Grant (CCGS) supported facility "Characterized Cell Line Core."

VI.B.2. Genetic Modification of Cell Lines

VI.B.2.a. ROR1 aAPC (clone#1)

Clone#9 aAPC was generated though enforced expression of CD19, CD64, CD86, and CD137L on K562 cells (June CH, UPenn). This aAPC was further modified to express IL15/IL15Rα fusion protein (**Chapter VI.A.2.b**) on their surfaces and was sub-cloned to generate clone#27. Then clone#27 was made to express dROR1 (**Chapter VI.A.1.a**), and single cell clones were isolated based on expression of ROR1, CD137L, and IL15. The clone#1 aAPC uniformly expressed CD19, CD32, CD64, CD86, CD137L, IL15, and ROR1 and was cleared for co-culture following negative testing for mycoplasma and other microbial pathogens.

VI.B.2.b. HLA$^{-/-}$ aAPC

Zinc-finger nuclease (ZFN) specific for HLA-C was used to remove all MHC Class I expression from K562 cell surface (Torakai H, Lee DA, Rosoff H, and Cooper LJN). Clone#4 aAPC expresses IL15, CD86, and CD137L, so in order to investigate the roles of these molecules on γδ T cell proliferation new aAPC were constructed on K562 background (**Figure 28**). SB transposon containing IL15/IL15Rα fusion protein and SB11 transposase were electro-transferred into K562 cells (CD86^{neg} and CD137L^{neg}) using Amaxa nucleofection and Kit V (cat#VCA-1003, Lonza, Basel, Switzerland). FACS was used to isolate IL15^{+} cells, which were electroporated with SB11 and SB transposons containing either CD86 or CD137L. Cells were sorted again by FACS to obtain IL15^{+}CD86^{+} or IL15^{+}CD137L^{+} as single cell clones A3 (IL15^{+}CD86^{+}CD137L^{neg}) and D4 (IL15^{+}CD86^{neg}CD137L^{+}), respectively. Single cell sorting FACS was also used to make a single cell clone (A6; IL15^{+}CD86^{neg}CD137L^{neg}) of cells electroporated once. Each cell line was negative for mycoplasma and microbial pathogens.

VI.B.2.c. Lenitviral Packaging and Gene Transduction

Lentivirus particles were packaged according to a modified version of a protocol described elsewhere.(357) Briefly, packaging cells (293-METR) were plated on flasks and transfected the following day with pCMV R8.2, VSV-G, and pLVU3G-effLuc-T2A-mKateS158A (**Figure 45**) plasmids in conjunction with Lipofectamine 2000 transfection reagent according to manufacturer's instructions (Invitrogen). Virus-like particles were harvested 48 and 72 hours post-transfection and were concentrated on 100 kDa NMWL filters (cat#UFC810096, MilliPore, Billerica, MA). CAOV3 cells were plated on wells of a 6 well plate, and the following day *ffluc*-mKate virus particles were added with 8 μg/ml polybrene then plate was spun at 1,800 rpm for 1.5 hours. The same was done for Kasumi2, except that polybrene was not added. Six hours later, the viral-conditioned supernatant was removed and the tissue culture media was immediately changed and changed the following day. Transduced CAOV3 were sub-cultured and single-cell clones were derived from limiting dilution that displayed the same morphology as the parental cell line and had uniform mKate fluorescence with high (>10^6 signal to noise ratio) *ffLuc* activity. CAOV3 clone 1C2 was used for mouse experiments. Kasumi2 were sorted for mKate and were used as a bulk population for mouse experiments (**Figure 17a and 17b**).

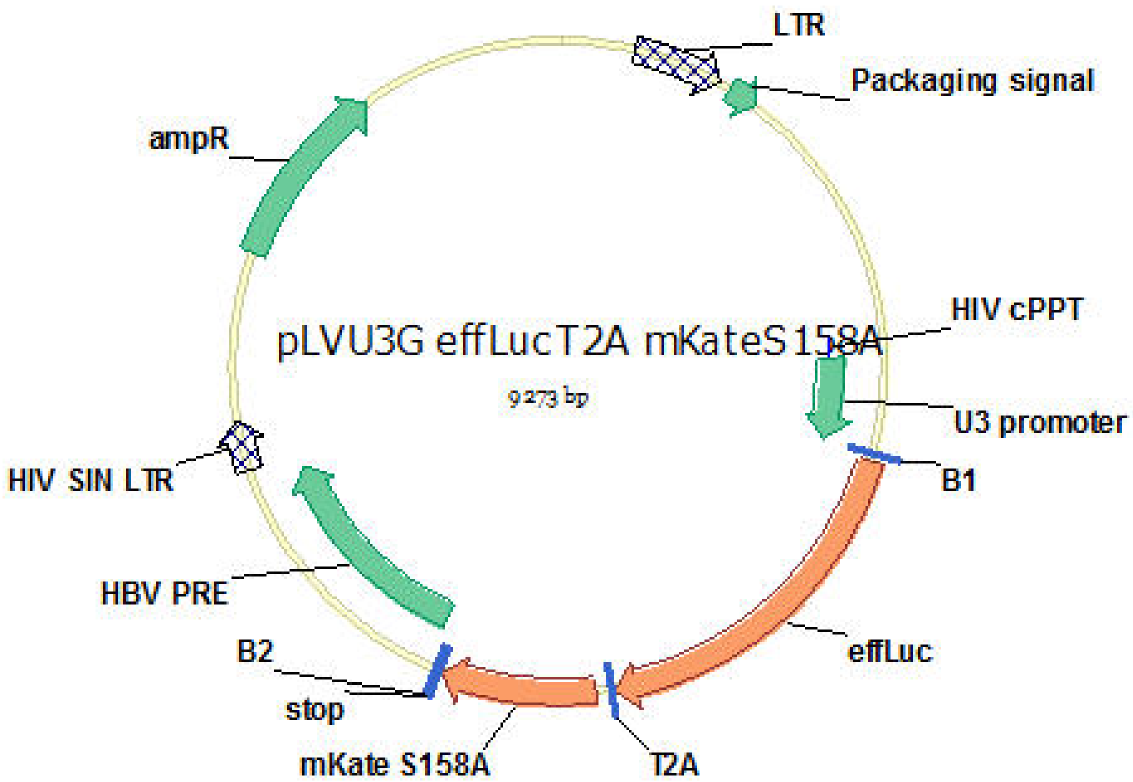

Figure 45. DNA Plasmid Map for pLVU3G-effLuc-T2A-mKateS158A. Annotations are, LTR: long terminal repeat; HIV cPPT: HIV central polypurine tract; B1: Gateway donor site B1; effLuc: enhanced *firefly Luciferase*; T2A: T2A ribosomal slip site; mKate S158A: enhanced mKate red fluorescence protein; B2: Gateway donor site B2; HBV PRE: Hepatitis B post-translational regulatory element; HIV SIN LTR: HIV self-inactivating long terminal repeat; ampR: ampicillin resistance (β-Lactamase).

VI.B.3. Primary Tumor Cells

PBMC were isolated by Ficoll-Hypaque (GE Healthcare) from patients with CLL diagnosis after informed consent was granted. Samples were cryopreserved and were thawed and used the day of the experiments. All cells frozen at the Cooper Lab were cryopreserved in 50% FBS, 40% RPMI, 10% dimethylsulfoxide (DMSO) termed "free media." All patient samples were maintained in RPMI-CM.

VI.B.4. Lymphocyte Cultures

All PBMC from adult donor blood or UCB used in this dissertation were obtained after informed consent and were isolated from whole blood by Ficoll-Hypaque or steady-state apheresis. PBMC were cryopreserved and thawed for experimental use whereas UCB were freshly isolated and immediately used. All aAPC were γ-irradiated (100 Gy) prior to co-culture and were then used immediately or were cryopreserved then thawed at the time of the co-culture. Validation of co-expression of cell surface markers (for example CD19, CD64, CD86, CD137L, and IL15 (co-expressed with eGFP) for clone #4) were performed before addition to T-cell cultures. All lymphocyte cultures were maintained in RPMI-CM.

VI.B.4.a. CAR^{neg} αβ T cells

γ-irradiated clone#4 aAPC were loaded with OKT3 antibody, which is agonistic for CD3 thereby leading to T cell proliferation independent of the TCR specificity, by

OKT3 antibody docking on CD64 (high-affinity Fc Receptor) expressed on aAPC. $CD3^+$ T cells were stimulated with an equal number of OKT3-loaded clone#4 cells in the presence of exogenous IL2 (50 U/mL; Aldeleukin; Novartis, Switzerland) and IL21 (30 ng/mL; cat#AF20021; Peprotech, Rocky Hill, NJ) unless otherwise stated. Exogenous IL2 and IL21 were added back to cultures every 2-3 days along with at least half of the current volume of RPMI-CM.

VI.B.4.b. CAR^+ $\alpha\beta$ T cells

CAR^+ T cells were propagated based on modified standard operating protocols as previously described.(57, 273) Cryopreserved PBMC were thawed the day of the electroporation (designated day 0) and rested for 2 hours in RPMI-CM at 37^{o}C. Cells for electroporation were spun at 200g for 10 minutes, enumerated, and $2x10^7$ cells were mixed with DNA (5 μg SB11 transposase and 15 μg SB transposon) in Human T cell Nucleofector Solution (cat#VPA-1002, Lonza) then added to a cuvette, which was then electroporated on the U-014 program of Amaxa Nucleofector II (Lonza). Transfected cells were then added to wells of a 6-well plate containing phenol-free RPMI, 20% FBS, and 1x Glutamax-100. The following day, electroporated T cells were phenotyped and stimulated with aAPC according to their CAR expression. A ratio of 2:1 of clone#4 to CD19-specific CAR^+ T cells was used and a 1:1 ratio of clone#1 to ROR1-specific CAR^+ T cells was used. Each co-culture was supplemented with IL21 during the first week (given every 2-3 days) and with both IL2 and IL21 for the subsequent weeks. CAR expression was evaluated each week in order to do the stimulation according to

CAR$^+$ T cells. If NK cells reached >10% of the total populations, they were depleted from co-cultures with paramagnetic CD56 microbeads (cat#130-050-401, Miltenyi Biotec, Auburn, CA) and LS columns (cat#130-042-401, Miltenyi Biotec). Stocks were made of CAR$^+$ T cells at days 14, 21, 28, and 35 (where applicable), and inferred cell numbers were calculated by the number of cells that were generated multiplied by the fold change from the previous week relative to the number of cells that were carried forward. Phenotyping and functional analyses were performed between days 21 – 28 unless otherwise stated. For ROR1-specific CAR$^+$ T cell studies, 3 normal donors were tested in 3 independent experiments.

VI.B.4.c. CAR$^+$ γδ T cells

CAR$^+$ γδ T cells were generated as previously described.(311) Briefly, 10^8 PBMC were electroporated as described above for CAR$^+$ αβ T cells (**Chapter VI.B.4.b.**), and were then sorted for γδ T cells using TCRγ/δ+ Isolation Kit (cat#130-092-892, Miltenyi Biotec). Co-cultures were established with clone#4 along with IL2 and IL21 from the start of the cultures where cytokines were added every 2-3 days and clone#4 aAPC was added every 7 days at a 2:1 ratio with CAR$^+$ γδ T cells. NK cells were depleted from co-cultures when they reached >10% of total cells as described above. T cells were phenotyped for CD3, Fc (CAR), CD56, and TCRγδ every week to monitor the co-cultures. Cells were cryopreserved at days 21, 28, and 35 and inferred cell numbers were calculated as described above. Functional assays were performed between the third and fifth weeks of stimulation. Six donors were tested in 3 independent experiments.

VI.B.4.d. Polyclonal γδ T cells

Experiments were initiated to expand γδ T cells on aAPC that did not express a CAR. Thawed PBMC (10^8) were depleted of NK cells as described above and were then labeled with TCRγ/δ+ T-cell isolation kit and placed on LS columns to separate γδ T cells in the unlabeled fraction from other cells attached to magnet. γδ T cells were stimulated at a ratio of one T cell to two aAPC (clone #4) in presence of exogenous IL2 and IL21. Cells were serially re-stimulated with addition of aAPC every 7 days for three weeks. FACS was used to isolate Vδ1 (TCRδ1^{+} TCRδ2^{neg}), Vδ2 (TCRδ1^{neg} TCRδ2^{+}), and Vδ3 (TCRδ1^{neg} TCRδ2^{neg}) populations, which were stimulated as above with clone#4 aAPC twice and then phenotyped and used for functional assays. UCB-derived mononuclear cells were isolated from fresh Ficoll-Hypaque gradients by FACS following staining for TCRγδ and CD3, and were stimulated for five weeks on aAPC as per PBMC. Ten PBMC donors were tested in six independent experiments and five UCB donors were tested in four independent experiments. Four donors were tested in 2 independent experiments for Vδ sorting assays.

VI.B.4.e. NK cells

As controls for killing and allogeneic reactivity, NK cells autologous to γδ T cells were separated from healthy donor PBMC with CD56 microbeads and LS columns and were then stimulated at a 1:2 ratio with clone#4 aAPC in cultures that were supplemented at the initiation of culture and every 2-3 days later with IL2 and IL21.

VI.B.4.f. γδ T cell Proliferation in Hypoxia

A dedicated incubator set to 1% O_2, 5% CO_2, and 37^oC under humidified conditions was used to assess proliferation in hypoxia in parallel to "normal" tissue culture incubators set at 20% O_2, 5% CO_2, and 37^oC under humidified conditions. Parallel co-cultures were added to the incubators and were analyzed after the reported times.

VI.B.5 γδ T cell Co-culture Deconstruction

Experiments were implemented to assess the relative contribution of co-culture molecules to γδ T cell proliferation. This was dissected by cytokine dependence and dependence upon molecules on the aAPC using new aAPC described in **Chapter VI.B.2.b.** (**Figure 28**).

VI.B.5.a. Effects of Cytokines on γδ T cell Proliferation

In order to assess the dependence of γδ T cells on cytokines for proliferation, co-cultures were initiated with 10^5 γδ T cells and $2x10^5$ clone#4 aAPC and then were added to an equal volume of (i) complete media (CM), (ii) CM and 100 U/mL IL2, (iii) CM and 60 ng/mL IL21, or (iv) CM, 100 U/mL IL2, and 60 ng/mL IL21. Co-cultures were counted 9 days after initiation to determine yields. Three donors were tested in two independent experiments.

VI.B.5.b. Effects of Co-Stimulation on γδ T cell Proliferation

$HLA^{-/-}$ aAPC (**Chapter VI.B.2.b**) were used to assess effects of co-stimulation on γδ T cell growth. Co-cultures were then initiated with 10^5 γδ T cells in CM, 100 U/mL IL2, and 60 ng/mL IL21 and were added to $2x10^5$ γ-irradiated (i) parental K562 cells, (ii) clone A6, (iii) clone A3, (iv) clone D4, (v) clone#4 aAPC, or (vi) no aAPC. Co-cultures were counted 9 days as above with cytokine experiments. Three donors were tested in two independent experiments.

VI.C. Multiplex Gene Expression Analysis

At Day 22 of co-culture on aAPC, $>10^5$ T cells were lysed at a ratio of 5 μL RLT Buffer (Qiagen) per $3x10^4$ cells and frozen at -80°C in replicate vials for one time use. RNA lysates were thawed and immediately analyzed using nCounter Analysis System

(NanoString Technologies, Seattle, WA) with "designer TCR expression array" (DTEA) as previously described or with "lymphocyte codeset array" (LCA; **Appendix A**).(290, 311) DTEA data was normalized to both spike positive control RNA and housekeeping genes (ACTB, G6PD, OAZ1, POLR1B, POLR2A, RPL27, Rps13, and TBP) where 2 normalization factors were calculated and applied to the raw coutns. Each normalization factor was calculated from the average of sums for all samples divided by the sum of counts for an individual sample. Reported expression of TCR frequencies for ROR1-specific T cells (**Figure 14**) was calculated as counts for each TCRα or TCRβ allele over the total sum of TCRα or TCRβ counts, respectively. Total counts for LCA genes described in ROR1-specific CAR$^+$ T cells (**Figures 12 and 13**) and for TCRδ and TCRγ alleles in polyclonal γδ T cells were directly reported as normalized counts (**Figure 30**). For Vδ sorted γδ T cells, the normalized counts were reported at frequencies of each Vδ population per donor for each TCRδ or TCRγ allele (**Figure 32**). For example, $\%V\delta1{*}01 = (V\delta1{*}01)_{V\delta1} / [(Vd1{*}01)_{V\delta1} + (V\delta1{*}01)_{V\delta2} + (V\delta1{*}01)_{V\delta3}]$.

VI.D. Immunostaining

Antibodies directly conjugated to FITC, PerCP/Cy5.5, PE, and APC were used at 1:20, 1:33, 1:40, and 1:40 dilutions, respectively, in 100 μL FACS buffer (PBS, 0.1% FBS, 0.1% sodium azide) unless otherwise stated. A complete list of antibodies, clonotypes, and vendors can be found in **Appendix B**. CAR detection was primarily performed with anti-human Fc antibody (Invitrogen). CD19-specific CAR was stained with an idiotypic antibody conjugated to AlexFluor-647 in some instances.(259) BD FACS CAlibur was used for most flow cytometry. Samples were analyzed with FlowJo software (version 7.6.5). BD FACS Aria Ilu II was used to sort cells where appropriate and was used to isolate single cell clones in 96 well plates for aAPC cloning strategies. Tumor cells transduced with *ffLuc*-mKate lentivirus particles were sorted for mKate expression on BD Influx for bulk populations or as single cell clones as appropriate.

VI.E. Cytokine Production

Expression of cytokines was assessed by intracellular cytokine staining (ICS) and secretion of cytokines into tissue culture supernatants was evaluated by Luminex multiplex analysis. Co-cultures were set up with T cells and targets as described for each experiment and were incubated at 37^{o}C. For ICS, Brefeldin-A (GolgiPlug; BD Biosciences) was added to co-cultures to block exocytosis and secretion of cytokines produced in response to agonists. All ICS experiments were incubated for 6 hours and were then (i) stained for surface markers, e.g. CD3 and CAR, (ii) fixed and permeabilized with BD Cytofix/Cytoperm (BD Biosciences), (iii) stained for intracellular proteins, e.g. IFNγ and TNFα, and (iv) analyzed by flow cytometry. Co-cultures to assess cytokine secretion were incubated for 24 hours and supernatants from triplicate wells were pooled and analyzed by Bio-Plex Human Cytokine Group I 27-plex Assay (#L50-0KCAF0Y, BioRad Technologies, Hercules, CA) using Luminex100 (xMap Technologies, Austin, TX).

VI.F. *In Vitro* Killing Assays

VI.F.1. Chromium Release Assay

In vitro specific lysis was assessed using a standard 4-hour CRA, as previously described.(57) Purified antibodies specific for NKG2D (clone 1D11; BD Biosciences), DNAM1 (clone DX11; BD Biosciences), TCRγδ (clone B1; BD Biosciences), and TCRγδ (clone IMMU510; Thermo Fisher, Pittsburg, PA) were used for neutralization experiments at 0.3, 1.0, and 3.0 μg/mL final concentrations in CRA at E:T ratios of 12:1. Normal mouse serum was used as a negative control at the same concentrations.

VI.F.2. Long-term Killing Assay

Tumor cells were seeded in wells of 12-well plates at a density of $4x10^4$ cells/well. The following day, $5x10^5$ γδ T cells were added to each well of the plate and an equal number was added to a well without tumor cells (media only). One well of tumor cells had an equal volume of RPMI-CM added as a positive control for growth. After 2 days, supernatants were harvested, wells were washed in PBS, and remaining tumor cells were harvested with trypsin-EDTA and were then enumerated. The frequencies of cells remaining were normalized to mock treated tumor cells.

VI.G. Mixed Lymphocyte Reactions

B cells from healthy donors were isolated with CD19 microbeads (cat#130-050-301, Miltenyi Biotec) the day of each assay and were used as target cells in proliferation, IFNγ production (ELISpot), and cytolysis assays. Standard 4-hour CRA were used for the latter as described above. For proliferation assays, effector cells were labeled with PKH26 red fluorescent dye according to manufacturer's instructions (Sigma) and were co-cultured with target cells for 4 days at 37^{o}C at an E:T ratio of 5:1. Co-cultures were stained for CD3, CD19, and CD56 then were analyzed by flow cytometry. Similarly, IFNγ ELISpot plate (Mabtech, Mariemont, OH) was set up with effector (γδ T cells) and target (B cells) at an E:T ratio of 0.3:1 and plate was incubated for 24 hours at 37^{o}C then stained according to manufacturer's instructions, and spots were counted on Immunospot (CTL, Shaker Heights, OH). OKT3-loaded aAPC were used as positive controls and mock treated were used as negative controls along with autologous B cells. Co-cultures of effectors and allogeneic PBMC (normalized to equal CD34^{+} cells) at a 4:1 ratio of effectors to CD34^{+} HSC were incubated at 37^{o}C for 4 hours and were then plated in wells of 6-well plates in semi-solid HSC-CFU Complete without EPO (Miltenyi Biotec). After 12 days, individual colonies were counted under inverted microscope. Colonies formed with effectors alone or targets alone were used to normalize the relative number of colonies formed for each donor.

VI.H. *In Vivo* Anti-tumor Activity

In vivo anti-tumor efficacy was assessed in NSG mice (NOD.Cg-Prkdc^{scid} Il2rγ^{tm1Wjl}/SzJ; Jackson Laboratories). Non-invasive BLI was performed during the course of the experiments to measure tumor burden of cell lines expressing *ffLuc* following subcutaneous D-Luciferin (cat#122796, Caliper, Hopkinton, MA) administration with IVIS-100 Imager (Caliper). BLI was analyzed using Living Image software (version 2.50, Xenogen, Caliper).

VI.H.1. ROR1-specific Anti-leukemia Effects

Kasumi-2-*ffLuc*-mKate cells (4×10^4 per mouse) were engrafted into NSG mice (n = 15) intravenously (i.v.) the day before the first T cell dose (designated Day -1). The following day (Day 0), treatment groups for mice with tumors were set up with (i) no treatment (n = 5), (ii) ROR1RCD28 T cells (n = 5), and (iii) ROR1RCD137 T cells (n = 5). Mice were injected with T cells only as controls for xenogeneic reactivity (one mouse per T cell type). T cell doses (10^7 total cells per mouse) were given on days 0, 7, and 14. Frequencies for CAR expression for ROR1RCD28 were 96%, 91%, and 90% and for ROR1RCD137 were 94%, 62%, and 46% on days 0, 7, and 14, respectively. Survival was the primary endpoint for the study and BLI from tumor *ffLuc* was monitored twice per week as above.

VI.H.2. CD19-specific Anti-leukemia Activity

The anti-tumor effects of CD19-specific CAR$^+$ γδ T cells were evaluated as previously described.(311)

VI.H.3. γδ T cells Clearance of Ovarian Cancer

CAOV3-*ffLuc*-mkate (clone 1C2; 3x10^6 cells/mouse) tumors were established by intraperitoneal (i.p.) injection and mice were randomly distributed into treatment groups. Eight days later (designated Day 0), a dose escalation regimen was initiated with polyclonal γδ T cells administered i.p. and PBS administered i.p. as a negative control. T cell doses infused were 3x10^6, 6x10^6, 10^7, and 1.5x10^7 on days 0, 7, 14, and 21, respectively. BLI was monitored during the course of the experiment by weekly monitoring of tumor *ffLuc* activity as above. Survival was the primary endpoint for the experiment.

APPENDICIES

Appendix A. Lymphocyte CodeSet Array

GENE ID	Access-ion	Target Region	Target Sequence
ABCB1	NM_000927.3	3910-4010	TATAGCACTAAAGTAGGAGACAAAGGAACTCAGCTCTCTGGTGGCCAGAAAC AACGCATTGCCATAGCTCGTGCCCTTGTTAGACAGCCTCATATTTTGC
ABCG2	NM_004827.2	285-385	AGGATTTAGGAACGCACCGTGCACATGCTTGGTGGTCTTGTTAAGTGGAAACT GCTGCTTTAGAGTTTGTTTGGAAGGTCCGGGTGACTCATCCCAACAT
ACTB	NM_001101.2	1010-1110	TGCAGAAGGAGATCACTGCCCTGGCACCCAGCACAATGAAGATCAAGATCAT TGCTCCTCCTGAGCGCAAGTACTCCGTGTGGATCGGCGGCTCCATCCT
ADAM19	NM_023038.3	1690-1790	GAGAAGGTGAATGTGGCAGGAGACACCTTTGGAAACTGTGGAAAGGACATG AATGGTGAACACAGGAAGTGCAACATGAGAGATGCGAAGTGTGGGAAGA
AGER	NM_001136.3	340-440	GAAAGGAGACCAAGTCCAACTACCGAGTCCGTGTCTACCAGATTCCTGGGAA GCCAGAAATTGTAGATTCTGCCTCTGAACTCACGGCTGGTGTTCCCAA
AHNAK	NM_001620.1	15420-15520	GGATTTGACCTGAATGTTCCTGGGGGTGAAATTGATGCCAGCCTCAAGGCTCC GGATGTAGATGTCAACATCGCAGGGCCGGATGCTGCACTCAAAGTCG
AIF1	NM_032955.1	315-415	AAAAGCGAGAGAAAAGGAAAAGCCAACAGGCCCCCCAGCCAAGAAAGCTAT CTCTGAGTTGCCCTGATTTGAAGGGAAAAGGGATGATGGGATTGAAGGG
AIM2	NM_004833.1	607-707	ACGTGCTGCACCAAAAGTCTCTCCTCATGTTAAGCCTGAACAGAAACAGATG GTGGCCCAGCAGGAATCTATCAGAGAAGGGTTTCAGAAGCGCTGTTTG
AKT1	NM_005163.2	1772-1872	TTCTTTGCCGGTATCGTGTGGCAGCACGTGTACGAGAAGAAGCTCAGCCCACC CTTCAAGCCCCAGGTCACGTCGGAGACTGACACCAGGTATTTTGATG
ALDH1A1	NM_000689.3	11-111	ATTGCTGAGCCAGTCACCTGTGTTCCAGGAGCCGAATCAGAAATGTCATCCTC AGGCACGCCAGACTTACCTGTCCTACTCACCGATTTGAAGATTCAAT
ANXA1	NM_000700.1	515-615	GAAATCAGAGACATTAACAGGGTCTACAGAGAGGAACTGAAGAGAGATCTG GCCAAAGACATAACCTCAGACACATCTGGAGATTTTCGGAACGCTTTGC
ANXA2P2	NR_003573.1	257-357	ATATTGTCTTCTCCTACCAGAGAAGGACCAAAAAGGAACTTGCATCAGCACT GAAGTCAGCCTTATCTGGCCACCTGGAGACGGTGATTTTGGGCCTATT
AP1	NM_002228.3	140-240	ACACAGCCAGCCAGCCAGGTCGGCAGTATAGTCCGAACTGCAAATCTTATTTT CTTTTCACCTTCTCTCTAACTGCCCAGAGCTAGCGCCTGTGGCTCCC
Apaf1	NM_181869.1	1160-1260	TTCTGATGAAACTGCAGAATCTTTGCACACGGTTGGATCAGGATGAGAGTTTT TCCCAGAGGCTTCCACTTAATATTGAAGAGGCTAAAGACCGTCTCCG
ARG1	NM_000045.2	505-605	AAGGAACTAAAAGGAAAGATTCCCGATGTGCCAGGATTCTCCTGGGTGACTC CCTGTATATCTGCCAAGGATATTGTGTATATTGGCTTGAGAGACGTGG
ATM	NM_000051.3	30-130	ACGCTAAGTCGCTGGCCATTGGTGGACATGGCGCAGGCGCGTTTGCTCCGAC GGGCCGAATGTTTTGGGGCAGTGTTTTGAGCGCGGAGACCGCGTGATA
ATP2B4	NM_001684.3	7640-7740	CTTCCCATAGTATCATCTGTCCTCTGGAATGACTCTCCTGTCCCTAAAGGGGTT AAGAGAGAGATCACCTAGAAATCCCTCTGGACACTTGTGGGTTCTT
B2M	NM_004048.2	25-125	CGGGCATTCCTGAAGCTGACAGCATTCGGGCCGAGATGTCTCGCTCCGTGGCC TTAGCTGTGCTCGCGCTACTCTCTCTTTCTGGCCTGGAGGCTATCCA
BACH2	NM_021813.2	3395-3495	TGTGGCACTGTTCATCTGCTGTCCCGAAGAAACCGAGAACACATTTGGTGCAC ACTACAGCGGTCTTAGCAGCAATACTGTTCCGAAGTATCCTCTCCTC
BAD	NM_004	195-295	CAGCTGTGCCTTGACTACGTAACATCTTGTCCTCACAGCCCAGAGCATGTTCC

	322.2		AGATCCCAGAGTTTGAGCCGAGTGAGCAGGAAGACTCCAGCTCTGCA
BATF	NM_006 399.3	825-925	CACTGTGGGTTGCAGGCCCAATGCAGAAGAGTATTAAGAAAGATGCTCAAGT CCCATGGCACAGAGCAAGGCGGGCAGGGAACGGTTATTTTTCTAAATA
BAX	NM_138 761.2	694-794	ATTTTTCTGGGAGGGGTGGGGATTGGGGGACATGGCATTTTTCTTACTTTTG TAATTATTGGGGGGTGTGGGGAAGAGTGGTCTTGAGGGGGTAATAAA
BCL10	NM_003 921.2	1250-1350	TGAAAATACCATCTTCTCTTCAACTACACTTCCCAGACCTGGGGACCCAGGGG CTCCTCCTTTGCCACCAGATCTACAGTTAGAAGAAGAAGGAACTTGT
Bcl2	NM_000 633.2	1525-1625	CCAAGCACCGCTTCGTGTGGCTCCACCTGGATGTTCTGTGCCTGTAAACATAG ATTCGCTTTCCATGTTGTTGGCCGGATCACCATCTGAAGAGCAGACG
BCL2L1	NM_138 578.1	1560-1660	CTAAGAGCCATTTAGGGGCCACTTTTGACTAGGGATTCAGGCTGCTTGGGATA AAGATGCAAGGACCAGGACTCCCTCCTCACCTCTGGACTGGCTAGAG
BCL2L11	NM_138 621.2	2825-2925	TGTTGGCACCAGAACTTAAAGCGATGACTGGATGTCTCTGTACTGTATGTATC TGGTTATCAAGATGCCTCTGTGCAGAAAGTATGCCTCCCGTGGGTAT
Bcl6	NM_001 706.2	675-775	GTTGTGGACACTTGCCGGAAGTTTATTAAGGCCAGTGAAGCAGAGATGGTTT CTGCCATCAAGCCTCCTCGTGAAGAGTTCCTCAACAGCCGGATGCTGA
Beta-arrestin (ARRB2 and ARRB2)	NM_004 313.3	1652-1752	CATTAATTTTTTGACTGCAGCTCTGCTTCTCCAGCCCCGCCGTGGGTGGCAAG CTGTGTTCATACCTAAATTTTCTGGAAGGGGACAGTGAAAAGAGGAG
BHLHE41	NM_030 762.2	655-755	CGCCCATTCAGTCCGACTTGGATGCGTTCCACTCGGGATTTCAAACATGCGCC AAAGAAGTCTTGCAATACCTCTCCCGGTTTGAGAGCTGGACACCCAG
BID	NM_197 966.1	2095-2195	GCTTAGCTTTAGAAACAGTGCAACACTGGTCTGCTGTTCCAGTGGTAAGCTAT GTCCCAGGAATCAGTTTAAAAGCACGACAGTGGATGCTGGGTCCATA
BIRC2	NM_001 166.3	1760-1860	TGGGATCCACCTCTAAGAATACGTCTCCAATGAGAAACAGTTTTGCACATTCA TTATCTCCCACCTTGGAACATAGTAGCTTGTTCAGTGGTTCTTACTC
BMI1	NM_005 180.5	1145-1245	CCTGGAGAAGGAATGGTCCACTTCCATTGAAATACAGAGTTCGACCTACTTGT AAAAGAATGAAGATCAGTCACCAGAGAGATGGACTGACAAATGCTGG
BNIP3	NM_004 052.2	325-425	CACCTCGCTCGCAGACACCACAAGATACCAACAGGGCTTCTGAAACAGATAC CCATAGCATTGGAGAGAAAAACAGCTCACAGTCTGAGGAAGATGATAT
C10RF24	NM_052 966.2	3526-3626	TGCCCAATAGATTCAAGAGAAGCTAAGCGGAAATGGAGGGTGGAAGGTGTG ATCTGTGGGACTGTCTGGGCCTGTTACTCATCCTGCTATCAATTTCTTA
C11ORF17	NM_020 642.3	570-670	GAACATCTCTAAGGACCTCTACATAGAAGTATATCCAGGGACCTATTCTGTCA CTGTGGGCTCAAATGACTTAACCAAGAAGACTCATGTGGTAGCAGTT
C5ORF13	NM_001 142474.1	990-1090	AAACTCATTGTTTCCTTGTGGTAAGTGACCGAGATGCTGCCACAGGACCTGAG ACACTGATGAATGGTGCTATTTTGGACTTTCAACATGCTCCTTGGCG
C80RF70	NM_016 010.2	665-765	ACGATTACCGCAGCCAAGTGGCGCTGGCAAAACTGTTGTAGGTGTTCCTTCAG GTAAAGTGTCTTCAAGTAGCAGCTCTTTGGGAAACAAACTTCAGACC
CA9	NM_001 216.2	960-1060	CAGGTCCCAGGACTGGACATATCTGCACTCCTGCCCTCTGACTTCAGCCGCTA CTTCCAATATGAGGGGTCTCTGACTACACCGCCCTGTGCCCAGGGTG
CASP1	NM_033 292.2	575-675	ACAGGCATGACAATGCTGCTACAAAATCTGGGGTACAGCGTAGATGTGAAAA AAAATCTCACTGCTTCGGACATGACTACAGAGCTGGAGGCATTTGCAC
Caspase 9	NM_052 813.2	1850-1950	CGCTGACTTGGCCTGGAACGAGGAATCTGGTGCCCTGAAAGGCCCAGCCGGA CTGCCGGGCATTGGGGCCGTTTGTTAAGCGGCACTCATTTTGCGGAGG
CAT	NM_001 752.2	1130-1230	ATGCTTCAGGGCCGCCTTTTTGCCTATCCTGACACTCACCGCCATCGCCTGGG ACCCAATTATCTTCATATACCTGTGAACTGTCCCTACCGTGCTCGAG
CCL3	NM_002 983.2	681-781	CTGTGTAGGCAGTCATGGCACCAAAGCCACCAGACTGACAAATGTGTATCGG ATGCTTTTGTTCAGGGCTGTGATCGGCCTGGGGAAATAATAAAGATGC

CCL4	NM_002984.2	35-135	TTCTGCAGCCTCACCTCTGAGAAAACCTCTTTGCCACCAATACCATGAAGCTCTGCGTGACTGTCCTGTCTCTCCTCATGCTAGTAGCTGCCTTCTGCTC
CCL5	NM_002985.2	280-380	AGTGTGTGCCAACCCAGAGAAGAAATGGGTTCGGGAGTACATCAACTCTTTGGAGATGAGCTAGGATGGAGAGTCCTTGAACCTGAACTTACACAAATTT
CCNB1	NM_031966.2	715-815	AACTTGAGGAAGAGCAAGCAGTCAGACCAAAATACCTACTGGGTCGGGAAGTCACTGGAAACATGAGAGCCATCCTAATTGACTGGCTAGTACAGGTTCA
CCND1	NM_053056.2	690-790	TTGAACACTTCCTCTCCAAAATGCCAGAGGCGGAGGAGAACAAACAGATCATCCGCAAACACGCGCAGACCTTCGTTGCCCTCTGTGCCACAGATGTGAA
CCR1	NM_001295.2	535-635	CATCATTTGGGCCCTGGCCATCTTGGCTTCCATGCCAGGCTTATACTTTTCCAAGACCCAATGGGAATTCACTCACCACACCTGCAGCCTTCACTTTCCT
CCR2	NM_001123041.2	20-120	ACATTCTGTTGTGCTCATATCATGCAAATTATCACTAGTAGGAGAGCAGAGAGTGGAAATGTTCCAGGTATAAAGACCCACAAGATAAAGAAGCTCAGAG
CCR4	NM_005508.4	35-135	GGTCCTTCTTAGCATCGTGCTTCCTGAGCAAGCCTGGCATTGCCTCACAGACCTTCCTCAGAGCCGCTTTCAGAAAAGCAAGCTGCTTCTGGTTGGGCCC
CCR5	NM_000579.1	2730-2830	TAGGAACATACTTCAGCTCACACATGAGATCTAGGTGAGGATTGATTACCTAGTAGTCATTTCATGGGTTGTTGGGAGGATTCTATGAGGCAACCACAGG
CCR6	NM_031409.2	935-1035	CTTTAACTGCGGGATGCTGCTCCTGACTTGCATTAGCATGGACCGGTACATCGCCATTGTACAGGCGACTAAGTCATTCCGGCTCCGATCCAGAACACTA
CCR7	NM_001838.2	1610-1710	TTCCGAAAACCAGGCCTTATCTCCAAGACCAGAGATAGTGGGGAGACTTCTTGGCTTGGTGAGGAAAAGCGGACATCAGCTGGTCAAACAAACTCTCTGA
CD11b	NM_000632.3	515-615	GCCCTCCGAGGGTGTCCTCAAGAGGATAGTGACATTGCCTTCTTGATTGATGGCTCTGGTAGCATCATCCCACATGACTTTCGGCGGATGAAGGAGTTTG
CD16	NM_000570.3	73-173	CCTATTCCTGTTCTATGGTGGGGCTCCATTGCGAGACTTCAGATTGAGAAATCAGATGAAGTTTCAAGAAAAGGAAACTGGCAGGTGACAGAGATGGGTG
CD160	NM_007053.2	500-600	TTGATGTTCACCATAAGCCAAGTCACACCGTTGCACAGTGGGACCTACCAGTGTTGTGCCAGAAGCCAGAAGTCAGGTATCCGCCTTCAGGGCCATTTTT
CD19	NM_001770.4	1770-1870	AGATTCACACCTGACTCTGAAATCTGAAGACCTCGAGCAGATGATGCCAACCTCTGGAGCAATGTTGCTTAGGATGTGTGCATGTGTGTAAGTGTGTGTG
CD19RCD28CAR	MDA_00002.1	2-102	CAGGTGTTCCTGAAGATGAACAGCCTGCAGACCGACGACACCGCCATCTACTACTGTGCCAAGCACTACTACTACGGCGGCAGCTACGCCATGGACTACT
CD2	NM_001767.2	1400-1500	TGGGTCTCACTACAAGCAGCCTATCTGCTTAAGAGACTCTGGAGTTTCTTATGTGCCCTGGTGGACACTTGCCCACCATCCTGTGAGTAAAAGTGAAATA
CD244	NM_016382.2	1150-1250	AAGAGGAACCACAGCCCTTCCTTCAATAGCACTATCTATGAAGTGATTGGAAAGAGTCAACCTAAAGCCCAGAACCCTGCTCGATTGAGCCGCAAAGAGC
CD247	NM_198053.1	1490-1590	TGGCAGGACAGGAAAAACCCGTCAATGTACTAGGATACTGCTGCGTCATTACAGGGCACAGGCCATGGATGGAAAACGCTCTCTGCTCTGCTTTTTTTCT
CD274	NM_014143.2	684-784	TAGGAGATTAGATCCTGAGGAAAACCATACAGCTGAATTGGTCATCCCAGAACTACCTCTGGCACATCCTCCAAATGAAAGGACTCACTTGGTAATTCTG
CD276	NM_001024736.1	2120-2220	ACATTTCTTAGGGACACAGTACACTGACCACATCACCACCCTCTTCTTCCAGTGCTGCGTGGACCATCTGGCTGCCTTTTTTCTCCAAAAGATGCAATAT
CD28	NM_006139.1	305-405	GCTTGTAGCGTACGACAATGCGGTCAACCTTAGCTGCAAGTATTCCTACAATCTCTTCTCAAGGGAGTTCCGGGCATCCCTTCACAAAGGACTGGATAGT
CD38	NM_001775.2	1035-1135	CCTTGACTCCTTGTGGTTTATGTCATCATACATGACTCAGCATACCTGCTGGTGCAGAGCTGAAGATTTTGGAGGGTCCTCCACAATAAGGTCAATGCCA
CD3D	NM_000732.4	110-210	TATCTACTGGATGAGTTCCGCTGGGAGATGGAACATAGCACGTTTCTCTCTGGCCTGGTACTGGCTACCCTTCTCTCGCAAGTGAGCCCCTTCAAGATAC

CD3E	NM_000733.2	75-175	AAGTAACAGTCCCATGAAACAAAGATGCAGTCGGGCACTCACTGGAGAGTTC TGGGCCTCTGCCTCTTATCAGTTGGCGTTTGGGGGCAAGATGGTAATG
CD4	NM_000616.3	835-935	AGACATCGTGGTGCTAGCTTTCCAGAAGGCCTCCAGCATAGTCTATAAGAAA GAGGGGGAACAGGTGGAGTTCTCCTTCCCACTCGCCTTTACAGTTGAA
CD40LG	NM_000074.2	1225-1325	GCATTTGATTTATCAGTGAAGATGCAGAAGGGAAATGGGGAGCCTCAGCTCA CATTCAGTTATGGTTGACTCTGGGTTCCTATGGCCTTGTTGGAGGGGG
CD44	NM_000610.3	2460-2560	GTGGGCAGAAGAAAAAGCTAGTGATCAACAGTGGCAATGGAGCTGTGGAGG ACAGAAAGCCAAGTGGACTCAACGGAGAGGCCAGCAAGTCTCAGGAAAT
CD58	NM_001779.2	478-578	GTGCTTGAGTCTCTTCCATCTCCCACACTAACTTGTGCATTGACTAATGGAAG CATTGAAGTCCAATGCATGATACCAGAGCATTACAACAGCCATCGAG
CD63	NM_001780.4	350-450	GTCATCATCGCAGTGGGTGTCTTCCTCTTCCTGGTGGCTTTTGTGGGCTGCTGC GGGGCCTGCAAGGAGAACTATTGTCTTATGATCACGTTTGCCATCT
CD69	NM_001781.1	460-560	AGGACATGAACTTTCTAAAACGATACGCAGGTAGAGAGGAACACTGGGTTGG ACTGAAAAAGGAACCTGGTCACCCATGGAAGTGGTCAAATGGCAAAGA
CD80	NM_005191.3	1288-1388	AAAGATCTGAAGGTCCCACCTCCATTTGCAATTGACCTCTTCTGGGAACTTCC TCAGATGGACAAGATTACCCCACCTTGCCCTTTACGTATCTGCTCTT
CD86	NM_006889.3	146-246	TATGGGACTGAGTAACATTCTCTTTGTGATGGCCTTCCTGCTCTCTGGTGCTGC TCCTCTGAAGATTCAAGCTTATTTCAATGAGACTGCAGACCTGCCA
CD8A	NM_001768.5	1320-1420	GCTCAGGGCTCTTTCCTCCACACCATTCAGGTCTTTCTTTCCGAGGCCCCTGTC TCAGGGTGAGGTGCTTGAGTCTCCAACGGCAAGGGAACAAGTACTT
CDH1	NM_004360.2	1230-1330	CGATAATCCTCCGATCTTCAATCCCACCACGTACAAGGGTCAGGTGCCTGAGA ACGAGGCTAACGTCGTAATCACCACACTGAAAGTGACTGATGCTGAT
CDK2	NM_001798.2	220-320	TCGCTGGCGCTTCATGGAGAACTTCCAAAAGGTGGAAAAGATCGGAGAGGGC ACGTACGGAGTTGTGTACAAAGCCAGAAACAAGTTGACGGGAGAGGTG
CDK4	NM_000075.2	1055-1155	ACTTTTAACCCACACAAGCGAATCTCTGCCTTTCGAGCTCTGCAGCACTCTTA TCTACATAAGGATGAAGGTAATCCGGAGTGAGCAATGGAGTGGCTGC
CDKN1A	NM_000389.2	1975-2075	CATGTGTCCTGGTTCCCGTTTCTCCACCTAGACTGTAAACCTCTCGAGGGCAG GGACCACACCCTGTACTGTTCTGTGTCTTTCACAGCTCCTCCCACAA
CDKN1B	NM_004064.2	365-465	GCTTCCGAGAGGGGTTCGGGCCGCGTAGGGGCGCTTTGTTTTGTTCGGTTTTG TTTTTTTGAGAGTGCGAGAGAGGCGGTCGTGCAGACCCGGGAGAAAG
CDKN2C	NM_001262.2	1295-1395	ATAATGTAAACGTCAATGCACAAAATGGATTTGGAAGGACTGCGCTGCAGGT TATGAAACTTGGAAATCCCGAGATTGCCAGGAGACTGCTACTTAGAGG
CEBPA	NM_004364.2	1320-1420	GAGCTGGGAGCCCGGCAACTCTAGTATTTAGGATAACCTTGTGCCTTGGAAAT GCAAACTCACCGCTCCAATGCCTACTGAGTAGGGGGAGCAAATCGTG
CFLAR	NM_003879.3	445-545	CAAGACCCTTGTGAGCTTCCCTAGTCTAAGAGTAGGATGTCTGCTGAAGTCAT CCATCAGGTTGAAGAAGCACTTGATACAGATGAGAAGGAGATGCTGC
CIITA	NM_000246.3	470-570	GCCTGAGCAAGGACATTTTCAAGCACATAGGACCAGATGAAGTGATCGGTGA GAGTATGGAGATGCCAGCAGAAGTTGGGCAGAAAAGTCAGAAAAGACC
CITED2	NM_006079.3	965-1065	AGGAGCTGCCCGAACTCTGGCTGGGGCAAAACGAGTTTGATTTTATGACGGA CTTCGTGTGCAAACAGCAGCCCAGCAGAGTGAGCTGTTGACTCGATCG
CLA	NM_003006.3	2297-2397	CATGGGCTGTTAGGTTGACTTCAGTTTTGCCTCTTGGACAACAGGGGGTCTTG TACATCCTTGGGTGACCAGGAAAAGTTCAGGCTATGGGGGGCCAAAG
CLIC1	NM_001288.4	310-410	GTGATGGGGCCAAGATTGGGAACTGCCCATTCTCCCAGAGACTGTTCATGGT ACTGTGGCTCAAGGGAGTCACCTTCAATGTTACCACCGTTGACACCAA
CMRF-35H	NM_007261.2	0-100	CGGGGAAGTGAGAGTCGGGGATCAGTCCTGCAAGCTACGGAGTCACTACAGG GAGAGGTCTCATCACTAGAAATAGCCGAAGAACCTGCAGCCTCAACCA

CREB1	NM_004379.3	4855-4955	TTTGATGGTAGGTCAGCAGCAGTGCTAGTCTCTGAAAGCACAATACCAGTCA GGCAGCCTATCCCATCAGATGTCATCTGGCTGAAGTTTATCTCTGTCT
CRIP1	NM_001311.4	269-369	CAACCACCCCTGCTACGCAGCCATGTTTGGGCCTAAAGGCTTTGGGCGGGGC GGAGCCGAGAGCCACACTTTCAAGTAAACCAGGTGGTGGAGACCCCAT
CSAD	NM_015989.4	205-305	TCAAATTCTTCTGCCTAGCCTTAGCCATTAGAGAGAGGTCCTGCTAAAGATGG ACTGCAAATGCGCTTGATGGAAGGAGATGTCAATTCCACTGAAGTCC
CSF2	NM_000758.2	475-575	AGATGAGGCTGGCCAAGCCGGGGAGCTGCTCTCTCATGAAACAAGAGCTAGA AACTCAGGATGGTCATCTTGGAGGGACCAAGGGGTGGGCCACAGCCAT
CSNK2A1	NM_177559.2	1930-2030	CCATTCCCACCATTGTTCCTCCACCGTCCCACACTTTAGGGGGTTGGTATCTCG TGCTCTTCTCCAGAGATTACAAAAATGTAGCTTCTCAGGGGAGGCA
CTGF	NM_001901.2	1100-1200	ACCACCCTGCCGGTGGAGTTCAAGTGCCCTGACGGCGAGGTCATGAAGAAGA ACATGATGTTCATCAAGACCTGTGCCTGCCATTACAACTGTCCCGGAG
CTLA4	NM_005214.3	405-505	AGTCTGTGCGGCAACCTACATGATGGGGAATGAGTTGACCTTCCTAGATGATT CCATCTGCACGGGCACCTCCAGTGGAAATCAAGTGAACCTCACTATC
CTNNA1	NM_001903.2	75-175	TCGCCCAGCTAGCCGCAGAAATGACTGCTGTCCATGCAGGCAACATAAACTT CAAGTGGGATCCTAAAAGTCTAGAGATCAGGACTCTGGCAGTTGAGAG
CTNNB1	NM_001098210.1	1815-1915	TCTTGCCCTTTGTCCCGCAAATCATGCACCTTTGCGTGAGCAGGGTGCCATTC CACGACTAGTTCAGTTGCTTGTTCGTGCACATCAGGATACCCAGCGC
CTNNBL1	NM_030877.3	855-955	TGATGCCAACAAACTGTATTGCAGTGAAGTGCTGGCCATATTGCTCCAGGAC AATGATGAAAACAGGGAATTGCTTGGGGAGCTGGATGGAATCGATGTG
CX3C1	NM_002996.3	140-240	AGCACCACGGTGTGACGAAATGCAACATCACGTGCAGCAAGATGACATCAAA GATACCTGTAGCTTTGCTCATCCACTATCAACAGAACCAGGCATCATG
CX3CR1	NM_001337.3	1040-1140	GGGCGCTCAGTCCACGTTGATTTCTCCTCATCTGAATCACAAAGGAGCAGGCA TGGAAGTGTTCTGAGCAGCAATTTTACTTACCACACGAGTGATGGAG
CXCCR1	NM_000634.2	1950-2050	GCAGCCACCAGTCCATTGGGCAGGCAGATGTTCCTAATAAAGCTTCTGTTCCG TGCTTGTCCCTGTGGAAGTATCTTGGTTGTGACAGAGTCAAGGGTGT
CXCL10	NM_001565.1	40-140	GCAGAGGAACCTCCAGTCTCAGCACCATGAATCAAACTGCGATTCTGATTTGC TGCCTTATCTTTCTGACTCTAAGTGGCATTCAAGGAGTACCTCTCTC
CXCL12	NM_199168.2	505-605	GGGCCTGAGGTTTGCCAGCATTTAGACCCTGCATTTATAGCATACGGTATGAT ATTGCAGCTTATATTCATCCATGCCCTGTACCTGTGCACGTTGGAAC
CXCL9	NM_002416.1	1975-2075	CACCATCTCCCATGAAGAAAGGGAACGGTGAAGTACTAAGCGCTAGAGGAA GCAGCCAAGTCGGTTAGTGGAAGCATGATTGGTGCCCAGTTAGCCTCTG
CXCR3	NM_001504.1	80-180	GTGAGTGACCACCAAGTGCTAAATGACGCCGAGGTTGCCGCCCTCCTGGAGA ACTTCAGCTCTTCCTATGACTATGGAGAAAACGAGAGTGACTCGTGCT
CXCR4	NM_001008540.1	135-235	GTCACTATGGGAAAAGATGGGGAGGAGAGTTGTAGGATTCTACATTAATTCT CTTGTGCCCTTAGCCCACTACTTCAGAATTTCCTGAAGAAAGCAAGCC
CYORF14	NR_001544.2	143-243	GAGGCTGTCTGCCAACATCTTTCATCACTCTGCCTGCAACTATGAAAAATTTA GTTCTAAAAAATGCAACCTTGCTAAATTGAGTACTAATAGGATTGGT
DAP10	NM_001007469.1	132-232	ATCCTCTTCCTGCTTTTGCTCCCAGTGGCTGCAGCTCAGACGACTCCAGGAGA GAGATCATCACTCCCTGCCTTTTACCCTGGCACTTCAGGCTCTTGTT
DAP12	NM_003332.2	457-557	CTGCACCTCATTCCAACTCCTACCGCGATACAGACCCACAGAGTGCCATCCCT GAGAGACCAGACCGCTCCCCAATACTCTCCTAAAATAAACATGAAGC
DEC1	NM_017418.2	190-290	AGGCCTTACTTTCCAGATCCAGATCCTTGTGCATACAACTGACTTGTGTGGGT GAGGCTTGCAGAAAAAATCAGCTAGAACAGCCTTGGGGGTAGTGGCA
DNAM-1	NM_006566.2	163-263	TAAACAGGATACGATAAAAGTCCTTAACCAAGACGCAGATGGGAAGAAGCG TTAGAGCGAGCAGCACTCACATCTCAAGAACCAGCCTTTCAAACAGTTT

DPP4	NM_001 935.3	2700-2800	CAGCAGTCAGCTCAGATCTCCAAAGCCCTGGTCGATGTTGGAGTGGATTTCCA GGCAATGTGGTATACTGATGAAGACCATGGAATAGCTAGCAGCACAG
EGLN1	NM_022 051.1	3975-4075	AGCAGCATGGACGACCTGATACGCCACTGTAACGGGAAGCTGGGCAGCTACA AAATCAATGGCCGGACGAAAGCCATGGT
EGLN3	NM_022 073.3	800-900	AAGCTACATGGTGGGATCCTGCGGATATTTCCAGAGGGGAAATCATTCATAG CAGATGTGGAGCCCATTTTTGACAGACTCCTGTTCTTCTGGTCAGATC
EIF1	NM_005 801.3	869-969	CCTGAACAGTCCTCGGTGAATCTGAGAGGAGAGGATGGGGTAAGGCAGAAG CACCAGCTGTACTACTAGAAGGGAGCTTTTGGTGGTAGATCCCCTGGTG
ELF4	NM_001 421.3	335-435	AGCTCTGGAGGGCTCTGATAATCCCGTTGTCAGCTCTCTGAAAAGACAGCATG GCTATTACCCTACAGCCCAGTGACCTGATCTTTGAGTTCGCAAGCAA
ENTPD1	NM_001 776.4	225-325	TTCGAGTAACTTTAGGAAAATGAGCTGCTGGACTCCTCAGTCAATCTGTCCTT TCTAGTCAATGAAAAAGACAGGGTTTGAGGTTCCTTCCGAAACGGGG
Eomes	NM_005 442.2	1670-1770	ATCCCATGCCCTGGGGTATTACCCAGACCCAACCTTTCCTGCAATGGCAGGGT GGGGAGGTCGAGGTTCTTACCAGAGGAAGATGGCAGCTGGACTACCA
EPHA4	NM_004 438.3	20-120	GCAGCGTTGGCACCGGCGAACCATGGCTGGGATTTTCTATTTCGCCCTATTTT CGTGTCTCTTCGGGATTTGCGACGCTGTCACAGGTTCCAGGGTATAC
ETV6	NM_001 987.4	3840-3940	GTATGAATATGAAATCAGAGACCAGGGCATGATGTTGCTAGGATTAGAGCCT CTCAGTCTGGCCTCTTCACCCAAGTGCAAGAACTCAGTCTCTTACTGT
FADD	NM_003 824.2	1560-1660	TGAGACTGCTAAGTAGGGGCAGTGATGGTTGCCAGGACGAATTGAGATAATA TCTGTGAGGTGCTGATGAGTGATTGACACACAGCACTCTCTAAATCTT
FANCC	NM_000 136.2	2130-2230	GACTCAGTCAGACATGTTCACTAATGACTCAAGTGAGCCTTCGGTACTCCTGG TGCCCGCCCGGCCAGACCGTCAGCTTGATAATTACTAAAGCAAAGGC
FAS	NM_000 043.3	90-190	CACCGGGGCTTTTCGTGAGCTCGTCTCTGATCTCGCGCAAGAGTGACACACAG GTGTTCAAAGACGCTTCTGGGGAGTGAGGGAAGCGGTTTACGAGTGA
FASLG	NM_000 639.1	625-725	TCCATGCCTCTGGAATGGGAAGACACCTATGGAATTGTCCTGCTTTCTGGAGT GAAGTATAAGAAGGGTGGCCTTGTGATCAATGAAACTGGGCTGTACT
FLT1	NM_002 019.2	5615-5715	TTCAACTGCTTTGAAACTTGCCTGGGGTCTGAGCATGATGGGAATAGGGAGA CAGGGTAGGAAAGGGCGCCTACTCTTCAGGGTCTAAAGATCAAGTGGG
FLT3LG	NM_001 459.2	927-1027	CCTCCCCAGAATGGAGGCAACGCCAGAATCCAGCACCGGCCCCATTTACCCA ACTCTGTACAAAGCCCTTGTCCCCATGAAATTGTATATAAATCATCCT
FOS	NM_005 252.2	1475-1575	ACTCAAGTCCTTACCTCTTCCGGAGATGTAGCAAAACGCATGGAGTGTGTATT GTTCCCAGTGACACTTCAGAGAGCTGGTAGTTAGTAGCATGTTGAGC
FOXP3	NM_014 009.3	1230-1330	GGGCCATCCTGGAGGCTCCAGAGAAGCAGCGGACACTCAATGAGATCTACCA CTGGTTCACACGCATGTTTGCCTTCTTCAGAAACCATCCTGCCACCTG
FYN	NM_002 037.3	765-865	GTCTTTGGAGGTGTGAACTCTTCGTCTCATACGGGGACCTTGCGTACGAGAGG AGGAACAGGAGTGACACTCTTTGTGGCCCTTTATGACTATGAAGCAC
FZD1	NM_003 505.1	2430-2530	GTGCCAATCCTGACATCTCGAGGTTTCCTCACTAGACAACTCTCTTTCGCAGG CTCCTTTGAACAACTCAGCTCCTGCAAAAGCTTCCGTCCCTGAGGCA
GAL3ST4	NM_024 637.4	1140-1240	CGAGCCCAAACCCTCAATCCCAATGCCCTCATCCATCCTGTTTCCACTGTTAC TGATCATCGCAGCCAGATATCAAGCCCTGCCTCTTTCGATTTGGGGT
GARNL4	NM_015 085.4	4140-4240	CCCACGGCTGGAAAGAGGCCTGTACGTTCTGGACGCGTTTTGTTGGCTGGGCT TCTGGAGGCACTGGCAAGGTCAAACTGCATTTCTTTAAGAACAGTTG
GAS2	NM_005 256.3	915-1015	GATCTCCCGTGTGGATGGCAAAACATCCCCTATCCAAAGCAAATCTCCAACTC TAAAGGACATGAATCCAGATAACTACTTGGTGGTCTCTGCCAGTTAT
GATA2	NM_032 638.3	1495-1595	GAAGAAGGAAGGGATCCAGACTCGGAACCGGAAGATGTCCAACAAGTCCAA GAAGAGCAAGAAAGGGGCGGAGTGCTTCGAGGAGCTGTCAAAGTGCATG

GATA3	NM_001002295.1	2835-2935	AAGAGTCCGGCGGCATCTGTCTTGTCCCTATTCCTGCAGCCTGTGCTGAGGGT AGCAGTGTATGAGCTACCAGCGTGCATGTCAGCGACCCTGGCCCGAC
Gfi1	NM_005263.2	2235-2335	TCATCACTGGAGGTAAAAGCACAAGCAATGCCTGTGGACAAGATGTCATTCA TTCACTCAGCAAATGTTCATGGATCACCGGCTACCAAGGTACCAGGCA
GILZ	NM_198057.2	1400-1500	TTAAGCAGAGGCAACCTCTCTCTTCTCCTCTGTTTCGTGAAGGCAGGGGACAC AGATGGGAGAGATTGAGCCAAGTCAGCCTTCTGTTGGTTAATATGGT
GLIPR1	NM_006851.2	255-355	CTGCGTTCGAATCCATAACAAGTTCCGATCAGAGGTGAAACCAACAGCCAGT GATATGCTATACATGACTTGGGACCCAGCACTAGCCCAAATTGCAAAA
GLO1	NM_006708.1	1240-1340	GGAAATGATATGGTACCCAGACACTGGGCTAGGCTGCAACTTTATCTCATTTA ATACTCCCAGCTGTCATGTGAGAAAGAAAGCAGGCTAGGCATGTGAA
GSK3B	NM_002093.2	925-1025	ACTGATTATACCTCTAGTATAGATGTATGGTCTGCTGGCTGTGTGTTGGCTGA GCTGTTACTAGGACAACCAATATTTCCAGGGGATAGTGGTGTGGATC
GZMA	NM_006144.2	155-255	AGACCCTACATGGTCCTACTTAGTCTTGACAGAAAAACCATCTGTGCTGGGGC TTTGATTGCAAAAGACTGGGTGTTGACTGCAGCTCACTGTAACTTGA
GZMB	NM_004131.3	540-640	ACACTACAAGAGGTGAAGATGACAGTGCAGGAAGATCGAAAGTGCGAATCT GACTTACGCCATTATTACGACAGTACCATTGAGTTGTGCGTGGGGGACC
GzmH	NM_033423.3	705-805	AAAAAAGGGACACCTCCAGGAGTCTACATCAAGGTCTCACACTTCCTGCCCT GGATAAAGAGAACAATGAAGCGCCTCTAACAGCAGGCATGAGACTAAC
HDAC1	NM_004964.2	785-885	CAAGCCGGTCATGTCCAAAGTAATGGAGATGTTCCAGCCTAGTGCGGTGGTC TTACAGTGTGGCTCAGACTCCCTATCTGGGGATCGGTTAGGTTGCTTC
HDAC2	NM_001527.1	930-1030	AAGCCTATTATCTCAAAGGTGATGGAGATGTATCAACCTAGTGCTGTGGTATT ACAGTGTGGTGCAGACTCATTATCTGGTGATAGACTGGGTTGTTTCA
HES1	NM_004649.5	1340-1440	TTGAGTTAATCAGCGTAAGGGGATTTCTAAAGCAGGCAATCCCTGTAGCCGC AGAGAATAAACGCCTTCCCAAAATGGCAACTTCCCACAGCCACATTTC
HLA-A	NM_002116.5	1000-1100	GGAAGAGCTCAGATAGAAAAGGAGGGAGTTACACTCAGGCTGCAAGCAGTG ACAGTGCCCAGGGCTCTGATGTGTCCCTCACAGCTTGTAAAGTGTGAGA
HOXA10	NM_018951.3	1503-1603	TTCTATAGAGATAGATATTGTCCTAAGTGTCAAGTCCTGACTGGGCTGGGTTT GCTGTCTTGGGGTCCCACTGCTCGAAATGGCCCCTGTCTTCGGCCGA
HOXA9	NM_152739.3	1015-1115	GGCTCTAAACCTCAGGCCACATCTTTTCCAAGGCAAACCCTGTTCAGGCTGGC TCGTAGGCCTGCCGCTTTGATGGAGGAGGTATTGTAAGCTTTCCATT
HOXB3	NM_002146.4	60-160	TGTCCGTTTAAATGCTGCTGGGAGACTCGTAAAAAAATCATCGTGGACCTGG AGGATGAGAGGGGCGAGCTTTATTTCGGTCGGATTGCGGTGTGGTGGT
HOXB4	NM_024015.4	1340-1440	CCTTTCTTTGTCCCCCACTCCCGATACCCAGCGAAAGCACCCTCTGACTGCCA GATAGTGCAGTGTTTTGGTCACGGTAACACACACACACTCTCCCTCA
HPRT1	NM_000194.1	240-340	TGTGATGAAGGAGATGGGAGGCCATCACATTGTAGCCCTCTGTGTGCTCAAG GGGGGCTATAAATTCTTTGCTGACCTGCTGGATTACATCAAAGCACTG
HRH1	NM_000861.2	3055-3155	GTGGCAGCTCAAAATGATATGTTTGAGTAGACGAACAGCTGACATGGAGTTC CCGTGCACCTACGGAAGGGGACGCTTTGAAGGAACCAAGTGCATTTTT
HRH2	NM_022304.1	600-700	GCGGTCCTCATCCTCATCACCGTTGCTGGCAATGTGGTCGTCTGTCTGGCCGT GGGCTTGAACCGCCGGCTCCGCAACCTGACCAATTGTTTCATCGTGT
IAP	NM_001777.3	897-997	GCCATATTGGTTATTCAGGTGATAGCCTATATCCTCGCTGTGGTTGGACTGAG TCTCTGTATTGCGGCGTGTATACCAATGCATGGCCCTCTTCTGATTT
ICOS	NM_012092.2	640-740	AACTCTGGCACCCAGGCATGAAGCACGTTGGCCAGTTTTCCTCAACTTGAAGT GCAAGATTCTCTTATTTCCGGGACCACGGAGAGTCTGACTTAACTAC
ICOSLG	NM_015259.4	1190-1290	CTGCTGGCGTTGGCTGTGATCCTGGAATGAGGCCCTTTCAAAAGCGTCATCCA CACCAAAGGCAAATGTCCCCAAGTGAGTGGGCTCCCCGCTGTCACTG

ID2	NM_002 166.4	505-605	CGGATATCAGCATCCTGTCCTTGCAGGCTTCTGAATTCCCTTCTGAGTTAATGT CAAATGACAGCAAAGCACTGTGTGGCTGAATAAGCGGTGTTCATGA
IFNa1	NM_024 013.1	585-685	ATCCCTCTCTTTATCAACAAACTTGCAAGAAAGATTAAGGAGGAAGGAATAA CATCTGGTCCAACATGAAAACAATTCTTATTGACTCATACACCAGGTC
IFNG	NM_000 619.2	970-1070	ATACTATCCAGTTACTGCCGGTTTGAAAATATGCCTGCAATCTGAGCCAGTGC TTTAATGGCATGTCAGACAGAACTTGAATGTGTCAGGTGACCCTGAT
IFNGR1	NM_000 416.1	1140-1240	CCCGGGCAGCCATCTGACTCCAATAGAGAGAGAGAGTTCTTCACCTTTAAGT AGTAACCAGTCTGAACCTGGCAGCATCGCTTTAAACTCGTATCACTCC
IGF1R	NM_000 875.2	455-555	TCGGGGGGCCATCAGGATTGAGAAAAATGCTGACCTCTGTTACCTCTCCACTG TGGACTGGTCCCTGATCCTGGATGCGGTGTCCAATAACTACATTGTG
IKZF1	NM_006 060.3	4485-4585	CCGCTGTGTACTACTGTGTGCCTAGATTCCATGCACTCTCGTTGTGTTTGAAGT AAATATTGGAGACCGGAGGGTAACAGGTTGGCCTGTTGATTACAGC
IL10	NM_000 572.2	230-330	AAGGATCAGCTGGACAACTTGTTGTTAAAGGAGTCCTTGCTGGAGGACTTTA AGGGTTACCTGGGTTGCCAAGCCTTGTCTGAGATGATCCAGTTTTACC
IL10RA	NM_001 558.2	150-250	TGCCCAGCCCTCCGTCTGTGTGGTTTGAAGCAGAATTTTTCCACCACATCCTC CACTGGACACCCATCCCAAATCAGTCTGAAAGTACCTGCTATGAAGT
IL12A	NM_000 882.2	775-875	CTTTCTAGATCAAAACATGCTGGCAGTTATTGATGAGCTGATGCAGGCCCTGA ATTTCAACAGTGAGACTGTGCCACAAAAATCCTCCCTTGAAGAACCG
IL12RB1	NM_005 535.1	1292-1392	AGGAAAAGTGTTACTACATTACCATCTTTGCCTCTGCGCACCCCGAGAAGCTC ACCTTGTGGTCTACGGTCCTGTCCACCTACCACTTTGGGGGCAATGC
IL12RB2	NM_001 559.2	1315-1415	CCTCCGTGGGACATTAGAATCAAATTTCAAAAGGCTTCTGTGAGCAGATGTAC CCTTTATTGGAGAGATGAGGGACTGGTACTGCTTAATCGACTCAGAT
IL13	NM_002 188.2	516-616	TTTCTTTCTGATGTCAAAAATGTCTTGGGTAGGCGGGAAGGAGGGTTAGGGA GGGGTAAAATTCCTTAGCTTAGACCTCAGCCTGTGCTGCCCGTCTTCA
IL15	NM_172 174.1	1685-1785	AGGGTGATAGTCAAATTATGTATTGGTGGGGCTGGGTACCAATGCTGCAGGT CAACAGCTATGCTGGTAGGCTCCTGCCAGTGTGGAACCACTGACTACT
IL15Ra	NM_002 189.2	39-139	CGCTCGCCCGGGGAGTCCAGCGGTGTCCTGTGGAGCTGCCGCCATGGCCCCG CGGCGGGCGCGCGGCTGCCGGACCCTCGGTCTCCCGGCGCTGCTACTG
IL17A	NM_002 190.2	240-340	TACTACAACCGATCCACCTCACCTTGGAATCTCCACCGCAATGAGGACCCTGA GAGATATCCCTCTGTGATCTGGGAGGCAAAGTGCCGCCACTTGGGCT
IL17F	NM_052 872.3	210-310	GCCCGCCTGTGCCAGGAGGTAGTATGAAGCTTGACATTGGCATCATCAATGA AAACCAGCGCGTTTCCATGTCACGTAACATCGAGAGCCGCTCCACCTC
IL17RA	NM_014 339.4	3020-3120	CTACTATGTGGCGGGCATTTGGGATACCAAGATAAATTGCATGCGGCATGGC CCCAGCCATGAAGGAACTTAACCGCTAGTGCCGAGGACACGTTAAACG
IL18	NM_001 562.2	48-148	GACAGTCAGCAAGGAATTGTCTCCCAGTGCATTTTGCCCTCCTGGCTGCCAAC TCTGGCTGCTAAAGCGGCTGCCACCTGCTGCAGTCTACACAGCTTCG
IL18R1	NM_003 855.2	2025-2125	GAATGAGGGGATTTTAAGTGTCTGAAGAGGCATTTTCTAGGGACCAGTGGGT GACTGAGTAACTGAAATGCTGCTTTCACTCCCTAACACCATGGATCTG
IL18RAP	NM_003 853.2	2412-2512	GCTTGATGGACAATGGAGTGGGATTGAGACTGTGGTTTAGAGCCTTTGATTTC CTGGACTGGACTGACGGCGAGTGAATTCTCTAGACCTTGGGTACTTT
IL2	NM_000 586.2	300-400	AGGATGCAACTCCTGTCTTGCATTGCACTAAGTCTTGCACTTGTCACAAACAG TGCACCTACTTCAAGTTCTACAAAGAAAACACAGCTACAACTGGAGC
IL21R	NM_021 798.2	2080-2180	CGTGTTTGTGGTCAACAGATGACAACAGCCGTCCTCCCTCCTAGGGTCTTGTG TTGCAAGTTGGTCCACAGCATCTCCGGGGCTTTGTGGGATCAGGGCA
IL22	NM_020 525.4	319-419	CTATCTGATGAAGCAGGTGCTGAACTTCACCCTTGAAGAAGTGCTGTTCCCTC AATCTGATAGGTTCCAGCCTTATATGCAGGAGGTGGTGCCCTTCCTG

IL23R	NM_144701.2	710-810	AACTGCAAATTCACCTGGATGATATAGTGATACCTTCTGCAGCCGTCATTTCCAGGGCTGAGACTATAAATGCTACAGTGCCCAAGACCATAATTTATTG
IL2RA	NM_000417.1	1000-1100	CTTGGTAAGAAGCCGGGAACAGACAACAGAAGTCATGAAGCCCAAGTGAAATCAAAGGTGCTAAATGGTCGCCCAGGAGACATCCGTTGTGCTTGCCTGC
IL2RB	NM_000878.2	1980-2080	GTCCTGCTGCCCGAGCCAGGAACTGTGTGTGTTGCAGGGGGGCAGTAACTCCCCAACTCCCTCGTTAATCACAGGATCCCACGAATTTAGGCTCAGAAGC
IL2RG	NM_000206.1	595-695	CCACAGCTGGACTGAACAATCAGTGGATTATAGACATAAGTTCTCCTTGCCTAGTGTGGATGGGCAGAAACGCTACACGTTTCGTGTTCGGAGCCGCTTT
IL4	NM_000589.2	625-725	GACACTCGCTGCCTGGGTGCGACTGCACAGCAGTTCCACAGGCACAAGCAGCTGATCCGATTCCTGAAACGGCTCGACAGGAACCTCTGGGGCCTGGCGG
IL4R	NM_000418.2	705-805	ATCATCTCACCTATGCAGTCAACATTTGGAGTGAAAACGACCCGGCAGATTTCAGAATCTATAACGTGACCTACCTAGAACCCTCCCTCCGCATCGCAGC
IL5	NM_000879.2	105-205	CCACAGAAATTCCCACAAGTGCATTGGTGAAAGAGACCTTGGCACTGCTTTCTACTCATCGAACTCTGCTGATAGCCAATGAGACTCTGAGGATTCCTGT
IL6	NM_000600.1	220-320	TGACAAACAAATTCGGTACATCCTCGACGGCATCTCAGCCCTGAGAAAGGAGACATGTAACAAGAGTAACATGTGTGAAAGCAGCAAAGAGGCACTGGCA
IL6R	NM_000565.2	993-1093	CTTTCTACATAGTGTCCATGTGCGTCGCCAGTAGTGTCGGGAGCAAGTTCAGCAAAACTCAAACCTTTCAGGGTTGTGGAATCTTGCAGCCTGATCCGCC
IL7R	NM_002185.2	1610-1710	TTGCTTTGACCACTCTTCCTGAGTTCAGTGGCACTCAACATGAGTCAAGAGCATCCTGCTTCTACCATGTGGATTTGGTCACAAGGTTTAAGGTGACCCA
IL9	NM_000590.1	300-400	AAGTACTAAAGAACAACAAGTGTCCATATTTTTCCTGTGAACAGCCATGCAACCAAACCACGGCAGGCAACGCGCTGACATTTCTGAAGAGTCTTCTGGA
INDO	NM_002164.3	50-150	CTATTATAAGATGCTCTGAAAACTCTTCAGACACTGAGGGGCACCAGAGGAGCAGACTACAAGAATGGCACACGCTATGGAAAACTCCTGGACAATCAGT
IRF1	NM_002198.1	510-610	CTGTGCGAGTGTACCGGATGCTTCCACCTCTCACCAAGAACCAGAGAAAAGAAAGAAAGTCGAAGTCCAGCCGAGATGCTAAGAGCAAGGCCAAGAGGAA
IRF2	NM_002199.2	1375-1475	CAGTACCTGGAGCTTCTCTTTAACTCAGGACTCCAGCCCATTGGTAGACGTGTGTTTCTAGAGCCTGCTGGATCTCCCAGGGCTACTCACTCAAGTTCAA
IRF4	NM_002460.1	325-425	GGGCACTGTTTAAAGGAAAGTTCCGAGAAGGCATCGACAAGCCGGACCCTCCCACCTGGAAGACGCGCCTGCGGTGCGCTTTGAACAAGAGCAATGACTT
ITGA1	NM_181501.1	1875-1975	AAGTGGCAAGACTATAAGGAAAGAGTATGCACAACGTATTCCATCAGGTGGGGATGGTAAGACACTGAAATTTTTTGGCCAGTCTATCCACGGAGAAATG
ITGA4	NM_000885.4	975-1075	GCCCACTGCCAACTGGCTCGCCAACGCTTCAGTGATCAATCCCGGGGCGATTTACAGATGCAGGATCGGAAAGAATCCCGGCCAGACGTGCGAACAGCTC
ITGA5	NM_002205.2	925-1025	AGAAGACTTTGTTGCTGGTGTGCCCAAAGGGAACCTCACTTACGGCTATGTCACCATCCTTAATGGCTCAGACATTCGATCCCTCTACAACTTCTCAGGG
ITGAL	NM_002209.2	3905-4005	GTGAGGGCTTGTCATTACCAGACGGTTCACCAGCCTCTCTTGGTTTCCTTCCTTGGAAGAGAATGTCTGATCTAAATGTGGAGAAACTGTAGTCTCAGGA
ITGB1	NM_033666.2	2000-2100	TTTTAACATTACCAAGGTAGAAAGTCGGGACAAATTACCCCAGCCGGTCCAACCTGATCCTGTGTCCCATTGTAAGGAGAAGGATGTTGACGACTGTTGG
ITK	NM_005546.3	3430-3530	GCCAGTAAAGAAGTCAGTATAGAACCACTAGCGAATAGTGTTGCTCTGGCACAGACCACTGTGGTTGATGGCATGGCCCTCCAACTTGGAATAGGATTTT
JAK1	NM_002227.1	285-385	GAGAACACCAAGCTCTGGTATGCTCCAAATCGCACCATCACCGTTGATGACAAGATGTCCCTCCGGCTCCACTACCGGATGAGGTTCTATTTCACCAATT
JAK2	NM_004972.2	455-555	CTCCTCCCGCGACGGCAAATGTTCTGAAAAAGACTCTGCATGGGAATGGCCTGCCTTACGATGACAGAAATGGAGGGAACATCCACCTCTTCTATATATC

JAK3	NM_000215.2	1715-1815	GTGCTGCTGAAGGTCATGGATGCCAAGCACAAGAACTGCATGGAGTCATTCCTGGAAGCAGCGAGCTTGATGAGCCAAGTGTCGTACCGGCATCTCGTGC
JunB	NM_002229.2	1155-1255	GCGCGCCTGGAGGACAAGGTGAAGACGCTCAAGGCCGAGAACGCGGGGCTGTCGAGTACCGCCGGCCTCCTCCGGGAGCAGGTGGCCCAGCTCAAACAGA
KIR2DL1 (NKAT1)/CD158a	NM_014218.2	881-981	GCAGGAAACAGAACAGCGAATAGCGAGGACTCTGATGAACAAGACCCTCAGGAGGTGACATACACACAGTTGAATCACTGCGTTTTCACACAGAGAAAAA
KIR2DL2 (NKAT6)/CD158b	NM_014219.2	814-914	TCTCCTTCATCGCTGGTGCTCCAACAAAAAAAATGCTGCGGTAATGGACCAAGAGTCTGCAGGGAACAGAACAGCGAATAGCGAGGACTCTGATGAACAA
KIR2DL3 (NKAT2)/CD158b	NM_015868.2	741-841	CTCCGAAACCGGTAACCCCAGACACCTGCATGTTCTGATTGGGACCTCAGTGGTCATCATCCTCTTCATCCTCCTCCTCTTCTTTCTCCTTCATCGCTGG
KIR2DL4 (p49 CD158d)	NM_002255.5	15-115	GCGTCCTGGCAGCAGAAGCTGCACCATGTCCATGTCACCCACGGTCATCATCCTGGCATGTCTTGGGTTCTTCTTGGACCAGAGTGTGTGGGCACACGTG
KIR2DL5A	NM_020535.3	1451-1551	GACACGTGCTGTTCCACCTTCCCTCATGCTGTTTCACCTTTCCTCAGACTATTTTCCAGCCTTCTGTCAGTCAGCAGTGAAACTTATAAAATTTTTTGTG
KIR2DS1	NM_014512.1	698-798	CTTCACCCACTGAACCAAGCTCCGAAACCGGTAACCCCAGACACCTACATGTTCTGATTGGGACCTCAGTGGTCAAAATCCCTTTCACCATCCTCCTCTT
KIR2DS2 (NKAT5)/CD158b	NM_012312.2	856-956	CAAGAGCCTGCAGGGAACAGAACAGTGAACAGCGAGGATTCTGATGAACAAGACCATCAGGAGGTGTCATACGCATAATTGGATCACTGTGTTTTCACAC
KIR2DS3 (NKAT7)	NM_012313.1	693-793	GGCCTTCACCCACTGAACCAAGCTCCAAAACCGGTAACCCCAGACACCTACACGTTCTGATTGGGACCTCAGTGGTCAAACTCCCTTTCACCATCCTCCT
KIR2DS4 (NKAT8)	NM_012314.3	1427-1527	ACATACAAGAGGCTGCCTCTTAACACAGCACTTAGACACGTGCTGTTCCACCTCCCTTCAGACTATCTTTCAGCCTTCTGCCAGCAGTAAAACTTATAAA
KIR2DS5 (NKAT9)	NM_014513.2	204-304	CTTCCTTCTGCACAGAGAGGGGACGTTTAACCACACTTTGCGCCTCATTGGAGAGCACATTGATGGGGTCTCCAAGGGCAACTTCTCCATCGGTCGCATG
KIR3DL1 (NKAT3/NKB1)	NM_013289.2	1054-1154	CCAAATCTGGTAACCCCAGACACCTGCACATTCTGATTGGGACCTCAGTGGTCATCATCCTCTTCATCCTCCTCCTCTTCTTTCTCCTTCATCTCTGGTG
KIR3DL2 (NKAT4)	NM_006737.2	884-984	TGCCACCCACGGAGGGACCTACAGATGCTTCGGCTCTTTCCGTGCCCTGCCCTGCGTGTGGTCAAACTCAAGTGACCCACTGCTTGTTTCTGTCACAGGA
KIR3DL3 (KIRC1 CD158z)	NM_153443.3	508-608	CCTTGCGCCTCGTTGGACAGCTCCACGATGCGGGTTCCCAGGTCAACTATTCCATGGGTCCCATGACACCTGCCCTTGCAGGGACCTACAGATGCTTTGG
KIR3DS1 (NKAT10)	NM_001083539.1	1000-1100	CTCCAAATCTGGTAACCTCAGACACCTGCACATTCTGATTGGGACCTCAGTGGTCAAAATCCCTTTCACCATCCTCCTCTTCTTTCTCCTTCATCGCTGG
KIT	NM_000222.1	5-105	CATCGCAGCTACCGCGATGAGAGGCGCTCGCGGCGCCTGGGATTTTCTCTGCGTTCTGCTCCTACTGCTTCGCGTCCAGACAGGCTCTTCTCAACCATCT
KLF10	NM_005655.1	570-670	GCTCAGGCAACAAGTGTGATTCGTCATACAGCTGATGCCCAGCTATGTAACCACCAGACCTGCCCAATGAAAGCAGCCAGCATCCTCAACTATCAGAACA
Klf2	NM_016270.2	1015-1115	GGAAGTTTGCGCGCTCAGACGAGCTCACGCGCCACTACCGAAAGCACACGGGCCACCGGCCATTCCAGTGCCATCTGTGCGATCGTGCCTTCTCGCGCTC
KLF4	NM_004235.4	1980-2080	CGAGCATTTTCCAGGTCGGACCACCTCGCCTTACACATGAAGAGGCATTTTTAAATCCCAGACAGTGGATATGACCCACACTGCCAGAAGAGAATTCAGT

KLF6	NM_001 008490.1	1165-1265	GGGATGCGTGTTCCAGCCAAAGCATGCCGTTCTGCACCCTACCCAGTTGCCTC CAGGGCCTCTCCTTGGAAGGTCTTTTGAGGGCTAAAAAGGTCCTGTA
KLRB1	NM_002 258.2	85-185	TGAGTTAAACTTACCCACAGACTCAGGCCCAGAAAGTTCTTCACCTTCATCTC TTCCTCGGGATGTCTGTCAGGGTTCACCTTGGCATCAATTTGCCCTG
KLRC1	NM_002 259.3	335-435	ACCTATCACTGCAAAGATTTACCATCAGCTCCAGAGAAGCTCATTGTTGGGAT CCTGGGAATTATCTGTCTTATCTTAATGGCCTCTGTGGTAACGATAG
KLRD1 (CD94)	NM_002 262.3	542-642	AGCCTGCTTCAGCTTCAAAACACAGATGAACTGGATTTTATGAGCTCCAGTCA ACAATTTTACTGGATTGGACTCTCTTACAGTGAGGAGCACACCGCCT
KLRG1	NM_005 810.3	45-145	TGCCTACGGCAACCCAAGCCCAGAATGACTATGGACCACAGCAAAAATCTTC CTCTTCCAGGCCTTCTTGTTCTTGCCTTGTGGCAATAGCTTTGGGGCT
LAIR1	NM_002 287.3	1195-1295	GCACCTGAGGGTAGAAAGTCACTCTAGGAAAAGCCTGAAGCAGCCATTTGGA AGGCTTCCTGTTGGATTCCTCTTCATCTAGAAAGCCAGCCAGGCAGCT
LCK	NM_005 356.2	1260-1360	ATTAAGTGGACAGCGCCAGAAGCCATTAACTACGGGACATTCACCATCAAGT CAGATGTGTGGTCTTTTGGGATCCTGCTGACGGAAATTGTCACCCACG
LDHA	NM_005 566.1	985-1085	CAGAATGGAATCTCAGACCTTGTGAAGGTGACTCTGACTTCTGAGGAAGAGG CCCGTTTGAAGAAGAGTGCAGATACACTTTGGGGGATCCAAAAGGAGC
Lef1	NM_016 269.3	1165-1265	CCGTCACACATCCCATCAGATGTCAACTCCAAACAAGGCATGTCCAGACATC CTCCAGCTCCTGATATCCCTACTTTTTATCCCTTGTCTCCGGGTGGTG
LGALS3	NM_002 306.2	120-220	CAGCCGTCCGGAGCCAGCCAACGAGCGGAAAATGGCAGACAATTTTTCGCTC CATGATGCGTTATCTGGGTCTGGAAACCCAAACCCTCAAGGATGGCCT
LNK	NM_005 475.2	4285-4385	CCTCCAGCCAGAAGTTAAACATCTGGGATATGACGTCTTCATGCCAGGGGCA CTCATTTCTTAGCAGCCTCTCTACATACATCTCTCAGGTGGTGCCAAG
LOC282997	NR_026 932.1	665-765	TGATCACATTCTACCTGGCATTATTTCATCTGAGTCCCTGTCCTAGCCCTTCTG CCCATTAGACTGTAACCTTGTTTAGGGAAAGACCTGTGTCTTACTC
LRP5	NM_002 335.1	2515-2615	TGGACACCAACATGATCGAGTCGTCCAACATGCTGGGTCAGGAGCGGGTCGT GATTGCCGACGATCTCCCGCACCCGTTCGGTCTGACGCAGTACAGCGA
LRP6	NM_002 336.1	2185-2285	CTTAGATTATCCAGAAGGCATGGCAGTAGACTGGCTTGGGAAGAACTTGTAC TGGGCAGACACAGGAACGAATCGAATTGAGGTGTCAAAGTTGGATGGG
LRRC32	NM_005 512.2	3470-3570	CACCCTGGTGTGGGTTCTCCTGTTCTCTCTGTGCTCTTGCATTCTCTCATTCCCT TTTCCTCTATTGAGCAGAGCCTGGAGTTTGAGACTATGGAATCCA
MAD1L1	NM_003 550.2	306-406	GAAGACCTGGGGGAAAACACCATGGTTTTATCCACCCTGAGATCTTTGAACA ACTTCATCTCTCAGCGTGTGGAGGGAGGCTCTGGACTGGATATTTCTA
MAP2K1	NM_002 755.2	970-1070	ACGGAATGGACAGCCGACCTCCCATGGCAATTTTTGAGTTGTTGGATTACATA GTCAACGAGCCTCCTCCAAAACTGCCCAGTGGAGTGTTCAGTCTGGA
MAPK14	NM_001 315.1	450-550	TGGGCTCTGGCGCCTATGGCTCTGTGTGTGCTGCTTTTGACACAAAAACGGGG TTACGTGTGGCAGTGAAGAAGCTCTCCAGACCATTTCAGTCCATCAT
MAPK3	NM_002 746.2	580-680	AACGTGCTCCACCGAGATCTAAAGCCCTCCAACCTGCTCATCAACACCACCTG CGACCTTAAGATTTGTGATTTCGGCCTGGCCCGGATTGCCGATCCTG
MAPK8	NM_139 049.1	945-1045	TCTCTGTAGATGAAGCTCTCCAACACCCGTACATCAATGTCTGGTATGATCCT TCTGAAGCAGAAGCTCCACCACCAAAGATCCCTGACAAGCAGTTAGA
MCL1	NM_021 960.3	1260-1360	GCTGTAACTTCCTAGAGTTGCACCCTAGCAACCTAGCCAGAAAAGCAAGTGG CAAGAGGATTATGGCTAACAAGAATAAATACATGGGAAGAGTGCTCCC
MIF	NM_002 415.1	319-419	TCCTACAGCAAGCTGCTGTGCGGCCTGCTGGCCGAGCGCCTGCGCATCAGCC CGGACAGGGTCTACATCAACTATTACGACATGAACGCGGCCAATGTGG
MMP14	NM_004 995.2	1470-1570	GACAAGATTGATGCTGCTCTCTTCTGGATGCCCAATGGAAAGACCTACTTCTT CCGTGGAAACAAGTACTACCGTTTCAACGAAGAGCTCAGGGCAGTGG

MPL	NM_005 373.2	895-995	CAGTGGCACTTGGACTGCAATGCTTTACCTTGGACCTGAAGAATGTTACCTGT CAATGGCAGCAACAGGACCATGCTAGCTCCCAAGGCTTCTTCTACCA
MYB	NM_005 375.2	3145-3245	AACTGTTGCATGGATCCTGTGTTTGCAACTGGGGAGACAGAAACTGTGGTTG ATAGCCAGTCACTGCCTTAAGAACATTTGATGCAAGATGGCCAGCACT
Myc	NM_002 467.3	1610-1710	TCGGACACCGAGGAGAATGTCAAGAGGCGAACACACAACGTCTTGGAGCGCC AGAGGAGGAACGAGCTAAAACGGAGCTTTTTTGCCCTGCGTGACCAGA
MYO6	NM_004 999.3	6655-6755	AAGTTGGGGAGATGGCACCTTCTCAGAGGATTGTGAAAATATGAGGAAGAAA CAAAACAGTGCATGTAGGAGCACAGGGCCACACAAAGGCATTCTATTG
NBEA	NM_015 678.3	8645-8745	CTGAGAGCCCTTGAAGGACCAGAAAACTGCTTATTCCCACGCTTGATATCTGT CTCCAGCGAAGGCCACTGTATCATATACTATGAACGAGGGCGATTCA
NCAM1	NM_000 615.5	1620-1720	GGTATTTGCCTATCCCAGTGCCACGATCTCATGGTTTCGGGATGGCCAGCTGC TGCCAAGCTCCAATTACAGCAATATCAAGATCTACAACACCCCCTCT
NCL	NM_005 381.2	1492-1592	GAACAGAGATCGATGGGCGATCTATTTCCCTGTACTATACTGGAGAGAAAGG TCAAAATCAAGACTATAGAGGTGGAAAGAATAGCACTTGGAGTGGTGA
NFAT5	NM_173 214.1	3290-3390	CCCTGACAACTATTCAAACCCAGGACATCTCACAGCCTGGTACTTTTCCAGCA GTTTCTGCTTCTAGTCAGCTGCCCAACAGCGATGCACTATTGCAGCA
NFATC1	NM_172 390.1	2510-2610	CCAGTACCAGCGTTTCACCTACCTTCCCGCCAACGGTAACGCCATCTTTCTAA CCGTAAGCCGTGAACATGAGCGCGTGGGGTGCTTTTTCTAAAGACGC
NFATC2	NM_012 340.3	1815-1915	GACGGACATTGGAAGAAAGAACACGCGGGTGAGACTGGTTTTCCGAGTTCAC ATCCCAGAGTCCAGTGGCAGAATCGTCTCTTTACAGACTGCATCTAAC
NFATC3	NM_004 555.2	2190-2290	GTCCTTGAAGTTCCTCCATATCATAACCCAGCAGTTACAGCTGCAGTGCAGGT GCACTTTTATCTTTGCAATGGCAAGAGGAAAAAAAGCCAGTCTCAAC
NKG2C	NM_002 260.3	942-1042	TATGTGAGTCAGCTTATAGGAAGTACCAAGAACAGTCAAACCCATGGAGACA GAAAGTAGAATAGTGGTTGCCAATGTCTCAGGGAGGTTGAAATAGGAG
NKG2D	NM_007 360.1	760-860	GGACCAGGATTTACTTAAACTGGTGAAGTCATATCATTGGATGGGACTAGTA CACATTCCAACAAATGGATCTTGGCAGTGGGAAGATGGCTCCATTCTC
NKG2E	NM_002 261.2	760-860	ACTCCTGAGCTCAAGAAATCAACACATCTTGGCCTCCCAAGTTGCTGGGATTA CTGACACAAGCCACCGCCCCTGAGTGCTCATGTACCATTTAGCTTGT
NKG2F	NM_013 431.2	29-129	TTATATTGGTCAACAGCAAAATGAACATTACTACTCAGCCTCCAACACATGCA GTTTGCCTATACCAGGGATCCTGTCAAAATATACACCACTTATAGCT
NKp30 (CD337)	NM_147 130.1	50-150	GCATCTGTCCTCTCTCCTCAGGGAGGCAAGCATTTGATGCTCGAGGTCCCTGG CAGTTGTGGTCCTTGGCAAGTGATGTGTGAGTCCCGTGTGTCATAGG
NKp44 (CD336)	NM_004 828.3	798-898	CTTCAACAGGTCACGGACCTTCCCTGGACCTCAGTTTCCTCACCTGTAGAGAG AGAAATATTATATCACACTGTTGCAAGGACTAAGATAAGCGATGATG
NKp46 (CD335)	NM_001 145457.1	145-245	TTTCATGGTTCCAAAGGAAAAGCAAGTGACCATCTGTTGCCAGGGAAATTAT GGGGCTGTTGAATACCAGCTGCACTTTGAAGGAAGCCTTTTTGCCGTG
NKp80	NM_016 523.1	275-375	AAAAAGGAAGTTGTTCAAATGCCACTCAGTATGAGGACACTGGAGATCTAAA AGTGAATAATGGCACAAGAAGAAATATAAGTAATAAGGACCTTTGTGC
NOS2	NM_000 625.4	605-705	TTGCCTGGGGTCCATTATGACTCCCAAAAGTTTGACCAGAGGACCCAGGGAC AAGCCTACCCCTCCAGATGAGCTTCTACCTCAAGCTATCGAATTTGTC
Notch1	NM_017 617.3	735-835	CTGCCAGGCTTCACCGGCCAGAACTGTGAGGAAAATATCGACGATTGTCCAG GAAACAACTGCAAGAACGGGGGTGCCTGTGTGGACGGCGTGAACACCT
NR3C1	NM_001 018077.1	1665-1765	GCTTTCTCCTCTGGCGGGAGAAGACGATTCATTCCTTTTGGAAGGAAACTCGA ATGAGGACTGCAAGCCTCTCATTTTACCGGACACTAAACCCAAAATT
NR4A1	NM_002 135.3	155-255	CGGCCGGGTAGGGTGCAGCCTGAGGCTTGTTCAGCAGAACAGGTGCAAGCCA CATTGTTGCCAAGACCTGCCTGAAGCCGGATTCTCCCCACTGCCTCCT

NRIP1	NM_003489.2	335-435	TGACTCATGGAGAAGAGCTTGGCTCTGATGTGCACCAGGATTCTATTGTTTTAACTTACCTAGAAGGATTACTAATGCATCAGGCAGCAGGGGGATCAGG
NT5E	NM_002526.2	1214-1314	ATTCGGGTTTTGAAATGGATAAACTCATCGCTCAGAAAGTGAGGGGTGTGGACGTCGTGGTGGGAGGACACTCCAACACATTTCTTTACACAGGCAATCC
OPTN	NM_001008211.1	625-725	TGAAGCTAAATAATCAAGCCATGAAAGGGAGATTTGAGGAGCTTTCGGCCTGGACAGAGAAACAGAAGGAAGAACGCCAGTTTTTTGAGATACAGAGCAA
P2RX7	NM_002562.4	340-440	AGTTGGTGCACAGTGTCTTTGACACCGCAGACTACACCTTCCCTTTGCAGGGGAACTCTTTCTTCGTGATGACAAACTTTCTCAAAACAGAAGGCCAAGA
p38	NM_006303.3	507-607	CCCTCTCCCTGCTTGTGCTGCACAGGCTGCTCTGTGAGCACTTCAGGGTCCTGTCCACGGTGCACACGCACTCCTCGGTCAAGAGCGTGCCTGAAAACCT
Pax5	NM_016734.1	2288-2388	CTCCAAGAGGAGCACACTTTGGGGAGATGTCCTGGTTTCCTGCCTCCATTTCTCTGGGACCGATGCAGTATCAGCAGCTCTTTTCCAGATCAAAGAACTC
PDCD1	NM_005018.1	175-275	CTTCTTCCCAGCCCTGCTCGTGGTGACCGAAGGGGACAACGCCACCTTCACCTGCAGCTTCTCCAACACATCGGAGAGCTTCGTGCTAAACTGGTACCGC
PDCD1LG2	NM_025239.3	235-335	TGTGGAGCTGTGGCAAGTCCTCATATCAAATACAGAACATGATCTTCCTCCTGCTAATGTTGAGCCTGGAATTGCAGCTTCACCAGATAGCAGCTTTATT
PDE3	NM_000921.3	3010-3110	CTGGCCAACCTTCAGGAATCCTTCATCTCTCACATTGTGGGGCCTCTGTGCAACTCCTATGATTCAGCAGGACTAATGCCTGGAAAATGGGTGGAAGACA
PDE4	NM_001111307.1	3855-3955	AATAATGGTGTATACCCTCATTCTCATTCCTGGGCAGCCCTTCCTTCCACCCTGGCACCAAAATAATTTCTCCTCCATCCGTACCTTGCCTAGCCTCTCC
PDE7	NM_002604.2	2210-2310	GTAGCTCAACAAGGAATAGAGGGAGGAGTGTAATTTTGGTAGCTGGTGTTGAATAGGGCCTTTGAGAATCAGACTGAACACAGTGAAATATGTGCCCAAA
PDK1	NM_002610.3	1170-1270	TGGATTGCCCATATCACGTCTTTACGCACAATACTTCCAAGGAGACCTGAAGCTGTATTCCCTAGAGGGTTACGGGACAGATGCAGTTATCTACATTAAG
PECAM1	NM_000442.3	1365-1465	ATCTGCACTGCAGGTATTGACAAAGTGGTCAAGAAAAGCAACACAGTCCAGATAGTCGTATGTGAAATGCTCTCCCAGCCCAGGATTTCTTATGATGCCC
PHACTR2	NM_001100164.1	8350-8450	GGCAGAATGCCACTCTACCCTCAGGTCAATTTTATGGTATATGAAAATGCCAGTAATATTTGTGCCACTTGCCAACTCGGGGGAGGAGGGGCTTTTCCCT
PHC1	NM_004426.2	2905-3005	ATACAGCTCCACCTACACCGGAATTACATGGCATCAACCCTGTGTTCCTGTCCAGTAATCCCAGCCGTTGGAGTGTAGAGGAGGTGTACGAGTTTATTGC
POP5	NM_015918.3	560-660	GCTTCAGGCCCACTTGTTGAACAGAACAATCTGGGTAGCAACAGCATCTTCCACAGTTTTCCAAACTGGATAGCTGCCAACCAGCAGACATTACCCACTT
PPARA	NM_001001928.2	5220-5320	GGGTGTGTTTGCTATACGAACATAATGGACGTGAAGTGGGGCAGAAACCCAGAACTCAGCATTCAAGGATGCCCAGGAGAGCTGTCCCTGTTTTAAAGAG
PPP2R1A	NM_014225.3	1440-1540	AACTTAACTCCTTGTGCATGGCCTGGCTTGTGGATCATGTATATGCCATCCGCGAGGCAGCCACCAGCAACCTGAAGAAGCTAGTGGAAAAGTTTGGGAA
PRDM1	NM_182907.1	310-410	CATCCCTGCCAACCAGGAACTTCTTGTGTGGTATTGTCGGGACTTTGCAGAAAGGCTTCACTACCCTTATCCCGGAGAGCTGACAATGATGAATCTCACA
PRF1	NM_005041.3	2120-2220	ACTGTTTTTCAGGGAGGTGGCTGGGTTTACACGCTAATCCCGATTCACCCTGTCCAAACTGCCTAAGCCCTCCGCCATTCTCAAGCCCTGCAGTCACAGC
PROM1	NM_006017.1	925-1025	AGCCTGCGGTCATCTCTCAATGACCCTCTGTGCTTGGTGCATCCATCAAGTGAAACCTGCAACAGCATCAGATTGTCTCTAAGCCAGCTGAATAGCAACC
PTGER2	NM_000956.2	1410-1510	GTCAGAAGGAGCTACAAAACCTACCCTCAGTGAGCATGGTACTTGGCCTTTGGAGGAACAATCGGCTGCATTGAAGATCCAGCTGCCTATTGATTTAAGC
PTK2	NM_005607.3	1005-1105	GGTTCAAGCTGGATTATTTCAGTGGAACTGGCAATCGGCCCAGAAGAAGGAATCAGTTACCTAACGGACAAGGGCTGCAATCCCACACATCTTGCTGACT

PTPRK	NM_001135648.1	4315-4415	GTGATCAACCGGATTTTTAGGATATGCAATCTAACAAGACCACAGGAAGGTTATCTGATGGTGCAACAGTTTCAGTACCTAGGATGGGCTTCTCATCGAG
RAC1	NM_198829.1	1250-1350	AAAGACCTTCGTCTTTGAGAAGACGGTAGCTTCTGCAGTTAGGAGGTGCAGACACTTGCTCTCCTATGTAGTTCTCAGATGCGTAAAGCAGAACAGCCTC
RAC2	NM_002872.3	1069-1169	GCTGCCACAACTTGTGTACCTTCAGGGATGGGGCTCTTACTCCCTCCTGAGGCCAGCTGCTCTAATATCGATGGTCCTGCTTGCCAGAGAGTTCCTCTAC
RAP46	NM_004323.3	1490-1590	CTCTTGTGATCGTGTAGTCCCATAGCTGTAAAACCAGAATCACCAGGAGGTTGCACCTAGTCAGGAATATTGGGAATGGCCTAGAACAAGGTGTTTGGCA
RARA	NM_000964.2	115-215	AGCCACCTAGCTGGGGCCCATCTAGGAGTGGCATCTTTTTTGGTGCCCTGAAGGCCAGCTCTGGACCTTCCCAGGAAAAGTGCCAGCTCACAGAACTGCT
RHOA	NM_001664.2	1230-1330	GGTACTCTGGTGAGTCACCACTTCAGGGCTTTACTCCGTAACAGATTTTGTTGGCATAGCTCTGGGGTGGGCAGTTTTTTGAAAATGGGCTCAACCAGAA
RORA	NM_134261.2	1715-1815	AAAATTAACCGAGACACTTTATATGGCCCTGCACAGACCTGGAGCGCCACACACTGCACATCTTTTGGTGATCGGGGTCAGGCAAAGGAGGGGAAACAAT
RORC	NM_001001523.1	1350-1450	CTCATCAATGCCCATCGGCCAGGGCTCCAAGAGAAAAGGAAAGTAGAACAGCTGCAGTACAATCTGGAGCTGGCCTTTCATCATCATCTCTGCAAGACTC
RUNX1	NM_001754.4	635-735	CAGCCATGAAGAACCAGGTTGCAAGATTTAATGACCTCAGGTTTGTCGGTCGAAGTGGAAGAGGGAAAAGCTTCACTCTGACCATCACTGTCTTCACAAA
RUNX2	NM_004348.3	1850-1950	GAAGCCACAGCAGTTCCCCAACTGTTTTGAATTCTAGTGGCAGAATGGATGAATCTGTTTGGCGACCATATTGAAATTCCTCAGCAGTGGCCCAGTGGTA
S100A4	NM_002961.2	263-363	CAGGGACAACGAGGTGGACTTCCAAGAGTACTGTGTCTTCCTGTCCTGCATCGCCATGATGTGTAACGAATTCTTTGAAGGCTTCCCAGATAAGCAGCCC
SATB1	NM_001131010.1	1335-1435	TTCCGAAATCTACCAGTGGGTACGCGATGAACTGAAACGAGCAGGAATCTCCCAGGCGGTATTTGCACGTGTGGCTTTTAACAGAACTCAGGGCTTGCTT
SCAP2	NM_003930.3	3374-3474	TTTTACAGTTAATCCAGGAGAGGGAGTCCTTTGCCAACTGATGACCAACAGTTCCAAGCCAGATAGTCTCGTGAACAGTGACAATACAGAAATAAGGTGT
SCML1	NM_001037540.1	925-1025	GCAACGTATGGTTCTTCTTCAGGGCTCTGCCTTGGCAACCCTCGGGCTGACAGCATCCACAACACTTACTCAACTGACCATGCTTCTGCAGCACCACCTT
SCML2	NM_006089.2	360-460	ATTGGAAGCCCGTGACCCTCGCAATGCCACTTCAGTATGTATTGCTACGGTTATTGGAATTACTGGGGCCAGGTTACGGTTACGACTGGATGGTAGTGAC
SEL1L	NM_005065.4	980-1080	GGGCAATCTAATAGCCCACATGGTTTTGGGTTACAGATACTGGGCTGGCATCGGCGTCCTCCAGAGTTGTGAATCTGCCCTGACTCACTATCGTCTTGTT
SELL	NM_000655.3	110-210	CTCCCTTTGGGCAAGGACCTGAGACCCTTGTGCTAAGTCAAGAGGCTCAATGGGCTGCAGAAGAACTAGAGAAGGACCAAGCAAAGCCATGATATTTCCA
SERPINE2	NM_006216.2	240-340	CGCTGCCTTCCATCTGCTCCCACTTCAATCCTCTGTCTCTCGAGGAACTAGGCTCCAACACGGGGATCCAGGTTTTCAATCAGATTGTGAAGTCGAGGCC
SHP-1	NM_002831.5	1734-1834	TGGTGCAGACGGAGGCGCAGTACAAGTTCATCTACGTGGCCATCGCCCAGTTCATTGAAACCACTAAGAAGAAGCTGGAGGTCCTGCAGTCGCAGAAGGG
SIT1	NM_014450.2	720-820	GCCCCAGCCCCCCGTAGCAGGGGCATGACTGTTTCCCAACCAGCACCCAAAGACGGGCGCCATTGCCAAGTCACAGGATGTGATCTACCCCGGACTTCCT
SLA2	NM_032214.2	1640-1740	AAAGGAAAGCTGAGATGATGTCTTACCGTAGCAGCAGATCTTGGATGGTCCAGGCTCTATGTGACCTCCAGAGCAAAGAGAAAGACTTCGGACAGTCTAG
SLAMF1	NM_003037.2	580-680	GTGTCTCTTGATCCATCCGAAGCAGGCCCTCCACGTTATCTAGGAGATCGCTACAAGTTTTATCTGGAGAATCTCACCCTGGGGATACGGGAAAGCAGGA
SLAMF7	NM_021181.3	215-315	GGGCACTATCATAGTGACCCAAAATCGTAATAGGGAGAGAGTAGACTTCCCAGATGGAGGCTACTCCCTGAAGCTCAGCAAACTGAAGAAGAATGACTCA

SLC2A1	NM_006516.2	2500-2600	AGGCTCCATTAGGATTTGCCCCTTCCCATCTCTTCCTACCCAACCACTCAAATT AATCTTTCTTTACCTGAGACCAGTTGGGAGCACTGGAGTGCAGGGA
SMAD3	NM_005902.3	4220-4320	TTAAAGGACAGTTGAAAAGGGCAAGAGGAAACCAGGGCAGTTCTAGAGGAG TGCTGGTGACTGGATAGCAGTTTTAAGTGGCGTTCACCTAGTCAACACG
SNAI1	NM_005985.2	63-163	GACCACTATGCCGCGCTCTTTCCTCGTCAGGAAGCCCTCCGACCCCAATCGGA AGCCTAACTACAGCGAGCTGCAGGACTCTAATCCAGAGTTTACCTTC
SOD1	NM_000454.4	35-135	GCCTATAAAGTAGTCGCGGAGACGGGGTGCTGGTTTGCGTCGTAGTCTCCTGC AGCGTCTGGGGTTTCCGTTGCAGTCCTCGGAACCAGGACCTCGGCGT
SPI1	NM_003120.1	730-830	CTCCGCAGCGGCGACATGAAGGACAGCATCTGGTGGGTGGACAAGGACAAG GGCACCTTCCAGTTCTCGTCCAAGCACAAGGAGGCGCTGGCGCACCGCT
STAT1	NM_007315.2	205-305	TTTGCTGTATGCCATCCTCGAGAGCTGTCTAGGTTAACGTTCGCACTCTGTGT ATATAACCTCGACAGTCTTGGCACCTAACGTGCTGTGCGTAGCTGCT
STAT3	NM_139276.2	4535-4635	AGACTTGGGCTTACCATTGGGTTTAAATCATAGGGACCTAGGGCGAGGGTTC AGGGCTTCTCTGGAGCAGATATTGTCAAGTTCATGGCCTTAGGTAGCA
STAT4	NM_003151.2	789-889	AGACAATGGATCAGAGTGACAAGAATAGTGCCATGGTGAATCAGGAAGTTTT GACACTGCAGGAAATGCTTAACAGCCTCGATTTCAAGAGAAAGGAGGC
STAT5A	NM_003152.2	3460-3560	GAGACAGAGAGAGAGAAAGAGAGAGTGTGTGGGTCTATGTAAATGCATCTGT CCTCATGTGTTGATGTAACCGATTCATCTCTCAGAAGGGAGGCTGGGG
STAT5B	NM_012448.3	200-300	AAGGAGAAGCCCTTCATCAGATGCAAGCGTTATATGGCCAGCATTTTCCCATT GAGGTGCGGCATTATTTATCCCAGTGGATTGAAAGCCAAGCATGGGA
Stat6	NM_003153.3	2030-2130	AGAACATCCAGCCATTCTCTGCCAAAGACCTGTCCATTCGCTCACTGGGGGAC CGAATCCGGGATCTTGCTCAGCTCAAAAATCTCTATCCCAAGAAGCC
STMN1	NM_203401.1	287-387	CGTGGGTGGCGGCAGGACTTTCCTTATCCCAGTTGATTGTGCAGAATACACTG CCTGTCGCTTGTCTTCTATTCACCATGGCTTCTTCTGATATCCAGGT
TBX21	NM_013351.1	890-990	ACACAGGAGCGCACTGGATGCGCCAGGAAGTTTCATTTGGGAAACTAAAGCT CACAAACAACAAGGGGGCGTCCAACAATGTGACCCAGATGATTGTGCT
TBXA2R	NM_001060.3	385-485	CACACGCGCTCCTCCTTCCTCACCTTCCTCTGCGGCCTCGTCCTCACCGACTTC CTGGGGCTGCTGGTGACCGGTACCATCGTGGTGTCCCAGCACGCCG
Tcf7	NM_003202.2	2420-2520	ATTCCATTTCCAGTTCATCTATGGCAGTCCAGCCAGCTCCTGGGCAGCTTGAG AGGGCAAACCCAAAACCTCATGACAGCCAGAGCCTGTCTTTCAGCAT
TDGF1	NM_003212.2	1567-1667	AAGGAAAGAAAACATCTTTAAGGGGAGGAACCAGAGTGCTGAAGGAATGGA AGTCCATCTGCGTGTGTGCAGGGAGACTGGGTAGGAAAGAGGAAGCAAA
TDO2	NM_005651.1	0-100	AAGGTCAATGATAGCATCTGCCTAGAGTCAAACCTCCGTGCTTCTCAGACAGT GCCTTTTCACCATGAGTGGGTGCCCATTTTTAGGAAACAACTTTGGA
TEK	NM_000459.2	615-715	CGAGTTCGAGGAGAGGCAATCAGGATACGAACCATGAAGATGCGTCAACAA GCTTCCTTCCTACCAGCTACTTTAACTATGACTGTGGACAAGGGAGATA
TERT	NM_198253.1	2570-2670	GGCTTCAAGGCTGGGAGGAACATGCGTCGCAAACTCTTTGGGGTCTTGCGGC TGAAGTGTCACAGCCTGTTTCTGGATTTGCAGGTGAACAGCCTCCAGA
TF	NM_001063.2	640-740	CTGCTCCACCCTTAACCAATACTTCGGCTACTCGGGAGCCTTCAAGTGTCTGA AGGATGGTGCTGGGGATGTGGCCTTTGTCAAGCACTCGACTATATTT
TFRC	NM_003234.1	1220-1320	CAGTTTCCACCATCTCGGTCATCAGGATTGCCTAATATACCTGTCCAGACAAT CTCCAGAGCTGCTGCAGAAAAGCTGTTTGGGAATATGGAAGGAGACT
TGFA	NM_003236.2	780-880	TGCCACAGACCTTCCTACTTGGCCTGTAATCACCTGTGCAGCCTTTTGTGGGC CTTCAAAACTCTGTCAAGAACTCCGTCTGCTTGGGGTTATTCAGTGT
TGFB1	NM_000660.3	1260-1360	TATATGTTCTTCAACACATCAGAGCTCCGAGAAGCGGTACCTGAACCCGTGTT GCTCTCCCGGGCAGAGCTGCGTCTGCTGAGGCTCAAGTTAAAAGTGG

TGFB2	NM_003238.2	1125-1225	AAGCCAGAGTGCCTGAACAACGGATTGAGCTATATCAGATTCTCAAGTCCAA AGATTTAACATCTCCAACCCAGCGCTACATCGACAGCAAAGTTGTGAA
TGFBR1	NM_004612.2	4280-4380	GGGGAAATACGACTTAGTGAGGCATAGACATCCCTGGTCCATCCTTTCTGTCT CCAGCTGTTTCTTGGAACCTGCTCTCCTGCTTGCTGGTCCCTGACGC
TIE1	NM_005424.2	2610-2710	CATCGGGGAGGGGAACTTCGGCCAGGTCATCCGGGCCATGATCAAGAAGGAC GGGCTGAAGATGAACGCAGCCATCAAAATGCTGAAAGAGTATGCCTCT
TLR2	NM_003264.3	180-280	CTGCTTTCAACTGGTAGTTGTGGGTTGAAGCACTGGACAATGCCACATACTTT GTGGATGGTGTGGGTCTTGGGGGTCATCATCAGCCTCTCCAAGGAAG
TLR8	NM_138636.3	2795-2895	GACAAAAACGTTCTCCTTTGTCTAGAGGAGAGGGATTGGGATCCGGGATTGG CCATCATCGACAACCTCATGCAGAGCATCAACCAAAGCAAGAAAACAG
TNF	NM_000594.2	1010-1110	AGCAACAAGACCACCACTTCGAAACCTGGGATTCAGGAATGTGTGGCCTGCA CAGTGAAGTGCTGGCAACCACTAAGAATTCAAACTGGGGCCTCCAGAA
TNFRSF18	NM_004195.2	445-545	AGGGGAAATTCAGTTTTGGCTTCCAGTGTATCGACTGTGCCTCGGGGACCTTC TCCGGGGGCCACGAAGGCCACTGCAAACCTTGGACAGACTGCACCCA
TNFRSF1B	NM_001066.2	835-935	CCCAGCTGAAGGGAGCACTGGCGACTTCGCTCTTCCAGTTGGACTGATTGTGG GTGTGACAGCCTTGGGTCTACTAATAATAGGAGTGGTGAACTGTGTC
TNFRSF4	NM_003327.2	200-300	CCGTGCGGGCCGGGCTTCTACAACGACGTGGTCAGCTCCAAGCCGTGCAAGC CCTGCACGTGGTGTAACCTCAGAAGTGGGAGTGAGCGGAAGCAGCTGT
TNFRSF7	NM_001242.4	330-430	CCAGATGTGTGAGCCAGGAACATTCCTCGTGAAGGACTGTGACCAGCATAGA AAGGCTGCTCAGTGTGATCCTTGCATACCGGGGGTCTCCTTCTCTCCT
TNFRSF9	NM_001561.4	255-355	AGATTTGCAGTCCCTGTCCTCCAAATAGTTTCTCCAGCGCAGGTGGACAAAGG ACCTGTGACATATGCAGGCAGTGTAAAGGTGTTTTCAGGACCAGGAA
TNFSF10	NM_003810.2	115-215	GGGGGGACCCAGCCTGGGACAGACCTGCGTGCTGATCGTGATCTTCACAGTG CTCCTGCAGTCTCTCTGTGTGGCTGTAACTTACGTGTACTTTACCAAC
TNFSF14	NM_003807.2	270-370	ATTTTCAGAAGCCTCTGGAAAGTCGTGCACAGCCCAGGAGTGTTGAGCAATTT CGGTTTCCTCTGAGGTTGAAGGACCCAGGCGTGTCAGCCCTGCTCCA
TOX	NM_014729.2	3950-4050	AATGAGCAGCTTTGACTTTGACAGGCGGTTTGTGCAGGAAAGCACAGTGCCG TGTTGTTTACAGCTTTTCTAGAGCAGCTGTGCGACCAGGGTAGAGAGT
TP53	NM_000546.2	1330-1430	GGGGAGCAGGGCTCACTCCAGCCACCTGAAGTCCAAAAAGGGTCAGTCTACC TCCCGCCATAAAAAACTCATGTTCAAGACAGAAGGGCCTGACTCAGAC
TRAF1	NM_005658.3	3735-3835	CGAGTGATGGGTCTAGGCCCTGAAACTGATGTCCTAGCAATAACCTCTTGATC CCTACTCACCGAGTGTTGAGCCCAAGGGGGGATTTGTAGAACAAGCC
TRAF2	NM_021138.3	1325-1425	GTGGCCCTTCAACCAGAAGGTGACCTTAATGCTGCTCGACCAGAATAACCGG GAGCACGTGATTGACGCCTTCAGGCCCGACGTGACTTCATCCTCTTTT
TRAF3	NM_145725.1	1795-1895	ATATGATGCCCTGCTTCCTTGGCCGTTTAAGCAGAAAGTGACACTCATGCTGA TGGATCAGGGGTCCTCTCGACGTCATTTGGGAGATGCATTCAAGCCC
TSLP	NM_033035.3	395-495	CCGTCTCTTGTAGCAATCGGCCACATTGCCTTACTGAAATCCAGAGCCTAACC TTCAATCCCACCGCCGGCTGCGCGTCGCTCGCCAAAGAAATGTTCGC
TYK2	NM_003331.3	485-585	TCATCGCTGACAGCTGAGGAAGTCTGCATCCACATTGCACATAAAGTTGGTAT CACTCCTCCTTGCTTCAATCTCTTTGCCCTCTTCGATGCTCAGGCCC
VEGFA	NM_001025366.1	1325-1425	GAGTCCAACATCACCATGCAGATTATGCGGATCAAACCTCACCAAGGCCAGC ACATAGGAGAGATGAGCTTCCTACAGCACAACAAATGTGAATGCAGAC
WEE1	NM_003390.2	5-105	TGCGTTTGAGTTTGCCGCGAGCCGGGCCAATCGGTTTTGCCAACGCATGCCCA CGTGCTGGCGAACAAATGTAAACACGGAGATCGTGTGCCGGGCACTT
ZAP70	NM_001079.3	1175-1275	GGAGCTCAAGGACAAGAAGCTCTTCCTGAAGCGCGATAACCTCCTCATAGCT GACATTGAACTTGGCTGCGGCAACTTTGGCTCAGTGCGCCAGGGCGTG

ZNF516	NM_014643.2	4830-4930	GGTGGGGGACGGCTTCATATACCTCTTCCTCAGTAATGCAAATGCGAGTTTTT GTGGTGGGGGTTAAGGCCCATAACAAAGGATCTTAAACCATGCAGTG
p16	NM_000077.3	975-1075	AAGCGCACATTCATGTGGGCATTTCTTGCGAGCCTCGCAGCCTCCGGAAGCTG TCGACTTCATGACAAGCATTTTGTGAACTAGGGAAGCTCAGGGGGGT
SHP2	NM_002834.3	4650-4750	TAGTCCCTAGGTTGCTACGGCTTATCATGTGCTTGGTAAAAGGTGATCGCAGG TTCTCAGACGAGTTTACTTTACATGAGATGGAATCAGGCAGAGAGGC
CD57/B3GAT1	NM_018644.3	145-245	CTGGACAGCGACCCCTTCTCAGACTCCAGTTGGGCCGGACTCTCCAAACCTGC TTCCGCAATGGGTGGGTTGTGAGTGCTGGTAATGAGGAGCCGTGGGT
CD85/LILRB1	NM_001081637.1	2332-2432	AGCTGAGAAAACTAAGTCAGAAAGTGCATTAAACTGAATCACAATGTAAATA TTACACATCAAGCGATGAAACTGGAAAACTACAAGCCACGAATGAATG
Neil1	NM_024608.2	1675-1775	TTAGCAGGAGGCTCTCCTTGCTTGCACTCACCCTTTCTTATTGTCTTGCCCTGC ATCTGGGGGTCTGAATTTTTGGGAGCAGGCAATATCTGAAGGTGCA
Neil2	NM_145043.2	2570-2670	GCCCGGTGGTGTGTAGAGAAAAGCTGCTTGTTTACTCCTTAAGTCAATGTATT GGTGACTGTTGATTTGTTGAACAATTCAGGAATCAAGGGCTGTGGAG
PNK	NM_003681.3	580-680	TCCCGGAGGACCTCCTTCCCGTCTACAAAGAAAAAGTGGTGCCGCTTGCAGA CATTATCACGCCCAACCAGTTTGAGGCCGAGTTACTGAGTGGCCGGAA
POLR2A	NM_000937.2	3775-3875	TTCCAAGAAGCCAAAGACTCCTTCGCTTACTGTCTTCCTGTTGGGCCAGTCCG CTCGAGATGCTGAGAGAGCCAAGGATATTCTGTGCCGTCTGGAGCAT
POLR1B	NM_019014.3	3320-3420	GGAGAACTCGGCCTTAGAATACTTTGGTGAGATGTTAAAGGCTGCTGGCTAC AATTTCTATGGCACCGAGAGGTTATATAGTGGCATCAGTGGGCTAGAA
IL-1alpha	NM_000575.3	1085-1185	ACTCCATGAAGGCTGCATGGATCAATCTGTGTCTCTGAGTATCTCTGAAACCT CTAAAACATCCAAGCTTACCTTCAAGGAGAGCATGGTGGTAGTAGCA
IL-1beta	NM_000576.2	840-940	GGGACCAAAGGCGGCCAGGATATAACTGACTTCACCATGCAATTTGTGTCTTC CTAAAGAGAGCTGTACCCAGAGAGTCCTGTGCTGAATGTGGACTCAA
IL-12p40	NM_002187.2	1435-1535	GCAAGGCTGCAAGTACATCAGTTTTATGACAATCAGGAAGAATGCAGTGTTC TGATACCAGTGCCATCATACACTTGTGATGGATGGGAACGCAAGAGAT
Raf-1	NM_002880.2	1990-2090	CCTATGGCATCGTATTGTATGAACTGATGACGGGGGAGCTTCCTTATTCTCAC ATCAACAACCGAGATCAGATCATCTTCATGGTGGGCCGAGGATATGC
IL-23p19	NM_016584.2	411-511	CAGGGACAACAGTCAGTTCTGCTTGCAAAGGATCCACCAGGGTCTGATTTTTT ATGAGAAGCTGCTAGGATCGGATATTTTCACAGGGGAGCCTTCTCTG
gBAD-1R_scfv	SCFV001.1	1-101	AGACAGACACCCTGCTCCTCTGGGTGTCCGGCACCTGTGGCGACATCGTGATG AGCAGAAGCCCCAGCAGCCTGGCCGTGTCCGTGGGCGAGAAAGTGAC
CD20_scfv_rutuximab	SCFV002.1	8-108	GCTGTCCCAGAGCCCCGCCATCCTGAGCGCCAGCCCTGGCGAGAAGGTGACC ATGACCTGCCGGGCCAGCAGCTCTGTGAGCTACATGCACTGGTATCAG
c-MET_scfv	SCFV004.1	138-238	CTGATCTACGCCGCCAGCAGCCTGAAGAGCGGCGTGCCCAGCCGGTTTAGCG GCTCTGGCTCTGGCGCCGACTTCACCCTGACCATCAGCAGCCTGCAGC
CD45R_scfv	SCFV006.1	222-322	TTCACCCTGAACATCCACCCCGTGGAGGAAGAGGACGCCGCCACCTACTACT GCCAGCACAGCAGAGAGCTGCCCTTCACCTTCGGCTCCGGCACCAAGC
Thymidine_kinase	SCFV007.1	100-200	TCTACGTACCCGAGCCGATGACTTACTGGCAGGTGCTGGGGGCTTCCGAGAC AATCGCGAACATCTACACCACACAACACCGCCTCGACCAGGGTGAGAT
CD56R_scfv	SCFV008.1	197-297	ATTCAGCGGCTCTGGCTCCGGCACCGACTTCACTCTGATGATCTCTCGGGTGG AGGCCGAGGACCTGGGCGTGTACTACTGCTTTCAGGGCAGCCACGTG
Human_CD19R_scfv	SCFV009.1	215-315	CTTCACCATCAGCAGCCTGCAGCCCGAGGACATCGCCACCTACTACTGCCAG CAGTACCAGAGCCTGCCCTACACCTTCGGCCAGGGCACCAAGCTGCAG
DECTIN-1R	SCFV010.1	270-370	CTGAAGATCGACAGCAGCAACGAGCTGGGCTTCATCGTGAAGCAGGTGTCCA GCCAGCCCGACAACTCCTTCTGGATCGGCCTGAGCAGGCCCCAGACCG

HERV-K_6H5_scfv	SCFV012.1	137-237	CGGCGGCACCAGCTACAACCAGAAGTTCAAGGACAAGGCCATCCTGACCGTG GACAAGAGCAGCAGCACCGCCTACATGGAACTGCGGAGCCTGACCAGC
CD19R_scfv	SCFV013.1	204-304	GGCACCGACTACAGCCTGACCATCTCCAACCTGGAGCAGGAGGACATCGCCA CCTACTTTTGCCAGCAGGGCAACACACTGCCCTACACCTTTGGCGGCG
HER2_scfv	SCFV014.1	64-164	CCTGCAGCGCCAGCAGCAGCGTGTCCTACATGCACTGGTATCAGCAGAAGTC CGGCACTAGCCCCAAGCGGTGGATCTACGACACCTACAAGCTCGCCAG
EGFR_scfv_NIMO_CAR	SCFV015.1	7-107	AGATGACCCAGAGCCCTAGCAGCCTGAGCGCCAGCGTGGGCGACAGAGTGA CCATCACCTGCCGGTCCAGCCAGAACATCGTGCACAGCAACGGCAACAC
RPL27	NM_000988.3	23-123	GGGCCGGGTGGTTGCTGCCGAAATGGGCAAGTTCATGAAACCTGGGAAGGTG GTGCTTGTCCTGGCTGGACGCTACTCCGGACGCAAAGCTGTCATCGTG
OAZ1	NM_004152.2	313-413	GGTGGGCGAGGGAATAGTCAGAGGGATCACAATCTTTCAGCTAACTTATTCT ACTCCGATGATCGGCTGAATGTAACAGAGGAACTAACGTCCAACGACA
GABPa	NM_002040.3	1160-1260	GACCAAGTCCTGCATTGGGTGGTTTGGGTAATGAAGGAATTCAGCATGACCG ATATAGACCTCACCACACTCAACATTTCGGGGAGAGAATTATGTAGTC
XBP-1	NM_005080.2	440-540	GGAGTTAAGACAGCGCTTGGGGATGGATGCCCTGGTTGCTGAAGAGGAGGCG GAAGCCAAGGGGAATGAAGTGAGGCCAGTGGCCGGGTCTGCTGAGTCC
MBD2	NM_003927.3	2015-2115	ATTTACATTCAACTCTGATCCCTGGGCCTTAGGTTTGACATGGAGGTGGAGGA AGATAGCGCATATATTTGCAGTATGAACTATTGCCTCTGGACGTTGT
Bcl6b	NM_181844.3	2135-2235	CTTTATTTGTTCTAGGGCAGCTCTGGGAACATGCGGGATTGTGGAATTGGGTC AGGAACCCTCTCTGGTATTCTGGATGTTGTAGGTTCTCTAGCAGTCT
TSLP-R	NM_022148.2	1420-1520	CAAGGCAGCACGTCCAAAATGCTGTAAAACCATCTTCCCACTCTGTGAGTCCC CAGTTCCGTCCATGTACCTGTTCCATAGCATTGGATTCTCGGAGGAT
BTLA	NM_001085357.1	890-990	GCACCAACAGAATATGCATCCATATGTGTGAGGAGTTAAGTCTGTTTCTGACT CCAACAGGGACCATTGAATGATCAGCATGTTGACATCATTGTCTGGG
HVEM	NM_003820.2	916-1016	CTCAGGGAGCCTCGTCATCGTCATTGTTTGCTCCACAGTTGGCCTAATCATAT GTGTGAAAAGAAGAAAGCCAAGGGGTGATGTAGTCAAGGTGATCGTC
LTbR	NM_002342.1	1435-1535	CTAACAGGGGCCCAAGGAACCAATTTATCACCCATGACTGACGGAGTCTGAG AAAAGGCAGAAGAAGGGGGGCACAAGGGCACTTTCTCCCTTGAGGCTG
CD43	NM_001030288.1	2798-2898	AAGCCAGGCTTCATGGAAAGATCGTATGTGTGACCCAAATATGAGTTCTTCA GCTCAGCCATGGTAATCCCTTCCTTGAAGTCTCCATTTCTGCAGTACA
mTOR	NM_004958.2	5095-5195	TTAGTGTTGCTCCTGGGAGTTGATCCGTCTCGGCAACTTGACCATCCTCTGCC AACAGTTCACCCTCAGGTGACCTATGCCTACATGAAAAACATGTGGA
AMPK	NM_006252.2	975-1075	ATAGTGGTGACCCTCAAGACCAGCTTGCAGTGGCTTATCATCTTATCATTGAC AATCGGAGAATAATGAACCAAGCCAGTGAGTTCTACCTCGCCTCTAG
SIP1	NM_001009182.1	537-637	ACAAGCAACAGTAACTAGTGTCTTGGAATATCTGAGTAATTGGTTTGGAGAA AGAGACTTTACTCCAGAATTGGGAAGATGGCTTTATGCTTTATTGGCT
EphA2	NM_004431.2	1525-1625	GAGCCGAGTGTGGAAGTACGAGGTCACTTACCGCAAGAAGGGAGACTCCAAC AGCTACAATGTGCGCCGCACCGAGGGTTTCTCCGTGACCCTGGACGAC
CD254	NM_003701.2	490-590	TACCTGATTCATGTAGGAGAATTAAACAGGCCTTTCAAGGAGCTGTGCAAAA GGAATTACAACATATCGTTGGATCACAGCACATCAGAGCAGAGAAAGC
BCLxL	NM_001191.2	260-360	ATCTTGGCTTTGGATCTTAGAAGAGAATCACTAACCAGAGACGAGACTCAGT GAGTGAGCAGGTGTTTTGGACAATGGACTGGTTGAGCCCATCCCTATT
Xbp1	NM_001079539.1	935-1035	ATTCATTGTCTCAGTGAAGGAAGAACCTGTAGAAGATGACCTCGTTCCGGAG CTGGGTATCTCAAATCTGCTTTCATCCAGCCACTGCCCAAAGCCATCT
IL27	NM_145659.3	143-243	CAGGAGCTGCGGAGGGAGTTCACAGTCAGCCTGCATCTCGCCAGGAAGCTGC TCTCCGAGGTTCGGGGCCAGGCCCACCGCTTTGCGGAATCTCACCTGC

IKZF2	NM_001079526.1	945-1045	CCATGTACCTCCTATGGAAGATTGTAAGGAACAAGAGCCTATTATGGACAAC AATATTTCTCTGGTGCCTTTTGAGAGACCTGCTGTCATAGAGAAGCTC
GNLY	NM_006433.2	305-405	CAGGAGCTGGGCCGTGACTACAGGACCTGTCTGACGATAGTCCAAAAACTGA AGAAGATGGTGGATAAGCCCACCCAGAGAAGTGTTTCCAATGCTGCGA
NFkB	NM_001165412.1	2305-2405	CTTGGGTAACTCTGTTTTGCACCTAGCTGCCAAAGAAGGACATGATAAAGTTC TCAGTATCTTACTCAAGCACAAAAAGGCAGCACTACTTCTTGACCAC
GADD45alpha	NM_001924.2	865-965	GTTACTCCCTACACTGATGCAAGGATTACAGAAACTGATGCCAAGGGGCTGA GTGAGTTCAACTACATGTTCTGGGGGCCCGGAGATAGATGACTTTGCA
GADD45beta	NM_015675.2	365-465	TGTGGACCCAGACAGCGTGGTCCTCTGCCTCTTGGCCATTGACGAGGAGGAG GAGGATGACATCGCCCTGCAAATCCACTTCACGCTCATCCAGTCCTTC
ATF3	NM_001030287.2	600-700	GGCTCAGAATGGGAGGACTCCAGAAGATGAGAGAAACCTCTTTATCCAACAG ATAAAAGAAGGAACATTGCAGAGCTAAGCAGTCGTGGTATGGGGGCGA
MAD	NM_002357.2	880-980	GAGAATAAAGCTGCAGGACAGTCACAAGGCGTGTCTTGGTCTCTAAGAGAGT GGGCACTGCGGCTGTCTCCTTGAAGGTTCTCCCTGTTGGTTCTGATTA
Crem	NM_001881.2	260-360	CTCCACCTCCTCGCGTCCGTAATCAGTGACGAGGTCCGCTACGTAAATCCCTT TGCGGCGGACAAATGACCATGGAAACAGTTGAATCCCAGCATGATGG
SOCS1	NM_003745.1	1025-1125	TTAACTGTATCTGGAGCCAGGACCTGAACTCGCACCTCCTACCTCTTCATGTT TACATATACCCAGTATCTTTGCACAAACCAGGGGTTGGGGGAGGGTC
SOCS3	NM_003955.3	1870-1970	GGAGGATGGAGGAGACGGGACATCTTTCACCTCAGGCTCCTGGTAGAGAAGA CAGGGGATTCTACTCTGTGCCTCCTGACTATGTCTGGCTAAGAGATTC
DUSP16	NM_030640.2	615-715	ATGGGTTTAACTCTCCTTTTGCCAGTCACCACCAGCCTGACCTCATACACTTTT AGTACAATGGAGTGGCTGAGCCTTTGAGCACACCACCATTACATCA
Rps13	NM_001017.2	331-431	GCATCTTGAGAGGAACAGAAAGGATAAGGATGCTAAATTCCGTCTGATTCTA ATAGAGAGCCGGATTCACCGTTTGGCTCGATATTATAAGACCAAGCGA
TBP	NM_003194.3	25-125	CGCCGGCTGTTTAACTTCGCTTCCGCTGGCCCATAGTGATCTTTGCAGTGACC CAGCAGCATCACTGTTTCTTGGCGTGTGAAGATAACCCAAGGAATTG
G6PD	NM_000402.2	1155-1255	ACAACATCGCCTGCGTTATCCTCACCTTCAAGGAGCCCTTTGGCACTGAGGGT CGCGGGGGCTATTTCGATGAATTTGGGATCATCCGGGACGTGATGCA
Rbpms	NM_001008710.1	842-942	AAACAGCCTGTAGGTTTTGTCAGTTTTGACAGTCGCTCAGAAGCAGAGGCTGC AAAGAATGCTTTGAATGGCATCCGCTTCGATCCTGAAATTCCGCAAA
KLF7	NM_001270943.1	1546-1646	GTACTATTGAGATCTTTCGCGTCGATCCCAACGGCCTTAGCGGCGGCAGACTG GAATAACACCTTACACCTTTCTGGCCTGCATTTCTGTAGACTTCACT
Vax2	NM_012476.2	871-971	CAGCGCCAGCAGCTGCAAGAAAGCTAACACTTAAGACTCCCACCCTGTGACA CTGAGTCCCGAGCACAGCACCTTCCCAGTCTCCTGTGCCCCAGCGGAC
RUNX3	NM_004350.1	2085-2185	GTGGTCTCATAATTCCATTTGTGGAGAGAACAGGAGGGCCAGATAGATAGGT CCTAGCAGAAGGCATTGAGGTGAGGGATCATTTTGGGTCAGACATCAA
ERK	NM_017449.2	785-885	CAAAGCAGGCTTCGAGGCCGTTGAGAATGGCACCGTCTGCCGAGGTTGTCCA TCTGGGACTTTCAAGGCCAACCAAGGGGATGAGGCCTGTACCCACTGT
ITCH	NM_031483.4	155-255	ACTGTGAGAACTTCAGGTTTTCCAACCTATTGGTGGTATGTCTGACAGTGGAT CACAACTTGGTTCAATGGGTAGCCTCACCATGAAATCACAGCTTCAG
CBLB	NM_170662.3	3195-3295	TAATGTCGAAGTTGCCCGGAGCATCCTCCGAGAATTTGCCTTCCCTCCTCCAG TATCCCCACGTCTAAATCTATAGCAGCCAGAACTGTAGACACCAAAA
DGKA	NM_001345.4	1375-1475	TTCCTAACACCCACCCACTTCTCGTCTTTGTCAATCCTAAGAGTGGCGGGAAG CAGGGGCAAAGGGTGCTCTGGAAGTTCCAGTATATATTAAACCCTCG
LTA	NM_000595.2	885-985	CTGATCAAGTCACCGGAGCTTTCAAAGAAGGAATTCTAGGCATCCCAGGGGA CCACACCTCCCTGAACCATCCCTGATGTCTGTCTGGCTGAGGATTTCA

FoxP1	NM_032682.5	6758-6858	CCTGAAAATCAGATTTACAATGCTGAAGGCATTTCTTGGGCCCAGTGTAGCTC ACGCAATCTCTGCTACCCATAAGCCTTGATGAAGATGATACAGTCCG
CD223 (LAG3)	NM_002286.5	1735-1835	CTTTTGGTGACTGGAGCCTTTGGCTTTCACCTTTGGAGAAGACAGTGGCGACC AAGACGATTTTCTGCCTTAGAGCAAGGGATTCACCCTCCGCAGGCTC
CD118	NM_002310.3	2995-3095	CCTATTGTCCACCCATCATTGAGGAAGAAATACCAAACCCAGCCGCAGATGA AGCTGGAGGGACTGCACAGGTTATTTACATTGATGTTCAGTCGATGTA
Txk	NM_003328.1	800-900	ATGACTCGTCTCCGATATCCAGTTGGGCTGATGGGCAGTTGTTTACCAGCCAC AGCTGGGTTTAGCTACGAAAAGTGGGAGATAGATCCATCTGAGTTGG
Prkcq	NM_006257.2	1325-1425	GATGGACGATGATGTTGAGTGCACGATGGTAGAGAAGAGAGTTCTTTCCTTG GCCTGGGAGCATCCGTTTCTGACGCACATGTTTTGTACATTCCAGACC
STS2 (Ubash3a)	NM_001001895.1	1970-2070	GAGATGCTGCTGTTTCCAGAGGCGTCTTAGTCTCACCCAATGTGATTTGTAGA AGCACGAGACGCACTTTTATATCCCGGAATATTTCCCTCCGGCTTTC
RNF125	NM_017831.3	790-890	GCAAGGTGTGTATGTCCCTTTTGTCAGAGGGAACTGTATGAAGACAGCTTGCT GGATCATTGTATTACTCATCACAGATCGGAACGGAGGCCTGTGTTCT
Lat	NM_001014987.1	1290-1390	TGTGTAATAGAATAAAGGCCTGCGTGTGTCTGTGTTGAGCGTGCGTCTGTGTG TGCCTGTGTGCGAGTCTGAGTCAGAGATTTGGAGATGTCTCTGTGTG
Skap1	NM_003726.3	1360-1460	AAGTGGGAAGAGGCACGTTCATCAAACCTGTTACTAAACCAGCCTAGTCATA GCTCATCCCCATCTCTAAATGTGTCCACACAACCACATCTGCCTTTTC
Dok2	NM_003974.2	650-750	GCCAGGGACCCAGCTGTACGACTGGCCCTACAGGTTTCTGCGGCGCTTTGGGC GGGACAAGGTAACCTTTTCCTTTGAGGCAGGCCGTCGCTGCGTCTCT
Axin2	NM_004655.3	1035-1135	CTTGTCCAGCAAAACTCTGAGGGCCACGGCGAGTGTGAGGTCCACGGAAACT GTTGACAGTGGATACAGGTCCTTCAAGAGGAGCGATCCTGTTAATCCT
Sh2d2a	NM_001161443.1	341-441	TGCTGGAGCCCAAGCCTCAGGGGTGCTACTTGGTGCGGTTCAGCGAGAGCGC GGTGACCTTCGTGCTGACTTACAGGAGCCGGACTTGCTGCCGCCACTT
Klra5 (Ly49E)	NR_028045.1	414-514	CCTTCAGAGTCACAGAATAGATTAAGGCCTGATGATACTCAAAGGCCTGGGA AAACTGATGACAAAGAATTTTCAGTGCCCTGGCACCTCATTGCAGTGA
CD7	NM_006137.6	440-540	CCTACACCTGCCAGGCCATCACGGAGGTCAATGTCTACGGCTCCGGCACCCT GGTCCTGGTGACAGAGGAACAGTCCCAAGGATGGCACAGATGCTCGGA
CD11c	NM_000887.3	700-800	CCCCTCAGCCTGTTGGCTTCTGTTCACCAGCTGCAAGGGTTTACATACACGGC CACCGCCATCCAAAATGTCGTGCACCGATTGTTCCATGCCTCATATG
Syk	NM_003177.3	1685-1785	CGGACTCTCCAAAGCACTGCGTGCTGATGAAAACTACTACAAGGCCCAGACC CATGGAAAGTGGCCTGTCAAGTGGTACGCTCCGGAATGCATCAACTAC
Lyn	NM_002350.1	1285-1385	TCCTGAAGAGCGATGAAGGTGGCAAAGTGCTGCTTCCAAAGCTCATTGACTTT TCTGCTCAGATTGCAGAGGGAATGGCATACATCGAGCGGAAGAACTA
Lat2	NM_014146.3	1863-1963	TGCAGAGCTGATTAAACAGTGTTGTGACTGTCTCATGGGAAGAGCTGGGGCC CAGAGGGACCTTGAGTCAGAAATGTTGCCAGAAAAAGTATCTCCTCCA
Clnk	NM_052964.2	1108-1208	GAAGGAGAACAAGGATGGTAGTTTCTTGGTCCGAGATTGTTCCACAAAATCC AAGGAAGAGCCCTATGTTTTGGCTGTGTTTTATGAGAACAAAGTCTAC
Car2	NM_000067.2	575-675	AGCTGTGCAGCAACCTGATGGACTGGCCGTTCTAGGTATTTTTTTGAAGGTTG GCAGCGCTAAACCGGGCCTTCAGAAAGTTGTTGATGTGCTGGATTCC
Fgl2	NM_006682.2	250-350	CAATTCAGCAGGATCGAGGAGGTGTTCAAAGAAGTCCAAAACCTCAAGGAAA TCGTAAATAGTCTAAAGAAATCTTGCCAAGACTGCAAGCTGCAGGCTG
cathepsinC	NM_001114173.1	260-360	TGCTCGGTTATGGGACCACAAGAAAAAAAAGTAGTGGTGTACCTTCAGAAGC TGGATACAGCATATGATGACCTTGGCAATTCTGGCCATTTCACCATCA
CathepsinD	NM_001909.3	1495-1595	GAAGCCGGCGGCCCAAGCCCGACTTGCTGTTTTGTTCTGTGGTTTTCCCCTCC CTGGGTTCAGAAATGCTGCCTGCCTGTCTGTCTCTCCATCTGTTTGG

Rab31	NM_006 868.3	3800-3900	TTTTGTAAAGAGCTTCCATCTGGGCTGGACCCAGTTCTTGCACATACAAGACA CCGCTGCAGTCAGCTAGGACCTTTCCGCCATGTATTCTATTCTGTAG
Spry2	NM_005 842.2	85-185	AAAGAGGAAATACTCCGCGTGCGCTTGTAGAAGGGGAGTCGTCTCCAGCTCC GAACCCCGGAGTGTTCATCAGCGGGGAATCTGGCTCCGAATTCTCTTT
S100A6	NM_014 624.3	539-639	TTCCTGGGGGCCTTGGCTTTGATCTACAATGAAGCCCTCAAGGGCTGAAAATA AATAGGGAAGATGGAGACACCCTCTGGGGGTCCTCTCTGAGTCAAAT
Lgals1	NM_002 305.3	60-160	GGTGCGCCTGCCCGGGAACATCCTCCTGGACTCAATCATGGCTTGTGGTCTGG TCGCCAGCAACCTGAATCTCAAACCTGGAGAGTGCCTTCGAGTGCGA
Hmgb2	NM_001 130688.1	125-225	CTGTCAACATGGGTAAAGGAGACCCCAACAAGCCGCGGGGCAAAATGTCCTC GTACGCCTTCTTCGTGCAGACCTGCCGGGAAGAGCACAAGAAGAAACA
HopX	NM_001 145460.1	1117-1217	AACAATAGGAAGCTATGTGTATCTTCTGTGTAAAGCAGTGGCTTCACTGGAA AAATGGTGTGGCTAGCATTTCCCTTTGAGTCATGATGACAGATGGTGT
Dock5	NM_024 940.6	630-730	TGCGAGATGACAATGGGAACATCCTAGACCCTGACGAAACCAGCACCATTGC CCTCTTCAAGGCCCATGAGGTGGCCTCCAAAAGGATTGAGGAAAAGAT
Ptpn4	NM_002 830.2	705-805	TCGAGGCTTTTTTTCTCCAGCCGAGAGGACGCGGCTGTGATATACGAAGACTT TGTGTGGACAGTAATGACCTCACGTTTCCGATTGCCTGCTGGCAGAA
PLZF	NM_006 006.4	1585-1685	TCCTGGATAGTTTGCGGCTGAGAATGCACTTACTGGCTCATTCAGCGGGTGCC AAAGCCTTTGTCTGTGATCAGTGCGGTGCACAGTTTTCGAAGGAGGA
Foxo1	NM_002 015.3	1526-1626	TCTCATCACCAACATCATTAACTGTTTCGACCCAGTCCTCACCTGGCACCATG ATGCAGCAGACGCCGTGCTACTCGTTTGCGCCACCAAACACCAGTTT
Foxo3	NM_001 455.2	1860-1960	CCGGAACGTGATGCTTCGCAATGATCCGATGATGTCCTTTGCTGCCCAGCCTA ACCAGGGAAGTTTGGTCAATCAGAACTTGCTCCACCACCAGCACCAA
ID3	NM_002 167.3	195-295	AGGAAGCCTGTTTGCAATTTAAGCGGGCTGTGAACGCCCAGGGCCGGCGGGG GCAGGGCCGAGGCGGGCCATTTTGAATAAAGAGGCGTGCCTTCCAGGC
ZEB2	NM_014 795.2	20-120	TCCCAGAGAGAAACTTGGCGATCACGTTTTCACATGATGCTCACGCTCAGGGC GCTTCAATTATCCCTCCCCACAAAGATAGGTGGCGCGTGTTTCAGGG
SMAD4	NM_005 359.3	1370-1470	AGGTTGCACATAGGCAAAGGTGTGCAGTTGGAATGTAAAGGTGAAGGTGATG TTTGGGTCAGGTGCCTTAGTGACCACGCGGTCTTTGTACAGAGTTACT
YAP	NM_139 118.2	755-855	ATGGGAGCTATGCAGCTGATTGAAGACTTCAGCACACATGTCAGCATTGACT GCAGCCCTCATAAAACTGTCAAGAAGACTGCCAATGAATTTCCCTGTT
E2A	NM_003 200.2	4325-4425	ATACGTGTCAACACAGCTGGCTGGATGATTGGGACTTTAAAACGACCCTCTTT CAGGTGGATTCAGAGACCTGTCCTGTATATAACAGCACTGTAGCAAT
Nanog	NM_024 865.2	1100-1200	CTACTCCATGAACATGCAACCTGAAGACGTGTGAAGATGAGTGAAACTGATA TTACTCAATTTCAGTCTGGACACTGGCTGAATCCTTCCTCTCCCCTCC
OCT4	NM_002 701.4	1225-1325	AAGTTCTTCATTCACTAAGGAAGGAATTGGGAACACAAAGGGTGGGGGCAGG GGAGTTTGGGGCAACTGGTTGGAGGGAAGGTGAAGTTCAATGATGCTC
Sox2	NM_003 106.2	151-251	CTTAAGCCTTTCCAAAAAATAATAATAACAATCATCGGCGGCGGCAGGATCG GCCAGAGGAGGAGGGAAGCGCTTTTTTTGATCCTGATTCCAGTTTGCC
TAL1	NM_003 189.2	4635-4735	ACAGCATCTGTAGTCAGCCGACAACTATTTCGGCCTTTTGGGGGTGGGTCTGG CCGTACTTGTGATTTCGATGGTACGTGACCCTCTGCTGAAGACTTGC
ELF1	NM_032 377.3	125-225	AGACCCAGTTCACCTGCCCCTTCTGCAACCACGAGAAATCCTGTGATGTGAAA ATGGACCGTGCCCGCAACACCGGAGTCATCTCTTGTACCGTGTGCCT
SOX13	NM_005 686.2	3039-3139	ATTTATTGAGTGCCCACTACGTGCCAGGCACTGTTGCTGAGTTCCTGTGGGTG TGTCTCTCGATGCCACTCCTGCTTCTCTGGGGGCCTCTTTCTGTGCT
Nrp1	NM_003 873.5	370-470	GCCTCGCTGCTTTCTTTTCTCCAAGACGGGCTGAGGATTGTACAGCTCTAGGC GGAGTTGGGGCTCTTCGGATCGCTTAGATTCTCCTCTTTGCTGCATT

Blk	NM_001715.2	990-1090	AGCTTCTTGCTCCAATCAACAAGGCCGGCTCCTTTCTTATCAGAGAGAGTGAA ACCAACAAAGGTGCCTTCTCCCTGTCTGTGAAGGATGTCACCACCCA
CCR10	NM_001296.3	1345-1445	GAACAGATGGGAACCAGCTCAATTGGGTGTCCACTCAAAGTGCTCTCTCCAG GGGCCTCAGTGACTGTGTTGCTAAACCCAGTGGTCAGTTCTCAGTTCT
ITGB7	NM_000889.1	1278-1378	CAACGTGGTACAGCTCATCATGGATGCTTATAATAGCCTGTCTTCCACCGTGA CCCTTGAACACTCTTCACTCCCTCCTGGGGTCCACATTTCTTACGAA
Sox5	NM_152989.2	1885-1985	TAGCCATGCAATGATGGATTTCAATCTGAGTGGAGATTCTGATGGAAGTGCTG GAGTCTCAGAGTCAAGAATTTATAGGGAATCCCGAGGGCGTGGTAGC
Bcl11b	NM_022898.1	3420-3520	GAGATGTAGCACTCATGTCGTCCCGAGTCAAGCGGCCTTTTCTGTGTTGATTT CGGCTTTCATATTACATAAGGGAAACCTTGAGTGGTGGTGCTGGGGG
SOX4	NM_003107.2	3040-3140	GTTCACGGTCAAACTGAAATGGATTTGCACGTTGGGGAGCTGGCGGCGGCGG CTGCTGGGCCTCCGCCTTCTTTTCTACGTGAAATCAGTGAGGTGAGAC
Tcf12	NM_207037.1	1105-1205	CACATGACCGCTTGAGTTATCCTCCACACTCAGTTTCACCAACAGACATAAAC ACGAGTCTTCCACCAATGTCCAGCTTTCATCGCGGCAGTACCAGCAG
Dapl 1	NM_001017920.2	190-290	CGAGAAAACAAGTGCCATTGCAAATGTTGCCAAAATACAGACACTGGATGCC CTGAATGACGCACTGGAGAAGCTCAACTATAAATTTCCAGCAACAGTG
Trf	NM_003218.3	1037-1137	CTGAAAGCAGAATACCTGTTTCAAAGAGTCAGCCGGTAACTCCTGAAAAACA TCGAGCTAGAAAAAGACAGGCATGGCTTTGGGAAGAAGACAAGAATTT
Cpt1	NM_020244.2	1303-1403	GATATGGTGATATACTTTAGTGCTTTGTGCCTGCAAATTTCAAGACACCTTCA TCTAAATATATTCAAGACTGCATGTCATCAAGCACCTGAACAGGTTC
Bim	NM_138621.4	257-357	CGGACTGAGAAACGCAAGAAAAAAAGACCAAATGGCAAAGCAACCTTCTGA TGTAAGTTCTGAGTGTGACCGAGAAGGTAGACAATTGCAGCCTGCGGAG
C-flip	NM_001127183.1	653-753	TAGAGTGCTGATGGCAGAGATTGGTGAGGATTTGGATAAATCTGATGTGTCCT CATTAATTTTCCTCATGAAGGATTACATGGGCCGAGGCAAGATAAGC

Appendix B. Antibodies Used in Dissertation

Antibody specificity	Clone	Vendor
Fc*	H10104	Invitrogen
anti-CD19scFv mAb**	136.20.1	Cooper Lab
ROR1	4A5	Kipps, TJ Lab (UCSD)
CD3	SK7	BD Biosciences
CD4	RPA-T4	BD Biosciences
CD8	RPA-T8	BD Biosciences
CD19	HIB19	BD Biosciences
CD25	M-A251	BD Biosciences
CD27	M-T271	BD Biosciences
CD28	L293	BD Biosciences
CD32	FLI8.26 (2003)	BD Biosciences
CD38	HB7	BD Biosciences
CD45RA	HI100	BD Biosciences
CD45RO	UCHL1	BD Biosciences
CD56	B159	BD Biosciences
CD57	NK-1	BD Biosciences
CD62L	Dreg 56	BD Biosciences

CD64	10.1	BD Biosciences
CD86	2331 FUN-1	BD Biosciences
CD95	DX2	BD Biosciences
CD122	TM-Beta 1	BD Biosciences
CD127	HIL-7R-M21	BD Biosciences
CD137	4B4-1	BD Biosciences
CD137L	C65-485	BD Biosciences
CCR7***	TG8	eBiosciences
CXCR4	12G5	BD Biosciences
CLA	HECA-452	BD Biosciences
CCR4	1G1	BD Biosciences
ICOS	ISA-3	eBiosciences
ICOS-L	MIH12	eBiosciences
OX40	ACT35	BD Biosciences
PD-1	MIH4	BD Biosciences
TCRαβ	WT31	BD Biosciences
TCRγδ	B1	BD Biosciences
TCRγδ	IMMU510	Thermo Fisher
TCRδ1	TS-1	Thermo/Pierce

TCRδ2	B6	BD Biosciences
TCRγ9	B3	BD Biosciences
invariant NKT	6B11	BD Biosciences
NMS	015-000-120	Jackson ImmunoResearch
DNAM1	DX11	BD Biosciences
NKG2D	1D11	BD Biosciences
IL15	34559	R&D Systems
IFNγ	4S.B3	BD Biosciences
TNFα	MAb11	BD Biosciences

* To detect CAR expression

** To detect CD19-specific CAR expression

*** Used at 1:67 dilution

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VITA

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