

8-2013

## T-CELL TREATMENTS FOR SOLID AND HEMATOLOGICAL TUMORS

Drew C. Deniger

Follow this and additional works at: [https://digitalcommons.library.tmc.edu/utgsbs\\_dissertations](https://digitalcommons.library.tmc.edu/utgsbs_dissertations)



Part of the [Cancer Biology Commons](#), [Immune System Diseases Commons](#), [Immunology and Infectious Disease Commons](#), and the [Medical Immunology Commons](#)

---

### Recommended Citation

Deniger, Drew C., "T-CELL TREATMENTS FOR SOLID AND HEMATOLOGICAL TUMORS" (2013). *The University of Texas MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences Dissertations and Theses (Open Access)*. 377.

[https://digitalcommons.library.tmc.edu/utgsbs\\_dissertations/377](https://digitalcommons.library.tmc.edu/utgsbs_dissertations/377)

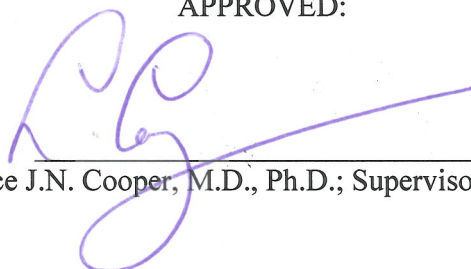
This Dissertation (PhD) is brought to you for free and open access by the The University of Texas MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences at DigitalCommons@TMC. It has been accepted for inclusion in The University of Texas MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences Dissertations and Theses (Open Access) by an authorized administrator of DigitalCommons@TMC. For more information, please contact [digitalcommons@library.tmc.edu](mailto:digitalcommons@library.tmc.edu).

T-CELL TREATMENTS FOR SOLID AND HEMATOLOGICAL TUMORS

by

Drew Caldwell Deniger, M.S.

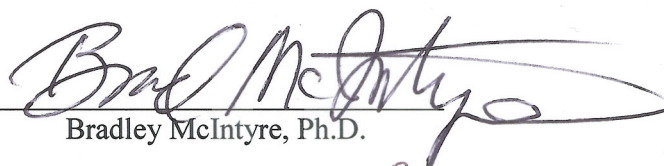
APPROVED:

A large, stylized signature in purple ink, appearing to be 'LJN Cooper', written over a horizontal line.

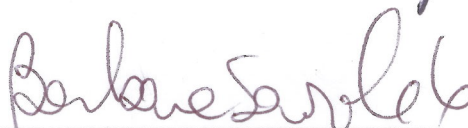
Laurence J.N. Cooper, M.D., Ph.D.; Supervisory Professor

A signature in black ink, appearing to be 'Vidya Gopalakrishnan', written over a horizontal line.

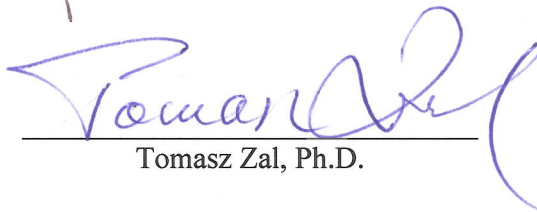
Vidya Gopalakrishnan, Ph.D.

A signature in black ink, appearing to be 'Brad McIntyre', written over a horizontal line.

Bradley McIntyre, Ph.D.

A signature in black ink, appearing to be 'Barbara Savoldo', written over a horizontal line.

Barbara Savoldo, M.D., Ph.D.

A signature in purple ink, appearing to be 'Tomasz Zal', written over a horizontal line.

Tomasz Zal, Ph.D.

\_\_\_\_\_  
Dean, The University of Texas  
Graduate School of Biomedical Sciences at Houston

T-CELL TREATMENTS FOR SOLID AND HEMATOLOGICAL TUMORS

A

DISSERTATION

Presented to the Faculty of  
The University of Texas  
Health Science Center at Houston  
and  
The University of Texas  
MD Anderson Cancer Center  
Graduate School of Biomedical Sciences  
in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

by

Drew Caldwell Deniger, M.S.

Houston, Texas

August 2013

## 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)





## ACKNOWLEDGEMENTS

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  $\gamma\delta$  T cell project, which is a major focus of this dissertation. The idea was that  $\gamma\delta$  T cells could kill malignant  $\alpha\beta$  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  $V\delta$  and  $V\gamma$  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 IL15R $\alpha$ , 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  $\gamma\delta$  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  $\gamma\delta$

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.

Members of my GSBS committees and other contributors have been invaluable to this process. Drs. Vidya Gopalakrishnan, Dean A Lee, Brad McIntyre, Prahlad Ram, Barbara Savoldo, Stephanie Watowich, and Tomasz Zal served on my advisory, examination, and/or supervisory committees and were instrumental in developing and challenging all ideas presented herein and in my academic development. They have also helped edit this document along with Dr. George McNamara, Dr. Spencer Stonier, Dr. Brian Friedrich, Dr. Maiti, Dr. Cooper, and Denise Crossland. Dr. Lee was kind to meet with many graduate students on Thursday night for writing advice, which was extremely useful, and was very helpful at lab meetings on Friday mornings. Dr. Thomas J Kipps was kind to give us the 4A5 monoclonal antibody specific for ROR1 and the corresponding genetic sequences in order to make the ROR1-specific CARs. Dr. William Wierda has pushed for ROR1-specific T cells for leukemia treatment, and provided primary CLL patient samples. Dr. Robert C. Bast, Jr. was gracious with his time and expertise in ovarian cancer as well as providing 12 ovarian cancer cell lines for

the Cooper Lab. The Immunology Program coordinator, Dr. Kimberly Schluns, also played a pivotal leadership role in guiding and preparing my immunology education. Bunny Perez in the GSBS Office Associate Dean for Academic Affairs answered many of my questions during my enrollment in both MS and PhD programs. I would also like to thank administration and Deans of the GSBS. In all, I have been surrounded by faculty who encouraged and enabled my professional and academic developments and I am extremely thankful to them.

Also in play were the core facilities at MD Anderson. The Immune Monitoring Core Lab (IMCL) performed and analyzed Luminex and ELISpot assays. Flow cytometry core lab assisted with FACS and mKate flow cytometry. STR fingerprinting was done by Center for General Cell Group System (CGCS) to corroborate cell line identity. I give my thanks to them for their expertise.

There have also been a number of funding agencies that have believed and supported many parts of the research in this dissertation. The GSBS Research Assistantship was awarded to me during my first 2 years of graduate school. Travel awards were generously provided by the UT-Houston GSBS, Immunology Program at UT-Houston GSBS, Society for Immunotherapy of Cancer (SITC), and American Society of Gene & Cell Therapy (ASGCT). An Excellence in Research Award was also granted by ASGCT at their 16<sup>th</sup> annual meeting. Scholarships in cancer research were given to support this dissertation by the American Legion Auxiliary (ALA) Foundation and Andrew Sowell-Wade Huggins and Cancer Answers Foundations. The Department of Defense Ovarian Cancer Research Program provided stipend and travel support in

the form of a Teal Pre-Doctoral Scholarship. This work could not have been completed without this generous support and I am forever grateful to you for your contributions.

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.** \_\_\_\_\_

**Drew Caldwell Deniger, M.S.**

**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  $\alpha\beta$  T cells (95% of T cells) and  $\gamma\delta$  T cells (<5% of T cells) express  $\alpha/\beta$  and  $\gamma/\delta$  TCR heterodimers, respectively.  $\gamma\delta$  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,  $\alpha\beta$  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 (CD3 $\zeta$ , CD28, CD137). A comparative study was done between  $\alpha\beta$  T cells re-directed with ROR1-specific CARs signaling through CD3 $\zeta$  and either CD28 (ROR1RCD28) or CD137 (ROR1RCD137) in the first specific aim of this dissertation. CAR<sup>+</sup> T cells proliferated to clinically significant numbers and ROR1<sup>+</sup> tumor cells were effectively targeted and killed by both ROR1-specific CAR<sup>+</sup> 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<sup>+</sup>  $\gamma\delta$  T cells with polyclonal TCR $\gamma\delta$  repertoire on CD19<sup>+</sup> artificial antigen presenting cells (aAPC). Enhanced cytotoxicity of CD19<sup>+</sup> leukemia was observed by CAR<sup>+</sup>  $\gamma\delta$  T cells compared to CAR<sup>neg</sup>  $\gamma\delta$  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  $\gamma\delta$  T cells expanded on aAPC without CAR<sup>+</sup> T cells, where V $\delta$ 1, V $\delta$ 2, and V $\delta$ 3 populations had naïve, effector memory, and central memory phenotypes and effector function strength in the following order: V $\delta$ 2>V $\delta$ 3>V $\delta$ 1. Polyclonal  $\gamma\delta$  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.

## TABLE OF CONTENTS

APPROVAL.....	i
TITLE PAGE.....	ii
DEDICATION.....	iii
ACKNOWLEDGEMENTS.....	iv
ABSTRACT.....	xii
TABLE OF CONTENTS.....	xiv
LIST OF FIGURES.....	xxii
LIST OF TABLES.....	xxv
ABBREVIATIONS.....	xxvi
CHAPTER I: INTRODUCTION.....	1
I.A. Cancer.....	1
I.A.1. Hematological Tumors.....	2
I.A.1.a. B-cell Acute Lymphoblastic Leukemia.....	2
I.A.1.b. T-cell Acute Lymphoblastic Leukemia.....	4
I.A.1.c. Chronic Lymphocytic Leukemia.....	4
I.A.2. Solid Tumors.....	5
I.A.2.a. Ovarian Cancer.....	6
I.A.2.b. Pancreatic Cancer.....	7
I.B. Tumor-associated Antigens.....	8
I.B.1. CD19.....	8
I.B.2. Receptor Tyrosine Kinase-like Orphan Receptor-1.....	9
I.C. T cell Immunity.....	13

I.C.1. $\alpha\beta$ T cells.....	14
I.C.1.a. T-cell Receptor Genetics.....	14
I.C.1.b. $\alpha\beta$ T cell Development .....	17
I.C.1.c. $\alpha\beta$ T cell Activation.....	17
I.C.1.d. $CD4^+$ $\alpha\beta$ T cell Subsets.....	19
I.C.1.e. $CD8^+$ $\alpha\beta$ T cell Subsets.....	20
I.C.2. $\gamma\delta$ T cells.....	24
I.C.2.a. Unique Characteristics of $\gamma\delta$ T cells.....	24
I.C.2.b. $V\delta 1$ $\gamma\delta$ T cells.....	25
I.C.2.c. $V\delta 2$ $\gamma\delta$ T cells.....	27
I.C.2.d. $V\delta 3$ $\gamma\delta$ T cells.....	28
I.D. Chimeric Antigen Receptors.....	28
I.D.1. Generation of CARs.....	29
I.D.2. Tumor-associated Antigens Targeted with CARs.....	31
I.D.3. Clinical Trials with $CAR^+$ T cells.....	33
I.E. <i>Ex vivo</i> Propagation of T cells.....	35
I.E.1. <i>Sleeping Beauty</i> Transposition-mediated Gene Transfer.....	35
I.E.2. Artificial Antigen Presenting Cells.....	39
I.E.2.a. Unique Features of K562 for Antigen Presentation.....	39
I.E.2.b. Established aAPC CellBanks and Clinical Trials with aAPC.....	40
I.F. Dissertation Specific Aims.....	41
I.F.1. Specific Aim#1: To evaluate whether ROR1-specific T cells can target ROR1+ tumor cells while sparing normal tissues.....	41

I.F.2. Specific Aim#2: To assess whether a CD19-specific CAR Expressed on $\gamma\delta$ T cells will render them bi-specific to tumors through their TCR and CAR.....	43
I.F.3. Specific Aim#3: To evaluate the inherent anti-tumor activity of aAPC-expanded $\gamma\delta$ T cells against solid and hematological cancers.....	44
CHAPTER II: Clinical Implications for ROR1-specific T cells.....	46
II.A. Hypothesis and Rationale.....	46
II.B. Introduction.....	46
II.C. Results.....	49
II.C.1. ROR1 Surface Expression on Tumor Cells.....	49
II.C.2. ROR1-specific CAR Plasmid Construction.....	52
II.C.3. Development of ROR1 <sup>+</sup> aAPC (clone#1).....	55
II.C.4. CAR <sup>+</sup> T-cell Expansion on Clone#1 aAPC.....	58
II.C.5. Immunophenotype of ROR1-specific T cells.....	63
II.C.5.a. T cell Immunophenotype of ROR1RCD28 and ROR1RCD137....	63
II.C.5.b. Memory Phenotype of ROR1-specific T cells.....	67
II.C.6. TCR Repertoire of ROR1-specific T cells.....	71
II.C.7. IFN $\gamma$ Production by CAR <sup>+</sup> T cells in Response to ROR1.....	73
II.C.7.a. TCR Stimulus with Leukocyte Activation Cocktail.....	73
II.C.7.b. Specific IFN $\gamma$ Production to ROR1 <sup>+</sup> Leukemia Cells.....	74
II.C.7.c. CAR <sup>+</sup> T cells Produce IFN $\gamma$ in Response to Primary ROR1 <sup>+</sup> Leukemia Cells but not Healthy ROR1 <sup>neg</sup> B cell LCL.....	74
II.C.8. ROR1-specific Cytotoxicity by CAR <sup>+</sup> T cells.....	77
II.C.8.a. CAR <sup>+</sup> T cells Lyse Leukemia but not Healthy B cells.....	77

II.C.8.b. ROR1-restricted Killing of Tumor Cell Lines.....	79
II.C.9. <i>In Vivo</i> Leukemia Clearance by ROR1-specific T cells.....	81
II.D. Discussion.....	84
II.D.1. Importance of Developing ROR1-specific T cells for Leukemia Patients.....	84
II.D.2. ROR1 as a Tumor Target and Safety Concerns in Immunotherapy.....	84
II.D.3. CD28 versus CD137 in CAR Design.....	86
II.D.4. Immediate Plans for ROR1-specific T cells in Leukemia Treatment.....	88
CHAPTER III: Bi-specific T cells Expressing Polyclonal Repertoire of Endogenous $\gamma\delta$ T-cell Receptors and Introduced CD19-specific Chimeric Antigen Receptor.....	89
III.A. Hypothesis and Rationale.....	89
III.B. Introduction.....	90
III.C. Results.....	92
III.C.1. CAR <sup>+</sup> $\gamma\delta$ T cells Numerically Expand on aAPC.....	92
III.C.2. Immunophenotype of Numerically Expanded CAR <sup>+</sup> $\gamma\delta$ T cells.....	96
III.C.3. Direct TCR Expression Assay to Reveal $\gamma$ and $\delta$ TCR Usage in CAR <sup>+</sup> $\gamma\delta$ T cells.....	99
III.C.4. T cells Produced Pro-inflammatory Cytokines in Response to Stimulation through Endogenous TCR $\gamma\delta$ and Introduced CAR.....	103
III.C.5. CAR <sup>+</sup> $\gamma\delta$ T cells Exhibit Enhanced Anti-tumor Effects against CD19 <sup>+</sup> Targets <i>in vitro</i> .....	105
III.C.6. CAR <sup>+</sup> $\gamma\delta$ T cells can Target CD19 <sup>+</sup> Tumor <i>in vivo</i> .....	107
III.D. Discussion.....	109

III.D.1. Polyclonal Bi-specific T cells for Immunotherapy.....	109
III.D.2. Changes Observed in V $\delta$ Populations Following Expansion on aAPC.....	110
III.D.3. Improvements upon CAR Expression on $\gamma\delta$ T cells.....	110
III.D.4. Improvements on Type of $\gamma\delta$ T cell used for CAR Immunotherapy....	114
III.D.5. Clinical Significance of Bi-specific T cells.....	115
CHAPTER IV: Artificial Antigen Presenting Cells Propagate Polyclonal Gamma Delta T cells with Broad Anti-tumor Activity.....	
IV.A. Hypothesis and Rationale.....	116
IV.B. Introduction.....	117
IV.C. Results.....	118
IV.C.1. Propagation of $\gamma\delta$ T cells on aAPC.....	118
IV.C.2. Roles for Co-stimulation and Cytokine Support in $\gamma\delta$ T cell Proliferation on aAPC.....	121
IV.C.3. UCB-derived $\gamma\delta$ T cells Expansion on aAPC.....	124
IV.C.4. Frequency of $\gamma$ and $\delta$ TCR Usage in aAPC-propagated $\gamma\delta$ T cells.....	126
IV.C.4.a. V $\delta$ and V $\gamma$ mRNA Expression.....	126
IV.C.4.b. TCR $\gamma\delta$ Surface Protein Expression.....	128
IV.C.4.c. Validation of V $\delta$ 3 Subset and V $\delta$ Lineage Propagation.....	130
IV.C.5. Immunophenotype of $\gamma\delta$ T cells Expanded on aAPC.....	133
IV.C.5.a. Immunophenotype of Polyclonal $\gamma\delta$ T cell Population.....	133
IV.C.5.b. Immunophenotype of V $\delta$ 1, V $\delta$ 2, and V $\delta$ 3 Subsets.....	136

IV.C.6. Polyclonal $\gamma\delta$ T cells Secrete Pro-inflammatory Cytokines and Chemokines.....	139
IV.C.7 TCR $\gamma\delta$ Involvement in V $\delta$ 1, V $\delta$ 2, and V $\delta$ 3 Production of IFN $\gamma$ .....	141
IV.C.8. Broad Anti-tumor Cytolysis by Polyclonal $\gamma\delta$ T cells.....	143
IV.C.8.a. Polyclonal $\gamma\delta$ T cells Lyse Hematological Tumors.....	143
IV.C.8.b. Polyclonal $\gamma\delta$ T cells Lyse Solid Tumors.....	145
IV.C.8.c. Mechanism of Tumor Cytolysis by $\gamma\delta$ T cells was Multi-factorial.....	147
IV.C.8.d. Importance for TCR $\delta$ in $\gamma\delta$ T cell Cytolysis.....	150
IV.C.9. Clearance of Established Tumor Xenografts by Polyclonal $\gamma\delta$ T cells.....	154
IV.D. Discussion.....	156
IV.D.1. Importance of Polyclonal $\gamma\delta$ T cells for Immunotherapy.....	156
IV.D.2. Potential Ligands for TCR $\gamma\delta$ on aAPC.....	157
IV.D.3. Co-stimulation in Polyclonal $\gamma\delta$ T cell Expansion.....	158
IV.D.4. Polyclonal $\gamma\delta$ T cells Apparently Lack Allogeneic Responses to Healthy Tissue.....	159
IV.D.5. Application of Polyclonal $\gamma\delta$ T cells for Immunotherapy.....	162
CHAPTER V: GENERAL DISCUSSION AND FUTURE DIRECTIONS.....	163
V.A. Dissertation Summary.....	163
V.B. Combinational Cellular Immunotherapies.....	164
V.C. Generation of IL17-producing T cells for Adoptive Immunotherapy.....	166
V.D. Importance of Polyclonal $\gamma\delta$ T cells to Immunology.....	167



V.E. Potential Benefits and Issues with Cellular Immunotherapy.....	168
V.F. Clinical Applications of Dissertation Immunotherapies.....	171
CHAPTER VI: Materials and Methods.....	173
VI.A. DNA Plasmids and Construct Cloning.....	173
VI.A.1. Tumor Antigens.....	173
VI.A.1.a. ROR1.....	173
VI.A.1.b. CD19.....	174
VI.A.2. Co-stimulatory Molecules.....	175
VI.A.2.a. CD86 and CD137L.....	175
VI.A.2.b. IL15-IL15R $\alpha$ Fusion Construct.....	175
VI.A.3. Chimeric Antigen Receptors.....	175
VI.B. Cell Culture.....	177
VI.B.1. Established Tumor Cell Lines.....	178
VI.B.2. Genetic Modification of Cell Lines.....	179
VI.B.2.a. ROR1 aAPC (clone#1).....	179
VI.B.2.b. HLA <sup>-/-</sup> aAPC.....	179
VI.B.2.c. Lenitviral Packaging and Gene Transduction.....	180
VI.B.3. Primary Tumor Cells.....	183
VI.B.4. Lymphocyte Cultures.....	183
VI.B.4.a. CAR <sup>neg</sup> $\alpha\beta$ T cells.....	183
VI.B.4.b. CAR <sup>+</sup> $\alpha\beta$ T cells.....	184
VI.B.4.c. CAR <sup>+</sup> $\gamma\delta$ T cells.....	185
VI.B.4.d. Polyclonal $\gamma\delta$ T cells.....	186

VI.B.4.e. NK cells.....	187
VI.B.4.f. $\gamma\delta$ T cell Proliferation in Hypoxia.....	187
VI.B.5 $\gamma\delta$ T cell Co-culture Deconstruction.....	187
VI.B.5.a. Effects of Cytokines on $\gamma\delta$ T cell Proliferation.....	188
VI.B.5.b. Effects of Co-Stimulation on $\gamma\delta$ T cell Proliferation.....	188
VI.C. Multiplex Gene Expression Analysis.....	188
VI.D. Immunostaining.....	189
VI.E. Cytokine Production.....	190
VI.F. <i>In Vitro</i> Killing Assays.....	191
VI.F.1. Chromium Release Assay.....	191
VI.F.2. Long-term Killing Assay.....	191
VI.G. Mixed Lymphocyte Reactions.....	192
VI.H. <i>In Vivo</i> Anti-tumor Activity.....	193
VI.H.1. ROR1-specific Anti-leukemia Effects.....	193
VI.H.2. CD19-specific Anti-leukemia Activity.....	194
VI.H.3. $\gamma\delta$ T cells Clearance of Ovarian Cancer.....	194
APPENDICES.....	195
APPENDIX A. Lymphocyte CodeSet Array.....	195
APPENDIX B. Antibodies Used in Dissertation.....	217
BIBLIOGRAPHY.....	220
VITA.....	278

## LIST OF FIGURES

Figure 1. ROR1 Protein Structure.....	12
Figure 2. Genetic Loci for TCR alleles .....	16
Figure 3. CD8 <sup>+</sup> Memory T cell subsets.....	23
Figure 4. Schematic Representation of CARs .....	30
Figure 5. Schematic of CAR <sup>+</sup> T cells Expansion on aAPC .....	38
Figure 6. Surface Expression of ROR1 on Tumors .....	51
Figure 7. ROR1-specific CAR Transposons .....	54
Figure 8. Surface Phenotype of Clone#1 aAPC Used for ROR1-specific T cell Expansion.....	56
Figure 9. CAR Expression in T cells Before and After Expansion on Clone#1 aAPC.....	60
Figure 10. rROR1 Antigen Binding by ROR1-specific T cells.....	61
Figure 11. Sustained Proliferation of CAR <sup>+</sup> T cells .....	62
Figure 12. Basic Immunophenotype of CAR <sup>+</sup> T cells.....	65
Figure 13. Memory Markers on CAR <sup>+</sup> T cell Surfaces.....	69
Figure 14. TCR $\alpha$ and TCR $\beta$ Expression in ROR1RCD28 and ROR1RCD137 cells....	72
Figure 15. IFN $\gamma$ Production by ROR1-specific T cells in Response to ROR1 <sup>+</sup> targets..	76
Figure 16. Specific Cytolysis of Primary ROR1 <sup>+</sup> B-cell CLL by CAR <sup>+</sup> T cells.....	78
Figure 17. Specific Cytolysis of Established ROR1 <sup>+</sup> Tumor Cell Lines by CAR <sup>+</sup> T cells.....	80
Figure 18. <i>In vivo</i> Tumor Clearance by ROR1-specific CAR <sup>+</sup> T cells.....	83

Figure 19. CAR <sup>+</sup> $\gamma\delta$ T cells Propagate on Designer aAPC.....	95
Figure 20. Immunophenotype of Electroporated, Separated, and Propagated CAR <sup>+</sup> $\gamma\delta$ T cells.....	98
Figure 21. Distribution of V $\delta$ and V $\gamma$ in $\gamma\delta$ T cells Expanded on Aminobisphosphonate.....	101
Figure 22. Distribution of V $\delta$ and V $\gamma$ in CAR <sup>+</sup> $\gamma\delta$ T cells.....	102
Figure 23. Bi-specific $\gamma\delta$ T cells Produce Pro-inflammatory Cytokines when Endogenous TCR and Introduced CAR are Stimulated.....	104
Figure 24. Specific Lysis of CD19 <sup>+</sup> Tumor Cell Lines by CAR <sup>+</sup> $\gamma\delta$ T cells.....	106
Figure 25. <i>In vivo</i> Anti-tumor Activity of CAR <sup>+</sup> $\gamma\delta$ T cells.....	108
Figure 26. Specific Lysis of CD19 <sup>+</sup> Tumor Cell Lines by CAR <sup>+</sup> , CAR <sup>++</sup> , and CAR <sup>+++</sup> $\gamma\delta$ T cells.....	113
Figure 27. Sustained Proliferation of $\gamma\delta$ T cells on aAPC and IL2/21.....	120
Figure 28. Co-stimulation and Cytokine Requirements for $\gamma\delta$ T cell Expansion on aAPC <i>ex vivo</i> .....	123
Figure 29. Expansion of UCB-derived $\gamma\delta$ T cells on aAPC.....	125
Figure 30. Pattern of V $\delta$ and V $\gamma$ mRNA Usage on aAPC-expanded $\gamma\delta$ T cells.....	127
Figure 31. TCR $\delta$ and TCR $\gamma$ Isotype Surface Expression on aAPC-expanded $\gamma\delta$ T cells.....	129
Figure 32. V $\delta$ Subset Separation, Propagation, and Resultant TCR Expression on Sorted T cells.....	132

Figure 33. Immunophenotype of Polyclonal $\gamma\delta$ T cells Propagated on aAPC, IL2, and IL21.....	135
Figure 34. Immunophenotype of V $\delta$ Lineages Propagated on aAPC, IL2, and IL21...	138
Figure 35. Cytokines and Chemokines Secreted by Polyclonal $\gamma\delta$ T cells.....	140
Figure 36. TCR $\gamma\delta$ -specific IFN $\gamma$ Production by V $\delta$ 1, V $\delta$ 2, and V $\delta$ 3 Subsets.....	142
Figure 37. <i>In vitro</i> Cytolysis of Hematological Tumor Cells by $\gamma\delta$ T cells.....	144
Figure 38. <i>In vitro</i> Cytolysis of Solid Tumor Cells by $\gamma\delta$ T cells.....	146
Figure 39. Neutralization of Polyclonal $\gamma\delta$ T cell Cytolysis.....	149
Figure 40. $\gamma\delta$ T cell Subset Acute Cytolysis.....	152
Figure 41. $\gamma\delta$ T cell Subset Long-term Killing.....	153
Figure 42. <i>In vivo</i> Tumor Clearance by Polyclonal $\gamma\delta$ T cells.....	155
Figure 43. Absence of Allogeneic Responses by Polyclonal $\gamma\delta$ T cells to Partially Mismatched Donors' Healthy Cells.....	161
Figure 44. Proliferation of $\gamma\delta$ T cells in Hypoxia Compared to Normoxia.....	170
Figure 45. DNA Plasmid Map for pLVU3G-effLuc-T2A-mKateS158A.....	182

## LIST OF TABLES

Appendix A. Lymphocyte CodeSet Array.....	208
Appendix B. Antibodies Used in Dissertation.....	230

## ABBREVIATIONS

aAPC: artificial Antigen Presenting Cell

$\alpha$ FR:  $\alpha$ -Folate Receptor

$\alpha$ GalCer:  $\alpha$ -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 CD3 $\zeta$  endodomains

CD19RCD137: CD19-specific CAR with CD137 and CD3 $\zeta$  endodomains

CDR: Complementarity Determining Regions

CEA: Carcinoembryonic Antigen

cGMP: current Good Manufacturing Practices

CLA: Cutaneous Lymphocyte Antigen

CK1 $\epsilon$ : Casein Kinase-1 $\epsilon$

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

IFN $\gamma$ : Interferon- $\gamma$

Ig: Immunoglobulin

IL: Interleukin

IL15/IL15R $\alpha$ : fusion protein of IL15 to IL15R $\alpha$

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

MIP1 $\alpha$ : Macrophage Inflammatory Protein-1 $\alpha$

MIP1 $\beta$ : Macrophage Inflammatory Protein-1 $\beta$

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 CD3 $\zeta$  endodomains

ROR1RCD137: ROR1-specific CAR with CD137 and CD3 $\zeta$  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<sub>C</sub>: Cytotoxic T cell

T<sub>H</sub>: Helper T cell

T<sub>H</sub>1: Type 1 CD4<sup>+</sup> Helper T cell

T<sub>H</sub>2: Type 2 CD4<sup>+</sup> Helper T cell

T<sub>H</sub>17: Type 17 CD4<sup>+</sup> Helper T cell

T<sub>REG</sub>: Regulatory CD4<sup>+</sup> Helper T cell

T<sub>CM</sub>: Central memory T cell

T<sub>EFF</sub>: Effector T cell

T<sub>EM</sub>: Effector memory T cell

T<sub>EMRA</sub>: Effector memory RA T cell

T<sub>M</sub>: Memory T cell

T<sub>N</sub>: Naïve T cell

TAA: Tumor-associated antigen

TCR: T-cell Receptor

TGF $\beta$ : Transforming Growth Factor- $\beta$

TIL: Tumor-infiltrating Lymphocytes

TFN $\alpha$ : Tumor Necrosis Factor- $\alpha$

UCB: Umbilical Cord Blood

UCSD: University of California at San Diego

UPenn: University of Pennsylvania

V $\delta$ 1:  $\gamma\delta$  T cell expressing TCR $\delta$ 1 isoform

V $\delta$ 2:  $\gamma\delta$  T cell expressing TCR $\delta$ 2 isoform

V $\delta$ 3:  $\gamma\delta$  T cell expressing TCR $\delta$ 3 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<sub>H</sub>) 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  $\beta_2$ -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

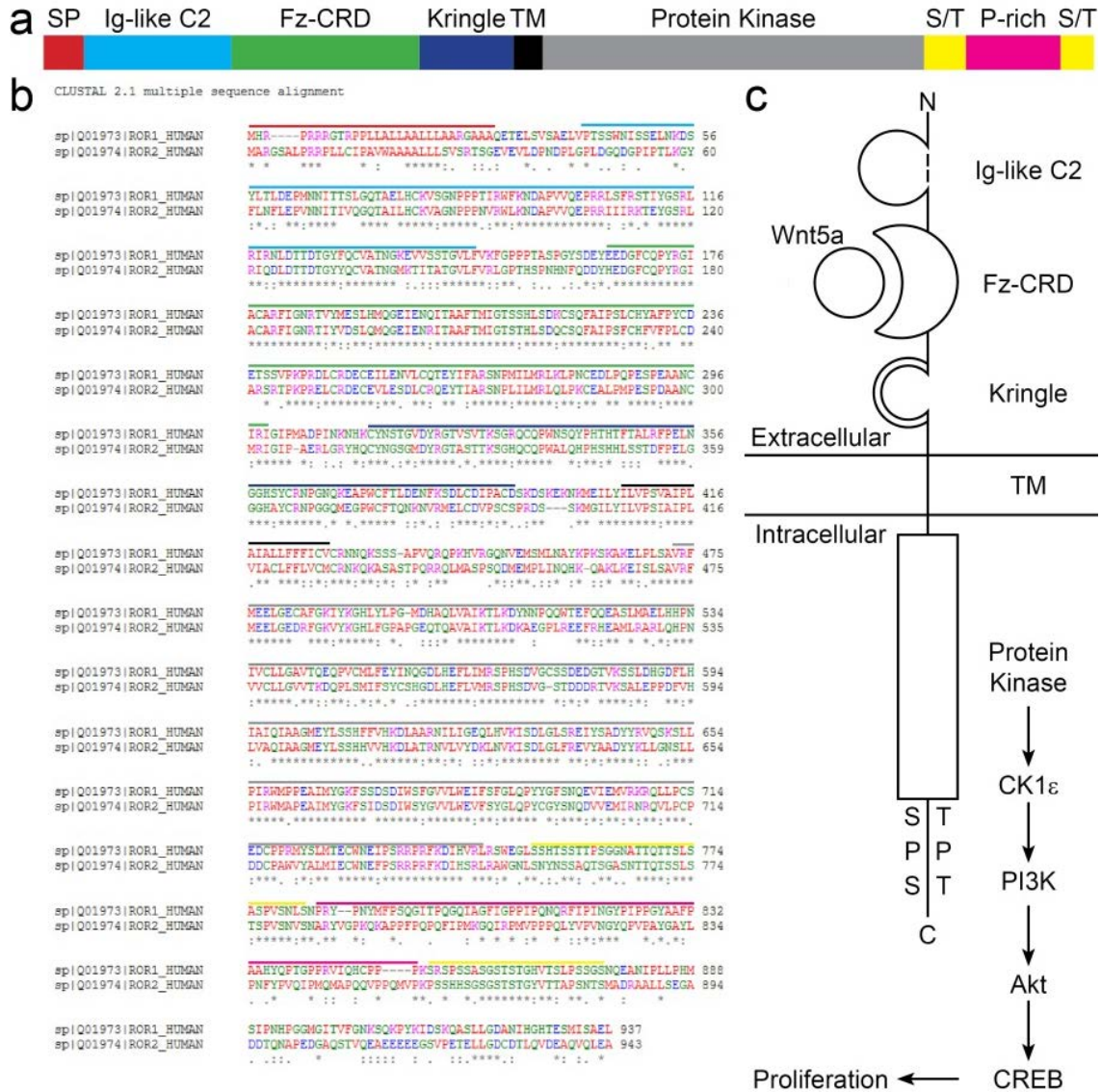
that of the BCR that are secreted into the circulation for opsonization and pathogen clearance.(59) The BCR complex is crucial for signal transduction, and is composed of CD19, CD21, and CD81, where CD19 is crucial for intracellular signaling.(60-62) CD19 is expressed from the early pro-B cell stage 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<sup>+</sup> 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<sup>-/-</sup> mice died of respiratory distress following birth, while ROR2<sup>-/-</sup> mice died of more advanced cardiovascular problems as well as skeletal abnormalities, and ROR1<sup>-/-</sup>ROR2<sup>-/-</sup> 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-1 $\epsilon$  (CK1 $\epsilon$ ) 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.

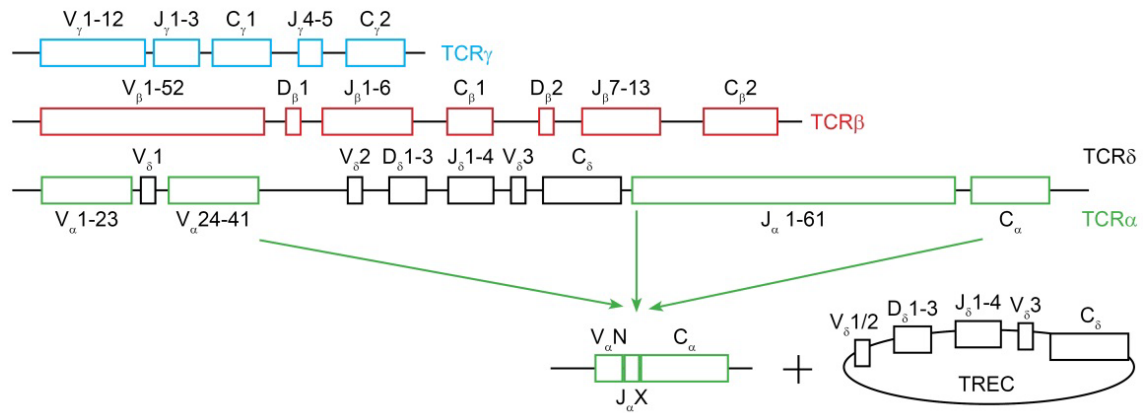
### I.C.1. $\alpha\beta$ T cells

The quintessential T cell lineage is the  $\alpha\beta$  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  $\alpha\beta$  T-cell receptor (TCR $\alpha\beta$ ). Effector functions are endowed upon  $\alpha\beta$  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 cytotoxicity (CD8). Therefore, it is important to understand the nuances of  $\alpha\beta$  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. TCR $\alpha$ , TCR $\beta$ , TCR $\gamma$ , and TCR $\delta$  exist in the human genome, which lead to two distinct T cell lineages based on TCR pairing.(92) More specifically, the  $\alpha\beta$  T cell lineage is defined by the pairing of TCR $\alpha$  and TCR $\beta$  chains whereas the  $\gamma\delta$  T cell lineage is defined by T cells expressing

TCR $\gamma$  and TCR $\delta$  heterodimers. Each TCR allele is further compartmentalized into variable (V), diversity (D), junction (J), and constant (C) regions.(93) TCR $\alpha$  and TCR $\gamma$  genes have V and J regions while TCR $\beta$  and TCR $\delta$  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. V $\alpha$  describes the variable region from the alpha locus or J $\delta$  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 TCR $\gamma$  (Gene ID: 6965) and TCR $\beta$  (Gene ID: 6957) loci are in distinct locations at 7p14 and 7q34, respectively, but TCR $\delta$  locus (Gene ID: 6964) exists within the TCR $\alpha$  (Gene ID: 6955) locus at 14q11.2 (**Figure 2**). Upon V(D)J recombination of the V $\alpha$  and J $\alpha$ , the entire  $\delta$ -chain locus is deleted from the T cell genome in a T-cell receptor excision circle (TREC).(97) Thus, once the  $\alpha$ -chain locus has recombined for a particular T cell, it can no longer become a  $\gamma\delta$  T cell. Programmed mutation of the T cell germline DNA allows for unbiased generation of many TCR specificities for extremely high combinatorial probabilities (at least  $10^{16}$  possible combinations for  $\alpha\beta$  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  $\beta$  and  $\delta$  chains for  $TCR\gamma$  (blue),  $TCR\beta$  (red),  $TCR\alpha$  (green), and  $TCR\delta$  (black). V(D)J recombination of  $V\alpha$ ,  $J\alpha$ , and  $C\alpha$  results in excision of the  $TCR\delta$  locus in a T cell Receptor Excision Circle (TREC).

*I.C.1.b.  $\alpha\beta$  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 TCR $\alpha\beta$  binding affinity to either MHC-I or MHC-II and subsequent TCR $\alpha\beta$  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.  $\alpha\beta$  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 TCR $\alpha\beta$  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 CD3 $\gamma$ /CD3 $\epsilon$  and CD3 $\delta$ /CD3 $\epsilon$  heterodimers and CD3 $\zeta$ /CD3 $\zeta$  homodimer. Each of the CD3 $\gamma$ , CD3 $\delta$ , and CD3 $\epsilon$  subunits has an immunoreceptor tyrosine activation domain (ITAM) motif and the CD3 $\zeta$  subunit has three ITAM motifs for a total of ten ITAMs surrounding each TCR. Upon TCR $\alpha\beta$  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) TCR $\alpha\beta$  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<sup>+</sup> $\alpha\beta$ T cell Subsets*

CD4<sup>+</sup> T cell subsets are numerous and typically described by the effector cytokines they release, and they can be stratified into T<sub>H</sub>0 (naïve), T<sub>H</sub>1, T<sub>H</sub>2, T<sub>H</sub>17, regulatory T cells (T<sub>REG</sub>), and natural killer T (NKT) cells.(124) Naïve T<sub>H</sub>0 cells can be polarized to differentiate based on environmental cues that then translate into distinct transcriptional programs and result in lineage commitment.(125) T<sub>H</sub>1 encourage inflammation and help promote CD8 memory responses by producing IL2, IL12, interferon- $\gamma$  (IFN $\gamma$ ), and tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) while T<sub>H</sub>2 cells inhibit inflammatory T<sub>C</sub> response and foster humoral immunity by secreting IL4, IL5, IL6, and IL10.(126) The primary role of T<sub>H</sub>17 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<sub>H</sub>17 cells and T<sub>REG</sub> cells as both require transforming growth factor- $\beta$  (TGF $\beta$ ) 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 TGF $\beta$ .(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 TCR $\alpha\beta$  alleles, e.g. V $\alpha$ 24/J $\alpha$ 18 with V $\beta$ 11, and are known to produce both  $T_H1$  and  $T_H2$  cytokines.(129) The best described antigen for NKT cells is  $\alpha$ -galactosylceramide ( $\alpha$ GalCer) 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.

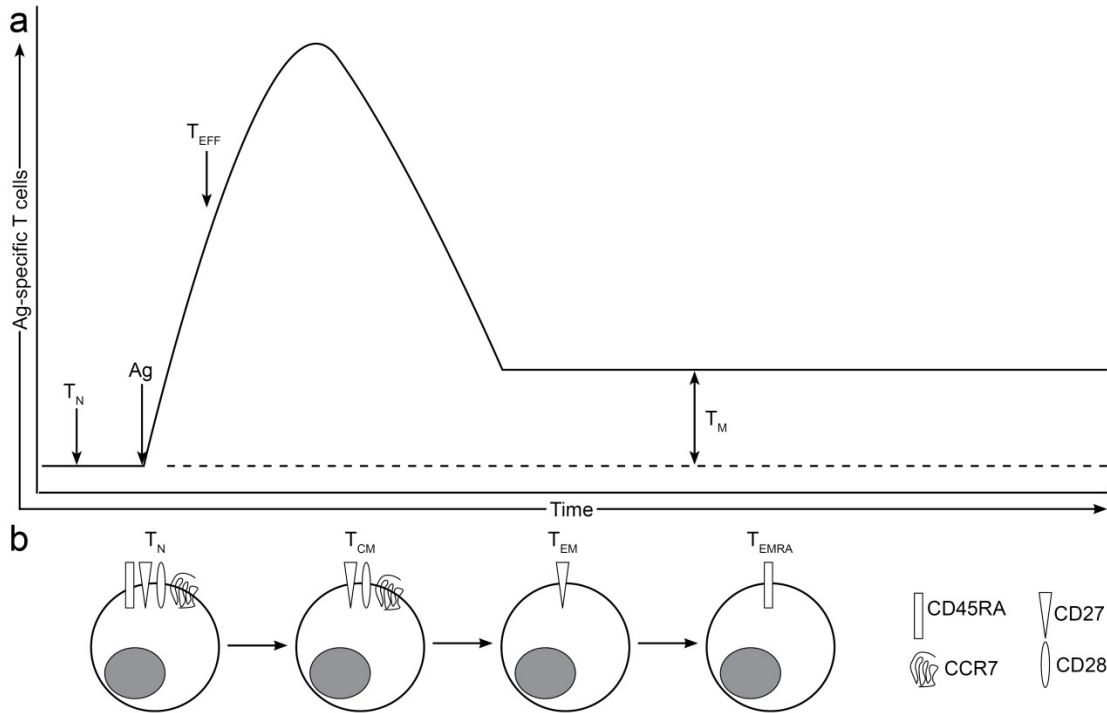
#### *I.C.1.e. $CD8^+$ $\alpha\beta$ T cell Subsets*

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 ( $T_{\text{EFF}}$ ). 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  $T_{\text{EFF}}$  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 ( $T_{\text{CM}}$ ), effector memory ( $T_{\text{EM}}$ ), and effector memory RA ( $T_{\text{EMRA}}$ ) T cells.(135)  $T_{\text{N}}$  express CD45RA, CD27, CD28, and CCR7 where CD45RA expression is lost on both  $T_{\text{CM}}$  and  $T_{\text{EM}}$  but is re-expressed on  $T_{\text{EMRA}}$  without CD27, CD28, and CCR7. The  $T_{\text{EM}}$  and  $T_{\text{CM}}$  groups can be distinguished by CD28 and CCR7 where the former expresses neither and the latter expresses both.  $T_{\text{CM}}$  cells have the greatest proliferative capacity with limited effector functions and serve as long-lasting antigen-specific pools. In contrast,  $T_{\text{EM}}$  have immediate effector functions, limited replicative capacity relative to  $T_{\text{CM}}$ , and serve as the main memory cytotoxicity mediators.(136) Lastly,  $T_{\text{EMRA}}$  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<sup>+</sup> 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. $\gamma\delta$ T cells

$\gamma\delta$  T cells are a completely separate T cell lineage from  $\alpha\beta$  T cells, and  $\gamma\delta$  T cells have both innate and adaptive immune cell functions.(140) In contrast to  $\alpha\beta$  T cells,  $\gamma\delta$  T cells have predictable inherent anti-tumor immunity mediated directly through their TCR.(141) However,  $\gamma\delta$  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  $\gamma\delta$  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 V $\delta$ 1, V $\delta$ 2, and V $\delta$ 3 subsets based on TCR $\gamma\delta$  alleles.(144) Targets of  $\gamma\delta$  T cells include tumor cells, viruses, bacteria, mycobacteria, and cell stress-associated proteins.(145, 146) Therefore,  $\gamma\delta$  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 $\gamma\delta$ T cells*

There are three variable TCR $\delta$  chains and 14 variable TCR $\gamma$  chains expressed in humans, and fewer unique TCR $\gamma\delta$  combinations are observed in  $\gamma\delta$  T cells compared to the immense combinatorial diversity seen with  $\alpha\beta$  T cells following V(D)J recombination.(92, 144) Expression of TCR $\gamma\delta$  heterodimers on the T cell surface in the thymus inhibits recombination of  $\beta$ -chain locus during the  $CD4^{neg}CD8^{neg}$  stage thereby committing the T cell to the  $\gamma\delta$  T cell lineage.(147) This double negative status is often

maintained after exit from the thymus, most likely because TCR $\gamma\delta$  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  $\gamma\delta$  T cell development, as many of these  $\gamma\delta$  T cells take up residence in peripheral tissues and exhibit immediate effector functions against pathogens.(149) Resident  $\gamma\delta$  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  $\gamma\delta$  T cells can be found in the blood and lymphoid organs, and are canonically dominated by  $\gamma\delta$  T cells expressing V $\delta$ 2 TCR isotype (called V $\delta$ 2 cells) with few  $\gamma\delta$  T cells expressing the V $\delta$ 1 TCR isotype (called V $\delta$ 1 cells) that are more frequently associated with resident  $\gamma\delta$  T cells.(146) Moreover, V $\delta$ 2 cells most commonly pair with V $\gamma$ 9, but V $\delta$ 1 and V $\delta$ 3 have broad  $\gamma$ -chain pairing potential.(141, 146) Therefore, the location of  $\gamma\delta$  T cells can lead to their subset diversity and effector functions that can be mediated through specific combinations of  $\gamma$  and  $\delta$  TCR chains to recognize pathogens upon encounter in their resident or circulating locations.

#### *I.C.2.b. V $\delta$ 1 $\gamma\delta$ T cells*

V $\delta$ 1 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 TCR $\gamma$  chains, and there are a variety of known ligands for V $\delta$ 1 cells.(140) In fact, the crystal structure

of V $\gamma$ 1V $\delta$ 1 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  $\gamma\delta$  T cells, NK cells, and, less frequently,  $\alpha\beta$  T cells.(155) Other non-classical MHC molecules and cell stress proteins are also recognized by  $\gamma\delta$  T cells. For instance, V $\gamma$ 4V $\delta$ 1 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  $\alpha\beta$  T cells, but  $\gamma\delta$  T cells have also been described to have direct NKT-like functions, enhance NKT  $\alpha\beta$  T cells reactivity to  $\alpha$ GalCer, and have even been shown to have specificity to cardiolipin with CD1d.(158-160) Also, murine V $\gamma$ 5V $\delta$ 1 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 V $\delta$ 1 T cells to have immunity towards cytomegalovirus (CMV) and human immunodeficiency virus (HIV).(164, 165) In aggregate, V $\delta$ 1 cells have immunity towards microbial pathogens, have antigen presenting capabilities, and can target proteins expressed on the tumor surface.

### *I.C.2.c. V $\delta$ 2 $\gamma\delta$ T cells*

The most extensively studied subset of  $\gamma\delta$  T cells is the V $\delta$ 2 lineage, which similar to V $\delta$ 1 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 V $\delta$ 2 cells when paired with V $\gamma$ 2.(167-169) In contrast to V $\delta$ 1, a strong preference towards V $\delta$ 2 heterodimerizing with V $\gamma$ 9 has been well documented. V $\gamma$ 9V $\delta$ 2 cells have been shown to react to phospho-antigens (isopentenyl pyrophosphate; IPP), F<sub>1</sub>-ATPase expressed on the cell surface, and *Mycobacterium tuberculosis*.(170-172) Furthermore, V $\gamma$ 9V $\delta$ 2 cells are reactive to cells treated with aminobisphosphonates, e.g. Zoledronic Acid (Zol), which is the only current means of propagating  $\gamma\delta$  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 V $\gamma$ 9V $\delta$ 2.(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 V $\gamma$ 9V $\delta$ 2 T cells, and aminobisphosphonates methods were subsequently translated into laboratory practice to expand V $\gamma$ 9V $\delta$ 2 cells *ex vivo*.(176) Thus, V $\delta$ 2 cells are the *only*  $\gamma\delta$  T cells that have been used for adoptive T cell therapy. Utility of the V $\delta$ 1 and V $\delta$ 3 lineages is appealing, but there are no current means to rapidly expand them to clinically-significant numbers and the existing polyclonal  $\gamma\delta$  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* V $\gamma$ 9V $\delta$ 2 expansions and/or (ii) infusions of

*ex vivo* expanded V $\gamma$ 9V $\delta$ 2 cells have generated objective clinical responses but complete responses have been unpredictable and have not always been directly correlated to the V $\gamma$ 9V $\delta$ 2 cells.(177-182) Thus, the extensive work studying V $\delta$ 2 cells has generated much interest in using  $\gamma\delta$  T cells for adoptive immunotherapy.

#### *I.C.2.d. V $\delta$ 3 $\gamma\delta$ T cells*

In contrast to V $\delta$ 1 and V $\delta$ 2 cells, very little is known about  $\gamma\delta$  T cells expressing V $\delta$ 3 TCR alleles (called V $\delta$ 3 cells). The limited quantities in peripheral blood and lack of commercially available reagents for V $\delta$ 3 inhibit attempts to study this subset. V $\delta$ 3 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**

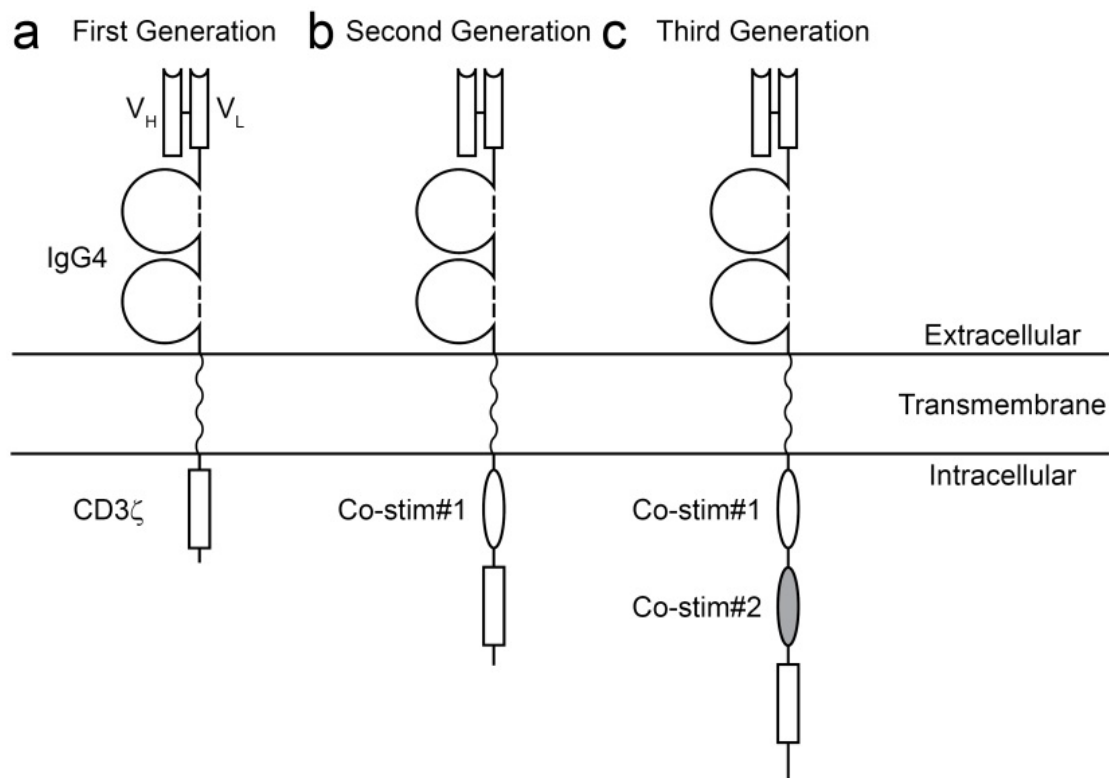
Chimeric Antigen Receptors (CARs) re-direct T cells to antigens independent of their endogenous TCR specificity.(184, 185) These recombinant molecules contain in order from N-terminus to C-terminus: (i) a single chain variable fragment (scFv) derived from a monoclonal antibody with specificity to a TAA, (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<sup>+</sup> 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 CD3 $\zeta$  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 CD3 $\zeta$  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 CD3 $\zeta$ .(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 CD3 $\zeta$  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), CD8 $\alpha$ , 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<sub>H</sub>) and light (V<sub>L</sub>) 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,  $\kappa$ -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<sup>+</sup> 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,  $\alpha$ -Folate Receptor ( $\alpha$ FR), 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<sup>+</sup> 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- $\alpha$ -2 (IL13R $\alpha$ 2) 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> T cells signaling through CD137 and CD3 $\zeta$ .(4, 7, 32) The exact reason why these cells out-performed other CARs signaling through CD28 and CD3 $\zeta$  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<sup>+</sup> 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<sup>+</sup> T cells. In this study, a third generation CAR (CD28, CD137, and CD3 $\zeta$ ) specific for HER2 (based on the monoclonal antibody trastuzumab) was used to treat breast cancer, and following infusion of 10<sup>10</sup> 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<sup>+</sup> 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<sup>+</sup> 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  $\alpha$ FR 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 IL13R $\alpha$ 2, 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<sup>+</sup> 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.

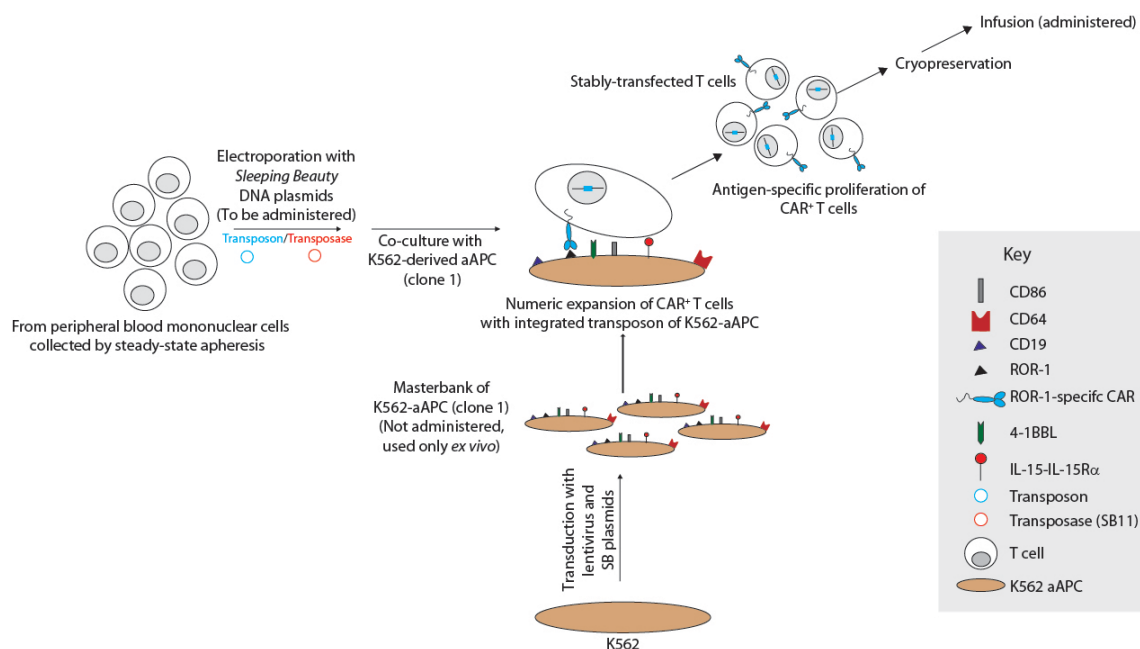
#### **I.E.1. *Sleeping Beauty* Transposition-mediated Gene Transfer**

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  $\gamma$ -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  $\gamma$ -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<sup>+</sup> 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/IL15R $\alpha$ . Following a month of co-culture on aAPC, stable CAR expression is achieved and clinically-relevant numbers of CAR<sup>+</sup> 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- $\alpha$

(IL15/IL15R $\alpha$ ) 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<sup>-/-</sup> K562 cells (Torikai H, Cooper LNJ, 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,  $\gamma$ -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 Pennsylvania (UPenn) was used successfully to expand CD19-specific CAR<sup>+</sup> 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.

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

ROR1-specific CARs will re-direct the specificity of T cells to target ROR1<sup>+</sup> 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<sup>+</sup> malignancies, and (iv) cancer patients treated with CAR<sup>+</sup> T cells have achieved complete responses.

*Sub-Aim 1.1. To generate ROR1-specific CAR<sup>+</sup> T cells.* Sequences from 4A5 antibody hybridoma will be constructed into second generation ROR1-specific CARs signaling through (i) CD28 and CD3 $\zeta$  (ROR1RCD28) or (ii) CD137 and CD3 $\zeta$  (ROR1RCD137), which will be part of SB transposons for stable CAR expression in T cells. CAR<sup>+</sup> T cells will be propagated on  $\gamma$ -irradiated ROR1<sup>+</sup> aAPC (clone#1), and CAR<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> T cell function is specific for ROR1.* Cytokine production and 4-hour chromium release assay (CRA) will be used to evaluate CAR<sup>+</sup> specificity in responding to ROR1<sup>+</sup> targets with ROR1<sup>neg</sup> targets as negative controls. ROR1<sup>+</sup> leukemia xenografts will be established in immunocompromised mice which will be treated with CAR<sup>+</sup> T cells to evaluate tumor clearance *in vivo*.

I.F.2. Specific Aim#2: To assess whether a CD19-specific CAR expressed on  $\gamma\delta$  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  $\gamma\delta$  T cells would stimulate them independent of their TCR $\gamma\delta$ , thus leading to expansion of  $\gamma\delta$  T cells with polyclonal TCR $\gamma\delta$  repertoire, and would amplify the anti-tumor effects from TCR $\gamma\delta$  towards TAA<sup>+</sup> malignancies through the CAR. The *rationale* for this specific aim is that (i)  $\gamma\delta$  T cells have inherent anti-tumor immunity through a number of combinations of TCR $\gamma$  and TCR $\delta$  pairings, (ii) the use of  $\gamma\delta$  T cells in the clinic is currently restricted to V $\gamma$ 9V $\delta$ 2 even though other  $\gamma\delta$  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  $\gamma\delta$  T cells, and (v) CD19-specific CAR transposon plasmids and CD19<sup>+</sup> 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<sup>+</sup>  $\gamma\delta$  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<sup>+</sup>  $\gamma\delta$  T cells will be propagated on CD19<sup>+</sup> aAPC. CAR expression and inferred cell counts will be used to evaluate CAR<sup>+</sup>  $\gamma\delta$  T cell numeric expansion. *Sub-Aim 2.2. To phenotype CAR<sup>+</sup>  $\gamma\delta$  T cells.* After a month of expansion on aAPC, CAR<sup>+</sup>  $\gamma\delta$  T cell surface phenotypes will be evaluated for T cell and memory molecules by flow cytometry and TCR $\gamma\delta$  allele expression will be assessed by nCounter gene expression analysis. *Sub-Aim 2.3. To determine the ability of CAR<sup>+</sup>  $\gamma\delta$  T cells to*

*functionally respond to tumors.* Cytokine production and 4-hour CRA assays will be tested against CD19<sup>+</sup> tumor targets with CD19<sup>neg</sup> targets serving as negative controls. Autologous CAR<sup>neg</sup>  $\gamma\delta$  T cells will be used to compare CAR-specific responses to CD19<sup>+</sup> tumors. CD19<sup>+</sup> leukemia xenografts will be established in immunocompromised mice which will be treated with CAR<sup>+</sup>  $\gamma\delta$  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  $\gamma\delta$  T cells against solid and hematological cancers. The *hypothesis* of this specific aim is that aAPC will expand polyclonal  $\gamma\delta$  T cells that will have broad anti-tumor immunity. The *rationale* for this specific aim is that (i) CAR<sup>neg</sup> polyclonal  $\gamma\delta$  T cells proliferated in parallel to CAR<sup>+</sup>  $\gamma\delta$  T cells described in specific aim#2 on aAPC, (ii) no current expansion protocols exist for polyclonal  $\gamma\delta$  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)  $\gamma\delta$  T cells expressing V $\delta$ 1 are correlated with long-term remissions in cancer therapy but have not been directly infused as an adoptive immunotherapy, (v)  $\gamma\delta$  T cells expressing V $\delta$ 2 have shown anti-tumor effects as direct adoptive immunotherapies, (vi)  $\gamma\delta$  T cells expressing V $\delta$ 3 have not been described to have direct anti-tumor immunity leaving a gap in the field of knowledge, and (vii) a polyclonal approach to  $\gamma\delta$  T cell immunotherapy could target multiple ligands on the tumor through a diverse repertoire of TCR $\gamma\delta$ . *Sub-Aim 3.1. To propagate  $\gamma\delta$  T cells on aAPC.* PBMC or UCB will be sorted for  $\gamma\delta$  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  $\gamma\delta$  T cells. Subsets of  $\gamma\delta$  T cells will also be sorted and expanded as co-cultures with clinical aAPC to assess differences in  $\gamma\delta$  T cell lineages.

*Sub-Aim 3.2. To phenotype  $\gamma\delta$  T cells expanded on aAPC.* After one month of co-culture on aAPC, the surfaces of polyclonal or sorted  $\gamma\delta$  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  $\gamma\delta$  T cells.* Polyclonal or sorted  $\gamma\delta$  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  $\gamma\delta$  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<sup>+</sup> 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<sup>+</sup> malignancies, and (iv) cancer patients treated with CAR<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> B cells in an elderly patient

treated with CD19-specific CAR<sup>+</sup> 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<sup>+</sup> T cells were generated in central memory T cells (T<sub>CM</sub>) that could then efficiently lyse ROR1<sup>+</sup> 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<sub>CM</sub> cells had short extracellular domains (14 amino acids) with CD137 and CD3 $\zeta$  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<sub>CM</sub> reported to have maximal efficacy as CAR<sup>+</sup> T cells,(131) (iii) propagation of CAR<sup>+</sup> T cells on aAPC containing membrane-bound IL15/IL15R $\alpha$  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<sup>+</sup> malignancies and could answer some of the same fundamental CAR questions in a broader set of peripheral T cells.

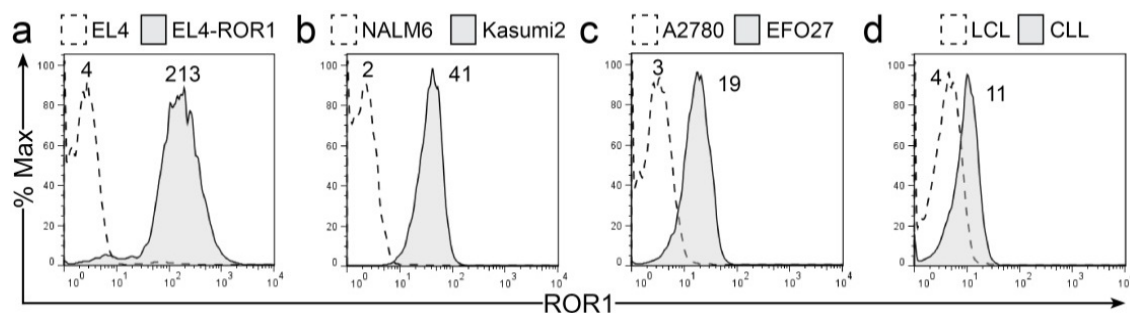
Clinical trials have not yet tested ROR1-specific CARs in humans, so this report of pre-clinical testing of ROR1-specific CARs aims to directly test CD28 and CD137 signaling CARs to streamline trial design and clinical efficacy for cancer treatments. “First-in-man” clinical trials open at MD Anderson translated (i) co-electro-transfer of CD19-specific CAR *Sleeping Beauty* (SB) transposon with SB transposase and (ii) expansion of CD19-specific CAR<sup>+</sup> T cell on CD19<sup>+</sup> 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<sup>+</sup> EFO27 cells and the only ROR1<sup>neg</sup> 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.



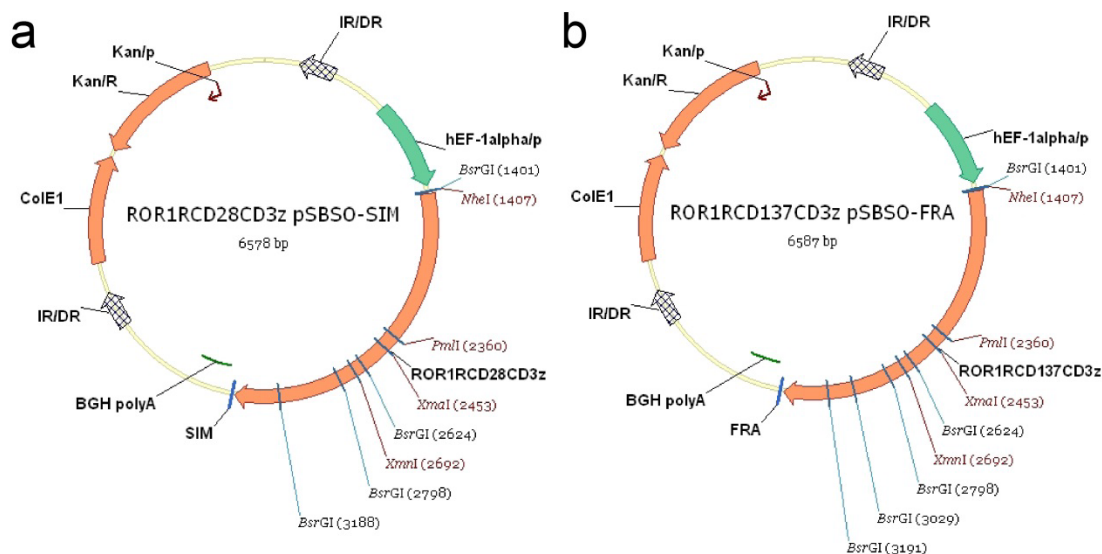
**Figure 6. Surface Expression of ROR1 on Tumors.** The 4A5 mAb specific for ROR1 was used to assess ROR1 expression on the surface of (a) EL4 parental (ROR1<sup>neg</sup>) and genetically modified EL4-ROR1 cells, (b) B-ALL cell lines NALM6 and Kasumi2, (c) OvCa cell lines A2780 and EFO27, and (d) primary 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



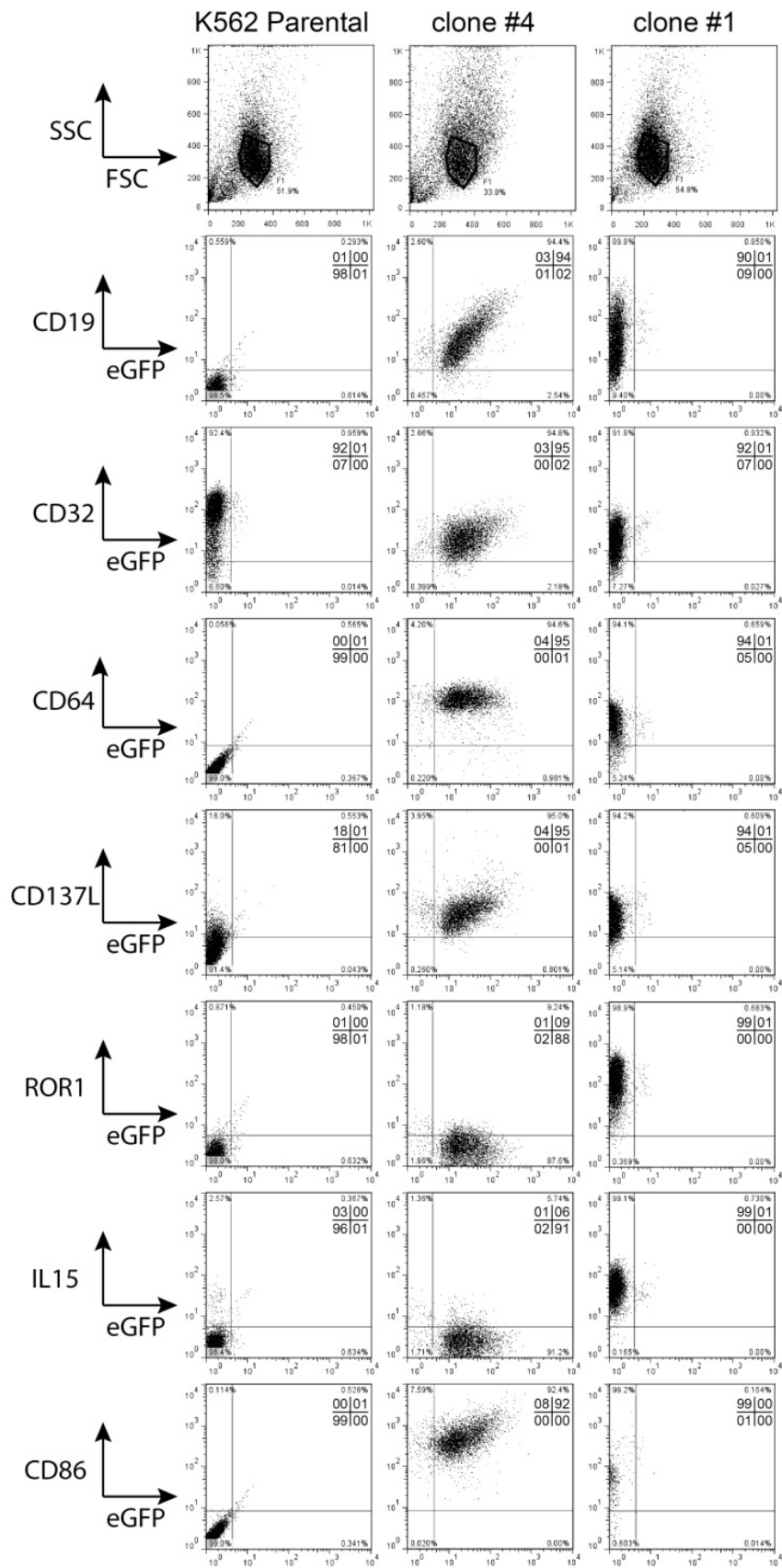
selectively amplify CAR plasmids in bacteria to large quantities (0.5 – 1.0 mg), which were cleared for transfection after testing negative for endotoxin. In summary, these two ROR1-specific CAR plasmids mimic current plasmids used for CD19-specific CAR clinical trials at MD Anderson and should be directly 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<sup>+</sup> 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- $\alpha$  (IL15/IL15R $\alpha$ ) along with the other molecules present on aAPC surfaces. Trans-presentation of IL15 by IL15R $\alpha$  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<sup>+</sup> 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  $\gamma$ -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<sup>+</sup> 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<sup>+</sup> T cell propagation.

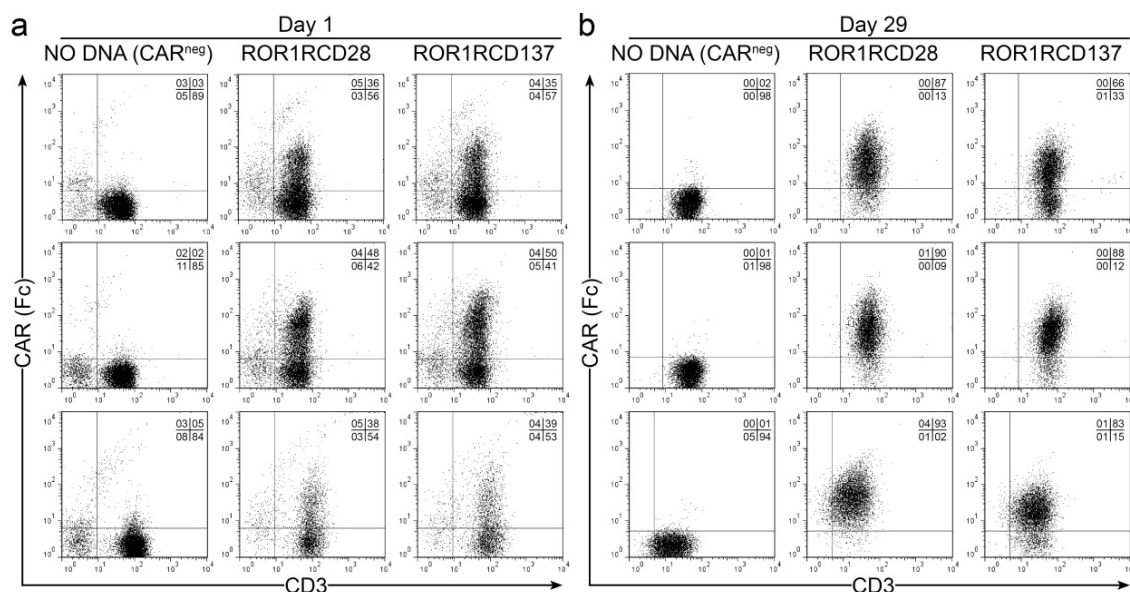


**Figure 8. Surface Phenotype of Clone#1 aAPC Used for ROR1-specific T cell Expansion.** Parental K562 (left), clone#4 aAPC (middle), and clone#1 aAPC (right) were stained for surface marker expression and were analyzed by flow cytometry. Top plots are forward scatter (FSC; x-axes) by side scatter (SSC; 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<sup>+</sup> T-cell Expansion on Clone#1 aAPC

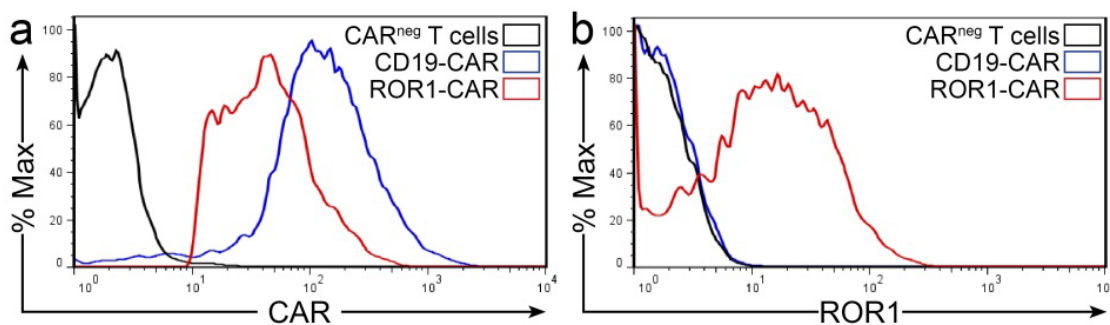
Healthy donor PBMC were electroporated with (i) no DNA as a negative control for CAR expression, (ii) SB11 transposase and ROR1RCD28 transposon plasmids, or (iii) SB11 transposase and ROR1RCD137 transposon plasmids. The following day, cells were phenotyped for CAR expression on their surfaces where “no DNA” and isotype antibodies served as negative controls. Transient expression of CAR was detected in T cells at  $41\% \pm 6\%$  and  $41\% \pm 8\%$  (mean  $\pm$  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  $\gamma$ -irradiated clone#1 aAPC and CAR<sup>+</sup> T cells at a 1:1 ratio. Similarly,  $\gamma$ -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  $90\% \pm 3\%$  and  $79\% \pm 11\%$  (mean  $\pm$  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 ( $51 \pm$

8 vs  $102 \pm 68$ , 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<sup>+</sup> T cells. CD19-specific CAR<sup>+</sup> 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<sup>neg</sup> 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<sup>+</sup> T cell counts ( $p = 0.74$ ). Total cell proliferation closely coincided with CAR<sup>+</sup> T cell proliferation kinetics for both ROR1RCD28 and ROR1RCD137 (**Figure 11**). ROR1RCD28 resulted in an average of  $2.5 \times 10^9$  total inferred cell counts (range  $1.4 \times 10^9 - 4.0 \times 10^9$ ) and  $2.2 \times 10^9$  CAR<sup>+</sup> T cells (range  $1.3 \times 10^9 - 3.6 \times 10^9$ ), and ROR1RCD137 resulted in an average of  $3.6 \times 10^9$  total inferred cell counts (range  $3.7 \times 10^9 - 8.2 \times 10^9$ ) and  $2.9 \times 10^9$  CAR<sup>+</sup> T cells (range  $2.4 \times 10^9 - 6.7 \times 10^9$ ). 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.

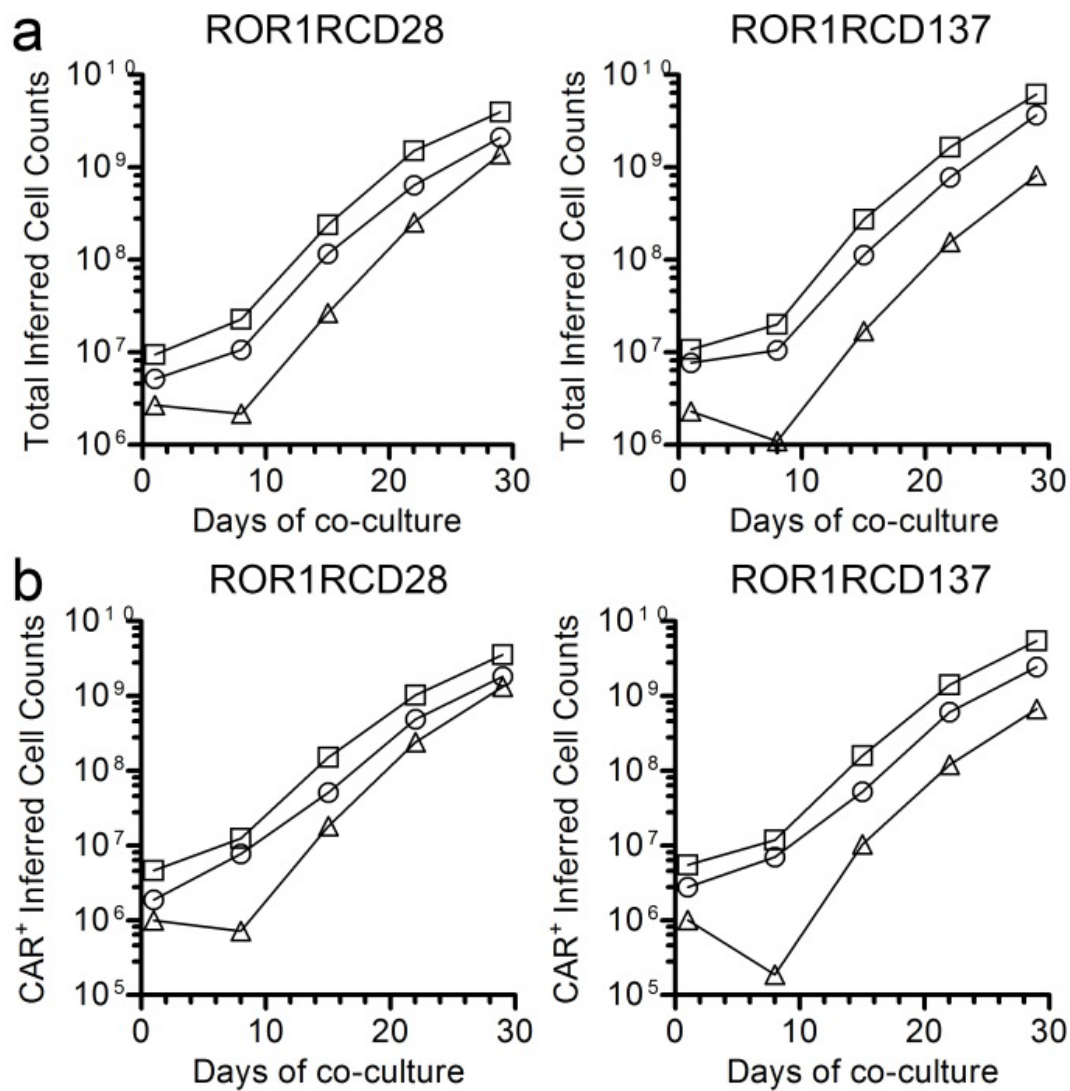


**Figure 9. CAR Expression in T cells Before and After Expansion on Clone#1 aAPC. (a)** Transient expression of ROR1RCD28 (middle) and ROR1RCD137 (right) T cells the day following electroporation where “no DNA” T cells (left) were used as negative controls. **(b)** Stable CAR expression in ROR1RCD28 (middle) and ROR1RCD137 (right) populations. T cells were marked by CD3 staining and CAR<sup>+</sup> 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<sup>+</sup> T cells (CD19RCD28) and “no DNA” CAR<sup>neg</sup> T cells were used as negative controls. (a) Fc detection of CARs and (b) rROR1 binding.



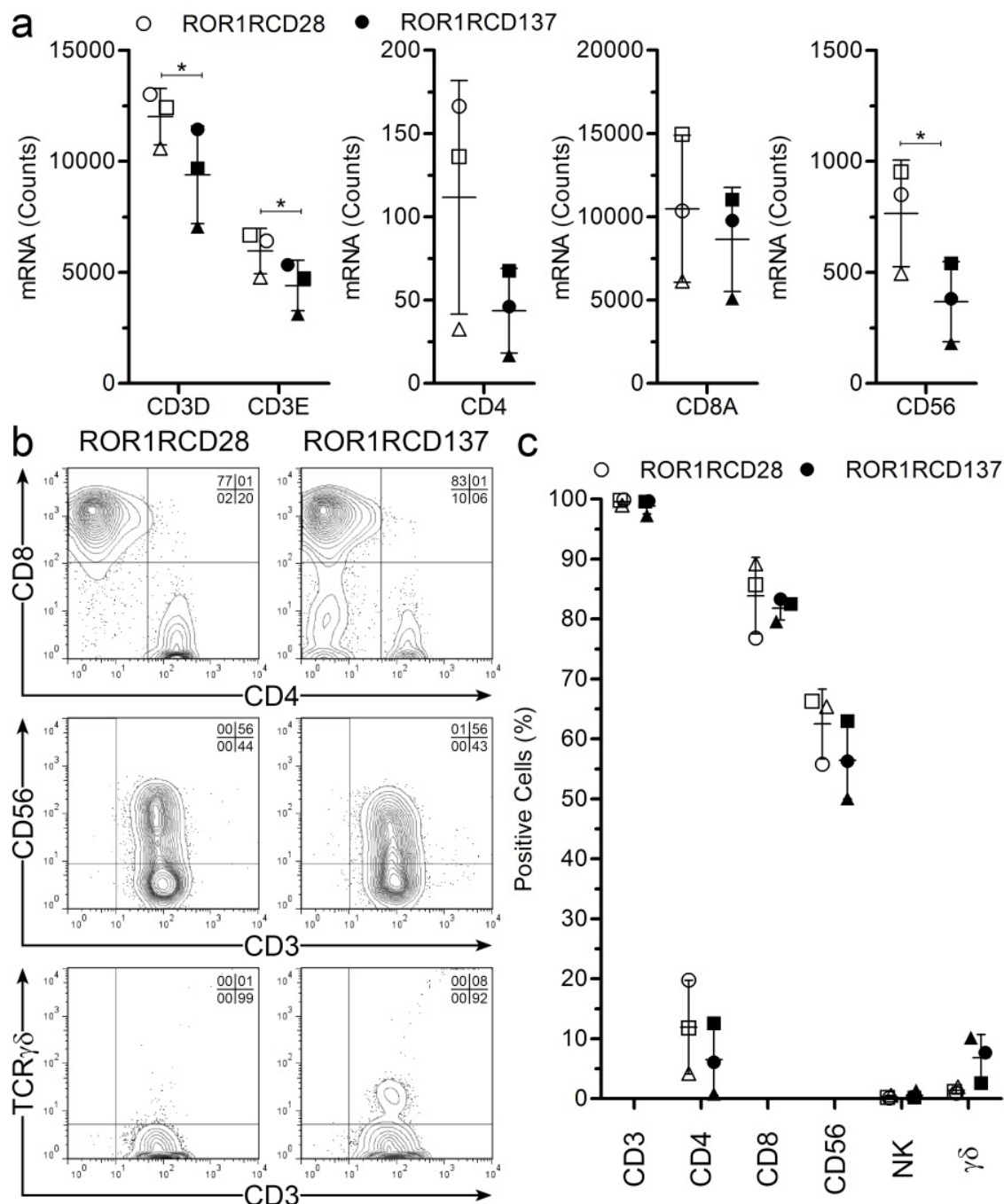
**Figure 11. Sustained Proliferation of CAR<sup>+</sup> T cells.** (a) Total cells and (b) CAR<sup>+</sup> T cell proliferation on clone#1 aAPC. ROR1RCD28 represented on the left and ROR1RCD137 shown on the right. Each symbol represents a different healthy donor.

## 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  $\delta$  and  $\epsilon$  isoforms of CD3 (CD3D and CD3E, respectively) were highly expressed by both CAR<sup>+</sup> T cell populations, and there was higher expression of both CD3D and CD3E in ROR1RCD28 cells (**Figure 12a**). Expression of CD3 $\zeta$  was not evaluated at the mRNA level because it could not be distinguished from CD3 $\zeta$  on CAR intracellular domains. Nonetheless, >97% of CAR<sup>+</sup> T cells were CD3<sup>+</sup> 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<sup>+</sup> T cells over CD4<sup>+</sup> 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<sup>+</sup> and CD8<sup>+</sup> T cells is most likely attributed to small frequencies of  $\gamma\delta$  T cells (identified by CD3<sup>+</sup>TCR $\gamma\delta$ <sup>+</sup>) that were present in the ROR1RCD137 cultures and not in the ROR1RCD28 cultures (**Figure 12b bottom panels**), because  $\gamma\delta$  T cells are commonly negative for both CD4 and CD8 but express

CD3.(286) Indeed,  $\gamma\delta$  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<sup>+</sup> 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<sup>neg</sup>CD56<sup>+</sup> 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 cytotoxicity (**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  $\gamma\delta$  T cells (**Figure 12c**). These results suggest that CAR<sup>+</sup> 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<sup>+</sup> 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

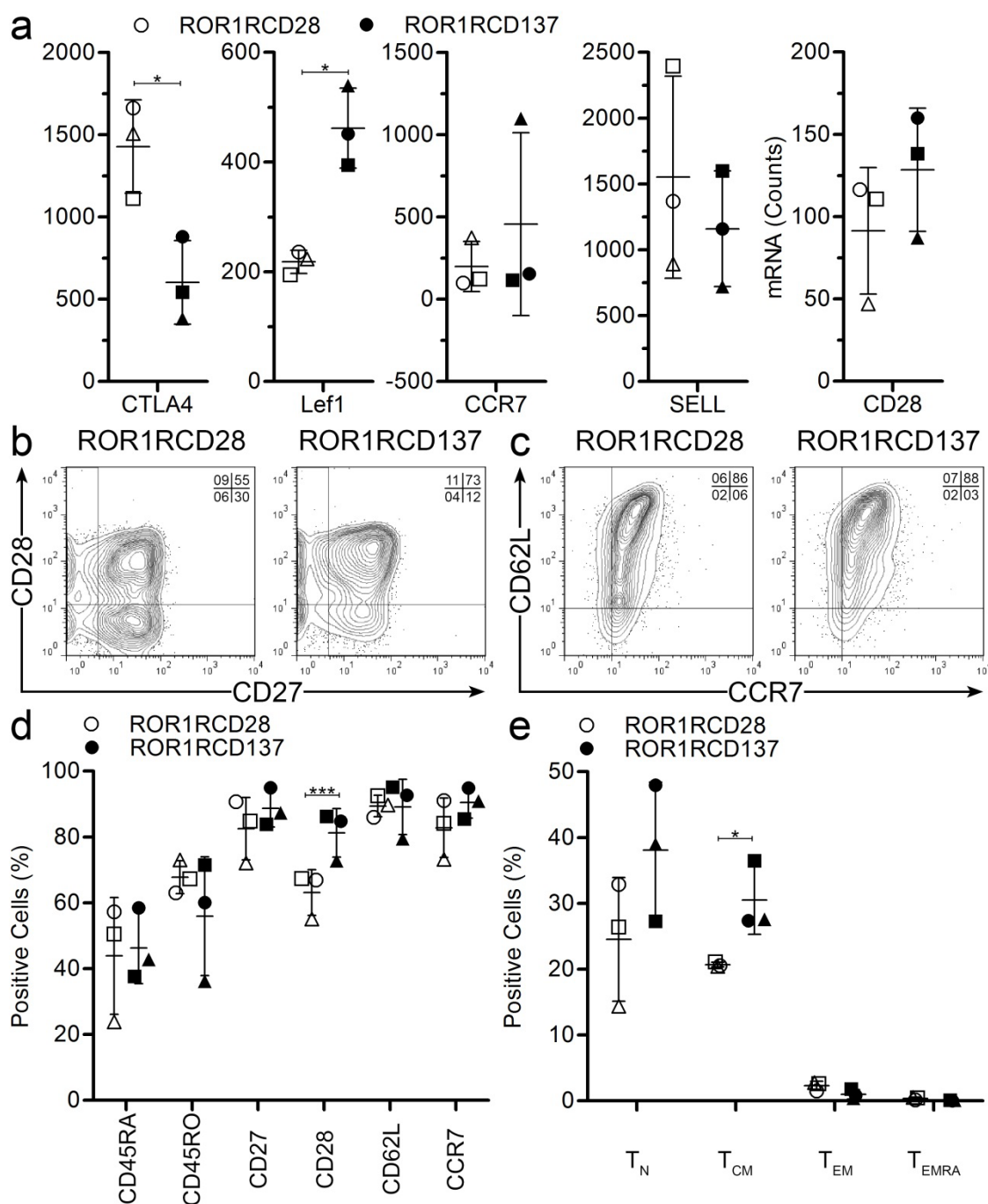
for statistical analysis ( $n = 3$ ).  $*p < 0.05$  **(b)** CD4 (x-axes) and CD8 (y-axes) expression (top panels), CD3 (x-axes) and CD56 (y-axes) expression (middle panels), and CD3 (x-axes) and TCR $\gamma\delta$  (y-axes) expression (bottom panels) of one of 3 representative donors. Gate frequencies 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<sup>neg</sup>CD56<sup>+</sup>,  $\gamma\delta$  T cells were defined as CD3<sup>+</sup>TCR $\gamma\delta$ <sup>+</sup>, and data are mean  $\pm$  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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> T cell populations suggesting they have not reached terminal differentiation (**Figure 13a, 13b, and 13d**). CAR<sup>+</sup> 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<sup>+</sup> malignancies.



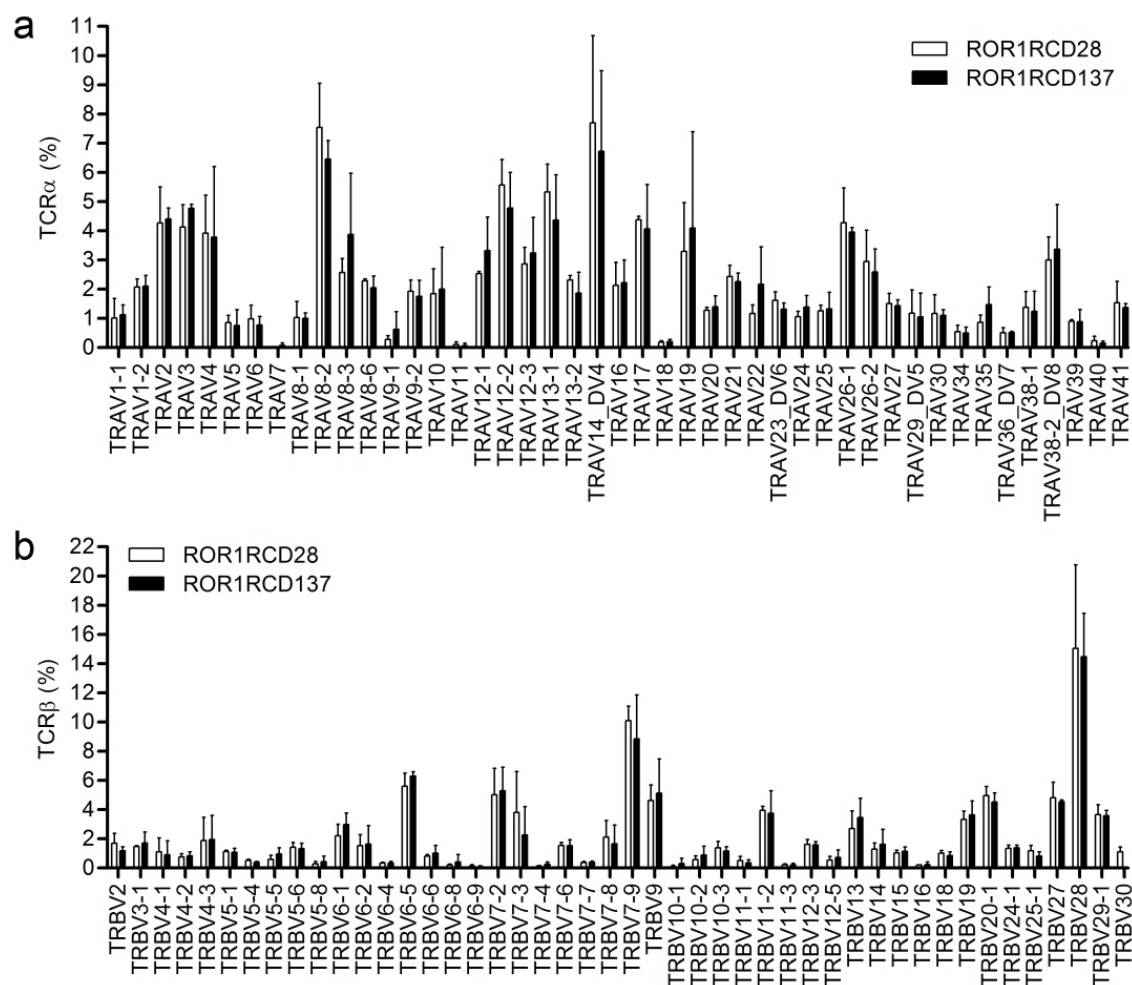


**Figure 13. Memory Markers on CAR<sup>+</sup> T cell Surfaces.** After 29 days of expansion on clone#1 aAPC, ROR1RCD28 and ROR1RCD137 cells were (i) lysed for mRNA expression analysis or (ii) phenotyped for T cell surface markers by flow cytometry. **(a)** RNA lysates were run on the nCounter LCA and 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  $\pm$  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 V $\alpha$  and 46 V $\beta$  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 TCR $\alpha$  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 TCR $\alpha$  (Student’s paired, two-tailed t-test) resulted in  $p$  values  $>0.05$  for all alleles (**Figure 14a**). Similarly, TCR $\beta$  isotypes were not significantly different between ROR1RCD28 and ROR1RCD137 when analyzed together ( $p = 0.33$ ) or as individual genes (**Figure 14b**). TCR $\alpha$  and TCR $\beta$  were both polyclonal suggesting that skewing to a particular TCR isotype did not occur. Additionally, DTEA measured TCR $\gamma$  and TCR $\delta$  expression where all V $\delta$  counts were 0.9% and 1.9% of the ROR1RCD28 and ROR1RCD137 total TCR frequencies, respectively, and V $\gamma$  counts were 6.2% and 8.1% of the ROR1RCD28 and ROR1RCD137 total TCR frequencies, respectively. These results showed that  $\gamma\delta$  T cells were minor contributors to the total CAR<sup>+</sup> T cell pools, which were mainly  $\alpha\beta$  T cells as determined by DTEA. Thus, CAR endodomain signaling was not preferential to a particular TCR $\alpha\beta$  clonotype but rather generated polyclonal  $\alpha\beta$  T cells.



**Figure 14. TCR $\alpha$  and TCR $\beta$  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)** TCR $\alpha$  and **(b)** TCR $\beta$  expression in ROR1RCD28 (filled bars) and ROR1RCD137 (open bars) T cells.

### II.C.7. IFN $\gamma$ Production by CAR<sup>+</sup> T cells in Response to ROR1

In order to assess whether CAR<sup>+</sup> T cells were functional and specific for ROR1<sup>+</sup> tumor cells, IFN $\gamma$  production was measured by flow cytometry after activation with leukemia cells or TCR agonists. Brefeldin-A was co-cultured with T cells to inhibit IFN $\gamma$  secretion. Collectively, the data suggest that CAR<sup>+</sup> T cells were specific and functional in responding to ROR1<sup>+</sup> 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<sup>2+</sup> 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 IFN $\gamma$  was measured in response to LAC as seen in example histograms (**Figure 15a**) and average mean fluorescence intensities (MFI) of IFN $\gamma$  staining (**Figure 15b**). There was a trend of higher production of IFN $\gamma$  by ROR1RCD28 compared to ROR1RCD137 that was not statistically different ( $p = 0.120$ ). These results established that IFN $\gamma$  was produced when CAR<sup>+</sup> T cells were activated through canonical TCR signaling pathways and suggested that ROR1RCD28 had higher propensity to express IFN $\gamma$  relative to ROR1RCD137 cells.

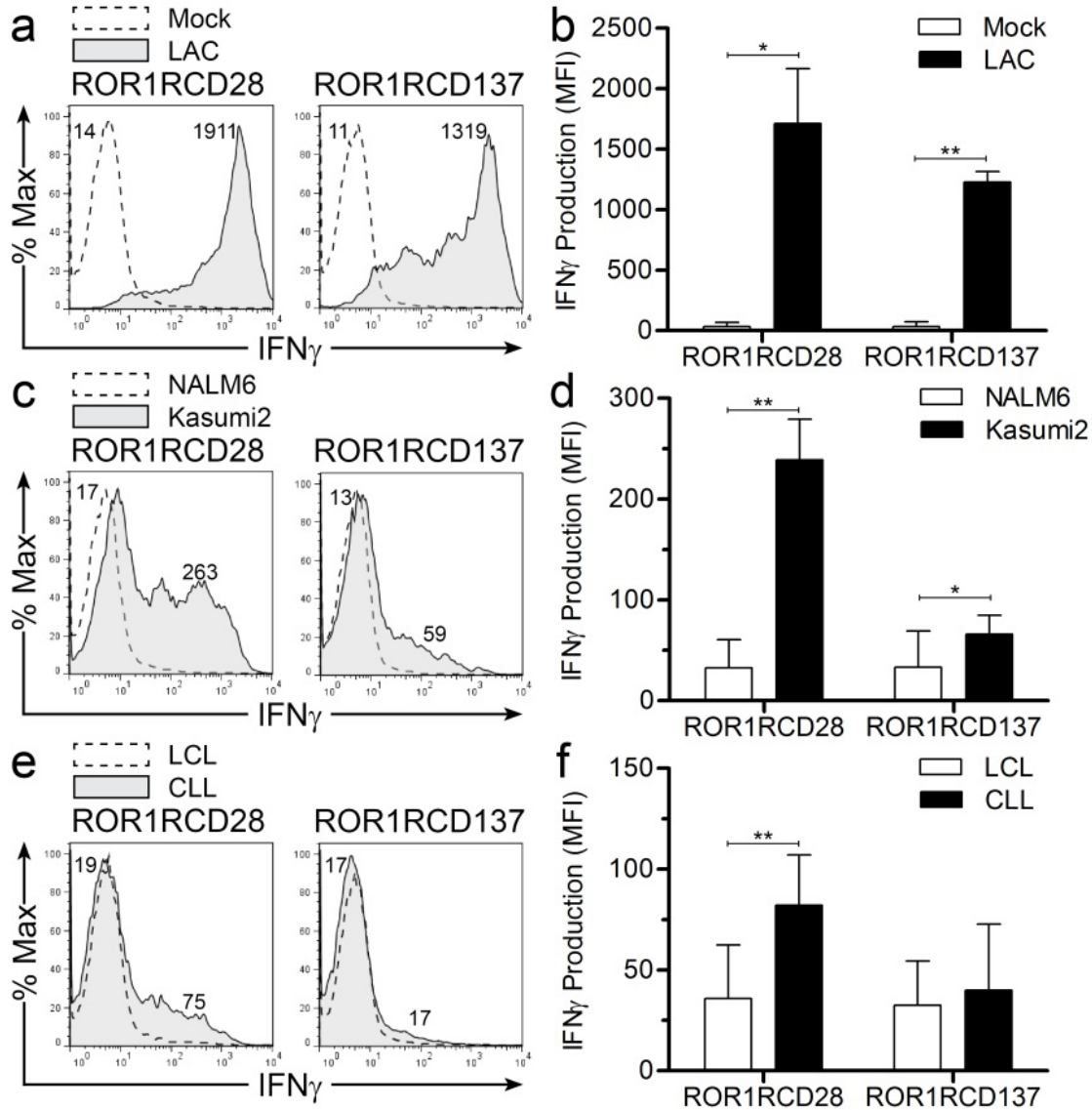
### *II.C.7.b. Specific IFN $\gamma$ Production to ROR1<sup>+</sup> Leukemia Cells*

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<sup>+</sup> T cells to human leukemia cells in 6 hours of co-culture. As expected, ROR1RCD28 and ROR1RCD137 T cells produced IFN $\gamma$  when co-cultured with Kasumi2 cells but not with NALM6 (**Figure 15c**). Similarly to LAC activation, ROR1RCD137 cells produced less IFN $\gamma$  than ROR1RCD28 cells (**Figure 15d**) in response to the ROR1<sup>+</sup> cell line. Nonetheless, ROR1-specific CAR<sup>+</sup> T cells responded specifically to ROR1<sup>+</sup> leukemia.

### *II.C.7.c. CAR<sup>+</sup> T cells Produce IFN $\gamma$ in Response to Primary ROR1<sup>+</sup> Leukemia Cells but not Healthy ROR1<sup>neg</sup> B cell LCL*

It was important to ensure that ROR1-specific T cells would respond to primary ROR1<sup>+</sup> 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 IFN $\gamma$  was produced by CAR<sup>+</sup> 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) IFN $\gamma$  was produced by ROR1RCD28 and there was a trend of increased IFN $\gamma$  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<sup>+</sup> T cells were functionally responsive to primary ROR1<sup>+</sup> leukemia and not to healthy B cells.



**Figure 15. IFN $\gamma$  Production by ROR1-specific T cells in Response to ROR1<sup>+</sup> Targets.** Brefeldin-A (GolgiPlug) was added to T cells to block IFN $\gamma$  secretion in order to measure functional responses to agonistic stimulation. At day 29 of co-culture, CAR<sup>+</sup> T cells were co-cultured for 6 hours at 37°C and cells were gated for CD3<sup>+</sup>Fc<sup>+</sup> 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<sup>neg</sup>) or Kasumi2 (ROR1<sup>+</sup>), or (e)/(f) healthy donor LCL cell line (ROR1<sup>neg</sup>) or CLL patient sample (ROR1<sup>+</sup>). Mean fluorescence intensities (MFI) are displayed next to histograms in (a), (c), and (e), which are representative of three CAR<sup>+</sup> T cell donors. Mean  $\pm$  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

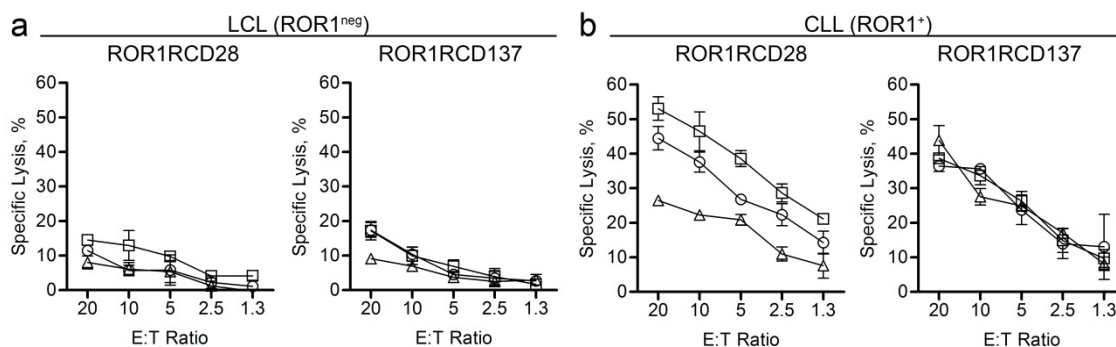


### II.C.8. ROR1-specific Cytotoxicity by CAR<sup>+</sup> T cells

Cytotoxicity was another important assessment of ROR1-specific CAR<sup>+</sup> 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<sup>+</sup> control cells, established tumor cell lines, and primary tumor cells. Significant lysis was only observed against ROR1<sup>+</sup> cells suggested that CAR<sup>+</sup> T cells were specific in their lytic abilities.

#### *II.C.8.a. CAR<sup>+</sup> T cells Lyse Leukemia but not Healthy B cells*

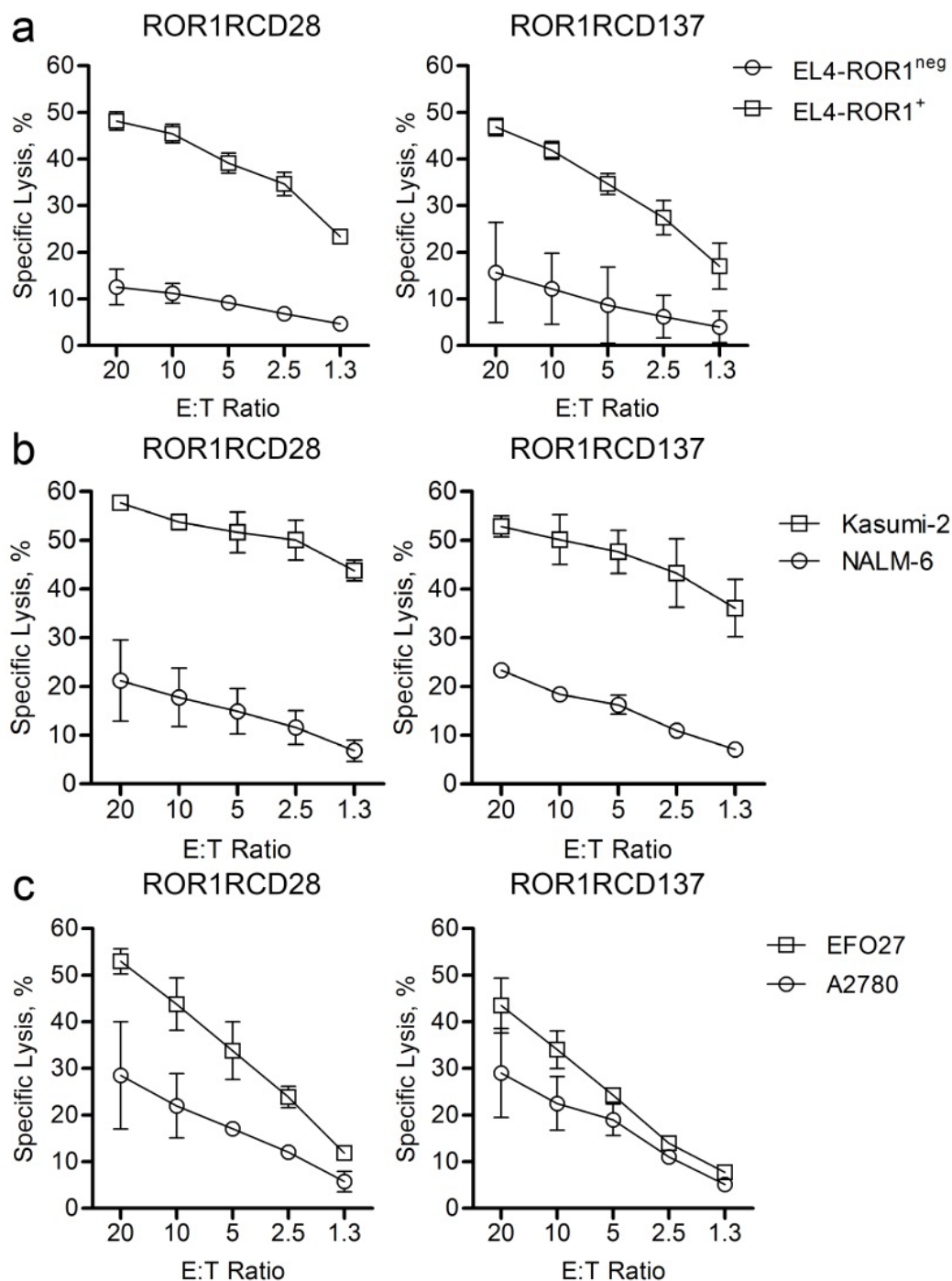
The clinical trial based on these data will treat patients with B-cell CLL, so primary B-cell CLL samples were tested as targets by allogeneic ROR1-specific CAR<sup>+</sup> T cells. ROR1<sup>neg</sup> LCLs were used for negative controls for CLL samples (**Figure 6a**). As expected, minimal lysis was 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<sup>+</sup> leukemia by CAR<sup>+</sup> T cells while sparing normal B cells.



**Figure 16. Specific Cytolysis of Primary ROR1<sup>+</sup> B-cell CLL by CAR<sup>+</sup> T cells.** (a) Established ROR1<sup>neg</sup> B-cell LCL and (b) Primary patient ROR1<sup>+</sup> CLL cells were tested for cytotoxicity by ROR1-specific CAR<sup>+</sup> 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  $\pm$  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<sup>+</sup> but showed minimal lysis of EL4-ROR1<sup>neg</sup> cells (**Figure 17a**). Similar to EL4 data, ROR1<sup>+</sup> B-ALL cell line Kasumi2 was lysed at significantly higher levels ( $p < 0.0001$ ) compared to ROR1<sup>neg</sup> 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<sup>+</sup> T cells, donor-matched CD19<sup>+</sup> specific CAR<sup>+</sup> T cells lysed all three cell lines, which were all CD19<sup>+</sup> (data not shown), and suggested that ROR1RCD28 and ROR1RCD137 were more discriminant in their killing abilities. Furthermore, ROR1<sup>+</sup> OvCa cell line EFO27 was lysed at significantly ( $p < 0.0001$ ) higher levels than ROR1<sup>neg</sup> OvCa cell line A2780 by both ROR1RCD28 and ROR1RCD137 (**Figure 17c**). In summary, ROR1-specific CAR<sup>+</sup> T cells demonstrated effective and specific lysis of ROR1<sup>+</sup> tumor cells *in vitro*.

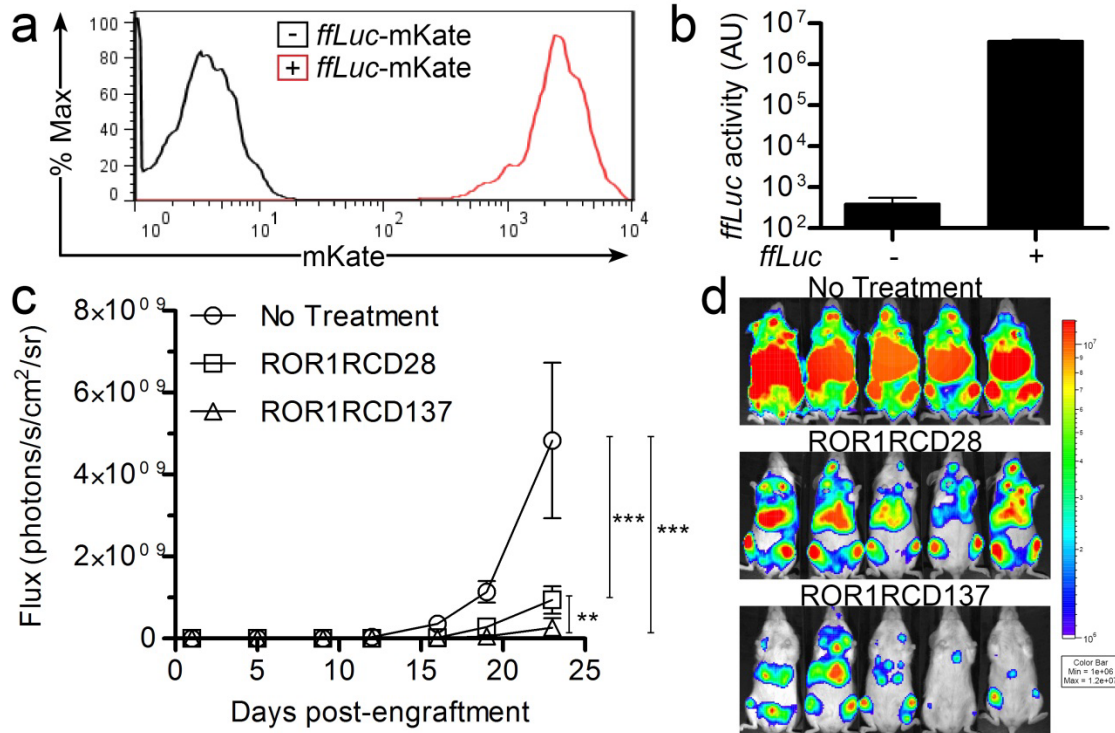


**Figure 17. Specific Cytolysis of Established ROR1<sup>+</sup> Tumor Cell Lines by CAR<sup>+</sup> T cells.** Standard 4-hour CRA were used to assess specific lysis of (a) EL4-ROR1<sup>neg</sup> (circles) or EL4-ROR1<sup>+</sup> (squares) cells, (b) ROR1<sup>neg</sup> NALM6 (circles) or ROR1<sup>+</sup> Kasumi2 (squares) cells, and (c) ROR1<sup>neg</sup> A2780 (circles) or ROR1<sup>+</sup> 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  $\pm$  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<sup>+</sup> T cells *in vivo*, a mouse model of MRD was implemented for leukemia and ROR1-specific CAR<sup>+</sup> 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.γ<sub>c</sub><sup>-/-</sup>* (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<sub>10</sub>-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<sup>+</sup> 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<sup>+</sup> T cells, which highlights their ability to outperform ROR1RCD28 in tumor killing *in vivo*. In summary, ROR1-specific CAR<sup>+</sup> T cells can efficiently treat ROR1<sup>+</sup> leukemia and, therefore, can now be moved into the clinic for testing in patients with ROR1<sup>+</sup> malignancies.



**Figure 18. *In vivo* Tumor Clearance by ROR1-specific CAR<sup>+</sup> T cells.** ROR1<sup>+</sup> 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  $4 \times 10^4$  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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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* IFN $\gamma$  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 cytotoxicity.(132, 135) It is consistent then that ROR1RCD137 cells produced less IFN $\gamma$  when challenged with ROR1<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> targets, with a minor exception of primary cell lines where ROR1RCD28 was highly variable in cytotoxicity 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<sup>+</sup> 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<sup>+</sup> 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<sub>N</sub> and T<sub>CM</sub> memory cells that are correlated with highest CAR<sup>+</sup> 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 IFN $\gamma$  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 $\gamma\delta$ 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  $\gamma\delta$  T cells will stimulate them independent of their TCR $\gamma\delta$ , thus leading to expansion of  $\gamma\delta$  T cells with polyclonal TCR $\gamma\delta$  repertoire, and would amplify the anti-tumor effects from TCR $\gamma\delta$  towards TAA<sup>+</sup> malignancies through the CAR. The *rationale* for this specific aim is that (i)  $\gamma\delta$  T cells have inherent anti-tumor immunity through a number of combinations of TCR $\gamma$  and TCR $\delta$  pairings, (ii) the use of  $\gamma\delta$  T cells in the clinic is currently restricted to V $\gamma$ 9V $\delta$ 2 even though other  $\gamma\delta$  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  $\gamma\delta$  T cells, and (v) CD19-specific CAR transposon plasmids and CD19<sup>+</sup> 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  $\gamma\delta$  T cells for CAR-based immunotherapy would allow for targeting the tumor through both CAR and multiple TCR $\gamma\delta$  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<sup>+</sup> 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<sup>+</sup> T cells co-expressing  $\alpha\beta$  T-cell receptor (TCR $\alpha\beta$ ) derived from a population that represents 95% of the peripheral T-cell pool. However, the remaining 1-5% of circulating T cells expressing TCR $\gamma\delta$  ( $\gamma\delta$  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 TCR $\gamma\delta$  repertoire (combination of V $\delta$ 1, V $\delta$ 2, or V $\delta$ 3 with one of fourteen V $\gamma$  chains).(300)

More specifically, T cells expressing V $\delta$ 1 and V $\delta$ 2 have been associated with anti-tumor immunity, but current adoptive immunotherapy approaches are limited to the V $\delta$ 2 sub-population due to limited expansion methods of V $\delta$ 1 to clinically-sufficient numbers of cells for human applications. For the most part,

$\gamma\delta$  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 V $\gamma$ 9V $\delta$ 2 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. V $\delta$ 1  $\gamma\delta$  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  $\alpha\beta$  T cell-depleted allogeneic hematopoietic stem-cell transplantation (HSCT).(302-305) As both of these sub-populations of  $\gamma\delta$  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 TCR $\gamma\delta$  repertoire.

Recognizing that a CD19-specific CAR can sustain the proliferation of  $\alpha\beta$  T cells on artificial antigen presenting cells (aAPC) independent of TCR $\alpha\beta$  usage,(280) we hypothesized that CAR<sup>+</sup>  $\gamma\delta$  T cells would expand on aAPC independent of TCR $\gamma\delta$ . Our approach was further stimulated by the observation that K562, the cell line from which the aAPC are derived, are a natural target for  $\gamma\delta$  T cells.(303) We report that CAR<sup>+</sup>  $\gamma\delta$  T cells can be propagated to clinically-relevant numbers on designer aAPC while maintaining a polyclonal population of TCR $\gamma\delta$  as assessed by our "direct TCR expression assay" (DTEA), a novel digital multiplexed gene expression analysis that we adapted to interrogate all TCR $\gamma\delta$  isotypes.(290) These CAR<sup>+</sup>  $\gamma\delta$  T cells

displayed enhanced killing of CD19<sup>+</sup> tumor cell lines *in vitro* compared to polyclonal  $\gamma\delta$  T cells not expressing CAR. Leukemia xenografts in immunocompromised mice were significantly reduced when treated with CAR<sup>+</sup>  $\gamma\delta$  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 TCR $\gamma\delta$  repertoire.

### III.C. Results

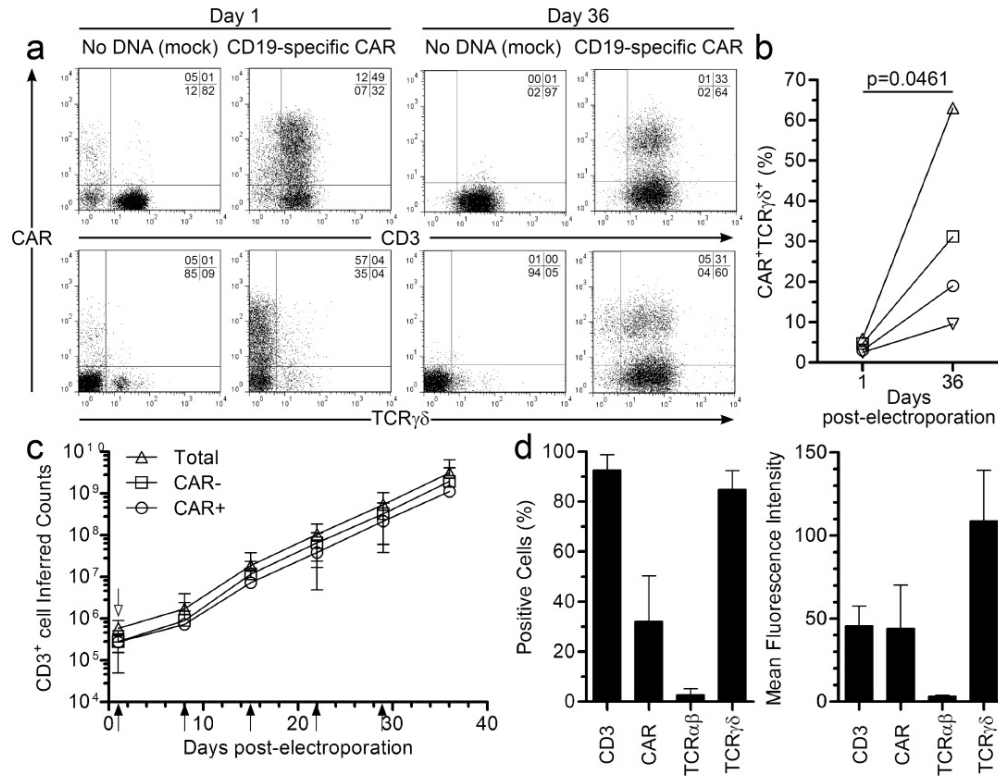
#### III.C.1. CAR<sup>+</sup> $\gamma\delta$ T cells Numerically Expand on aAPC

To date, it has been problematic to synchronously manipulate and expand multiple  $\gamma\delta$  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 V $\delta$ 2 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  $\gamma\delta$  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 TCR $\gamma\delta$  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 CD3 $\zeta$ . Electroporated cells were sorted using paramagnetic beads to separate the 4.0%  $\pm$  1.5% (mean  $\pm$  standard deviation (SD); n = 4) CAR<sup>+</sup>  $\gamma\delta$  T cells from the majority of CAR<sup>+</sup>  $\alpha\beta$  T cells. The CAR<sup>+</sup>  $\gamma\delta$  T cells were selectively propagated by the recursive additions of  $\gamma$ -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<sup>+</sup>  $\alpha\beta$  T cells so it was used to propagate CAR<sup>+</sup>  $\gamma\delta$  T cells.(57) Prior experiments predicted that IL2 and IL15 enhance the proliferative potential of  $\gamma\delta$  T cells, and synergy between IL2 and IL21 has led to improved anti-tumor activity compared with  $\gamma\delta$  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<sup>neg</sup>  $\alpha\beta$  T cells.(310) As expected, CAR was expressed on the day following electroporation (Day 1) in most of the T cells, including  $\gamma\delta$  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 TCR $\gamma\delta$  with 30.7%  $\pm$  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<sup>+</sup>  $\gamma\delta$  T cells at Day 1 (**Figure 19b**). As we have demonstrated, our aAPC co-culture system enforces CAR expression in  $\alpha\beta$  T cells (>90% CAR<sup>+</sup> T cells by 28 days of co-culture),(57) but the apparent lack of

the same degree of selective pressure when combined with  $\gamma\delta$  T cells was attributed to an inherent ability of  $\text{CAR}^{\text{neg}}$   $\gamma\delta$  T cells to sustain proliferation on aAPC derived from K562. Continuous proliferation of both  $\text{CAR}^{\text{neg}}$  and  $\text{CAR}^+$   $\gamma\delta$  T cells was observed over the tissue culture period. Even so, we could generate up to  $1.5 \times 10^9 \pm 1.2 \times 10^9$  ( $n = 3$ )  $\text{CAR}^+$   $\gamma\delta$  T cells from the  $2.8 \times 10^5 \pm 1.5 \times 10^5$  ( $n = 3$ )  $\text{CAR}^+$   $\gamma\delta$  T cells at the start of the culture (**Figure 19c**). Most of the propagated cells co-expressed CD3 and  $\text{TCR}\gamma\delta$ , but did not express  $\text{TCR}\alpha\beta$  (**Figure 19d**). These data demonstrate that aAPC could be used to sustain proliferation of  $\text{CAR}^+$  T cells co-expressing  $\text{TCR}\gamma\delta$ .

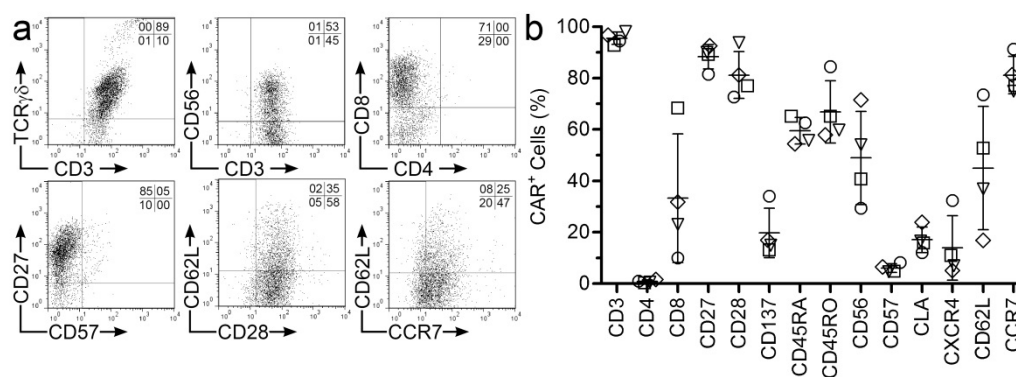


**Figure 19. CAR<sup>+</sup> γδ T cells Propagate on Designer aAPC.** (a) Transient (Day 1) and stable (Day 36) expression of CAR in T cells (top) and γδ T cells (bottom) in mock electroporated (“no DNA”) or CD19-specific CAR electroporated cells (CD19RCD28). (b) Percentage of CAR<sup>+</sup> γδ T cells 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<sup>neg</sup> γδ T cells (squares), and CAR<sup>+</sup> 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<sup>+</sup> $\gamma\delta$ T cells

Multi-parameter flow cytometry was used to gate on CAR<sup>+</sup> 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<sup>neg</sup>CD56<sup>+</sup> NK cells and <1% CD3<sup>+</sup>V $\alpha$ <sub>25</sub>TCR<sup>+</sup> NKT cells (data not shown). In contrast to  $\alpha\beta$  T cells, no CAR<sup>+</sup>  $\gamma\delta$  T cells expressed CD4, some were CD8<sup>+</sup>, but most were CD4<sup>neg</sup>CD8<sup>neg</sup>, 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<sup>+</sup>  $\gamma\delta$  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<sup>+</sup>CD27<sup>+</sup>CD28<sup>+</sup>CCR7<sup>+</sup>; 26.5%  $\pm$  6.2%), central memory (CD45RA<sup>neg</sup>CD27<sup>+</sup>CD28<sup>+</sup>CCR7<sup>+</sup>; 7.8%  $\pm$  3.6%), effector memory (CD45RA<sup>neg</sup>CD27<sup>+</sup>CD28<sup>neg</sup>CCR7<sup>neg</sup>; 10.1%  $\pm$  5.4%), and EMRA (CD45RA<sup>+</sup>CD27<sup>neg</sup>CD28<sup>neg</sup>CCR7<sup>neg</sup>; 7.6%  $\pm$  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<sup>+</sup>  $\gamma\delta$  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<sup>+</sup>  $\gamma\delta$  T cells suggesting that they could migrate to sites known to harbor leukemia. In sum, propagated CAR<sup>+</sup>  $\gamma\delta$  T cells expressed T cell-associated surface markers that indicate desired potential for memory and homing.



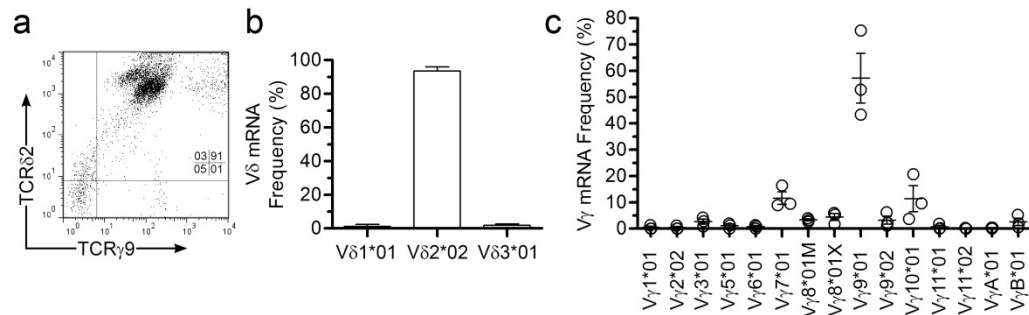
**Figure 20. Immunophenotype of Electroporated, Separated, and Propagated CAR $^+$   $\gamma\delta$  T cells.** (a) Expression by flow cytometry of cell-surface markers associated with T cells and memory as gated on CD3 $^+$ CAR $^+$  cells. (b) Percentages of CAR $^+$  T cells expressing T-cell markers where each shape represents a different donor. Data are mean  $\pm$  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 $\gamma$ and $\delta$ TCR Usage in $\text{CAR}^+$ $\gamma\delta$ T cells

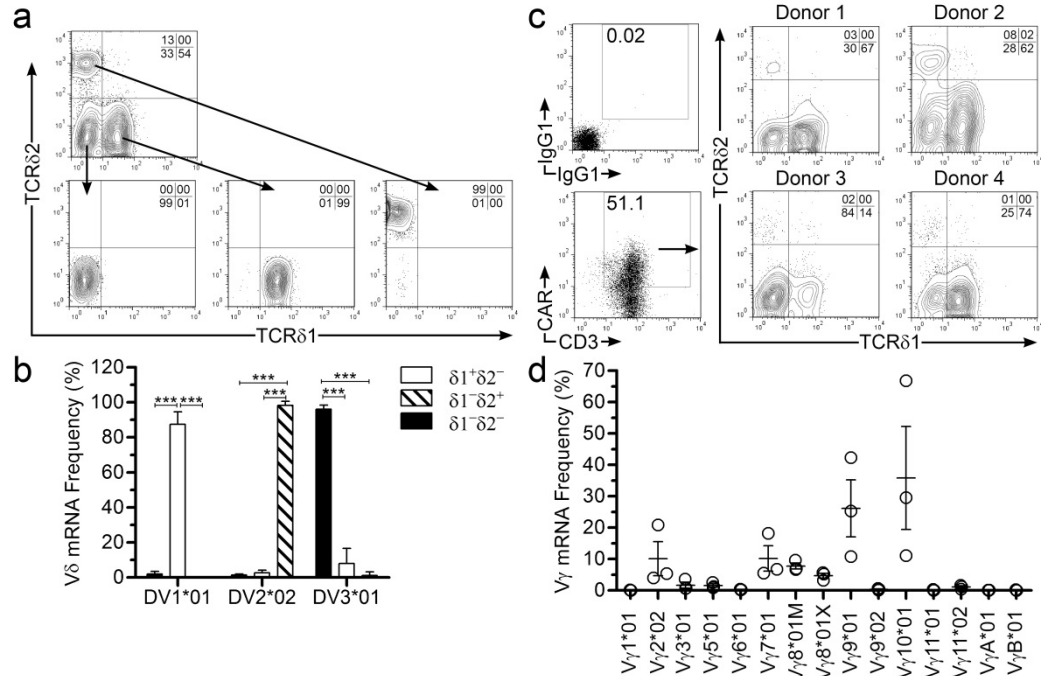
We sought to determine that aAPC-propagated  $\text{CAR}^+$  T cells were indeed bi-specific as defined by the presence of a polyclonal population of  $\text{TCR}\gamma\delta$  alleles. Up to now, it has been difficult to determine the pattern of expression of the  $\gamma$  and  $\delta$  TCR chains. Therefore, we adapted our DTEA to assess the complete  $\text{TCR}\gamma\delta$  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.<sup>(312)</sup> A multiplexed CodeSet was designed with two sequence-specific probes for each allele to evaluate  $\text{TCR}\gamma\delta$  isotypes. The DTEA was initially validated using Zol to preferentially propagate  $\text{V}\gamma 9\text{V}\delta 2$  cells from PBMC and, as expected, the resultant TCR usage was dominated by both  $\text{V}\delta 2$  and  $\text{V}\gamma 9$  at protein (**Figure 21a**) and mRNA levels (**Figure 21b and 21c**). A second validation employed antibodies directed against  $\gamma\delta$  T-cell subsets ( $\text{V}\delta 1$  and  $\text{V}\delta 2$ ; no commercially available antibodies to  $\text{V}\delta 3$ ) to measure their mRNA expression.  $\text{V}\delta 1^{\text{neg}}\text{V}\delta 2^{\text{neg}}$ ,  $\text{V}\delta 1^+\text{V}\delta 2^{\text{neg}}$ , and  $\text{V}\delta 1^{\text{neg}}\text{V}\delta 2^+$  cells were sorted from  $\text{CAR}^{\text{neg}}$  T cells (to maximize the number of  $\text{V}\delta 2$  cells recovered by fluorescence-activated cell sorting, FACS) and subjected to DTEA (**Figure 22a**). As expected,  $\text{V}\delta 1^+\text{V}\delta 2^{\text{neg}}$ ,  $\text{V}\delta 1^{\text{neg}}\text{V}\delta 2^+$ , and  $\text{V}\delta 1^{\text{neg}}\text{V}\delta 2^{\text{neg}}$  expressed  $\text{V}\delta 1^*01$ ,  $\text{V}\delta 2^*02$ , and  $\text{V}\delta 3^*01$  mRNA species, respectively (**Figure 22b**). These two strategies supported the validity of the DTEA panel enabling the identity of  $\text{TCR}\gamma\delta$  to be determined in  $\text{CAR}^+$  T

cells. Therefore, we measured the mRNA levels for all three V $\delta$  alleles as present in electroporated, separated, and propagated CAR<sup>+</sup>  $\gamma\delta$  T cells which correlated with multi-parameter flow cytometry on gated CAR<sup>+</sup> T cells to reveal the frequencies of V $\delta$  subsets based on protein expression. The three V $\delta$  populations were present in ascending frequency (V $\delta$ 1>V $\delta$ 3>>>V $\delta$ 2) in the electroporated and propagated T cells (**Figure 22c**). CAR<sup>neg</sup>  $\gamma\delta$  T cells displayed similar frequencies of V $\delta$  TCR usage as CAR<sup>+</sup>  $\gamma\delta$  T cells. DTEA array also assessed V $\gamma$  usage, which is of particular utility because only one antibody against V $\gamma$ 9 is commercially available, thus limiting the tools with which to detect V $\gamma$  usage. Of note, V $\gamma$ 2, V $\gamma$ 7, V $\gamma$ 8 (both alleles), V $\gamma$ 9, and V $\gamma$ 10 were present in CAR<sup>+</sup> T-cell cultures (**Figure 22d**). A lack of commercially-available antibodies prevented assessment of pairing between individual V $\delta$  and V $\gamma$  chains on the T cells. The TCR usage described for  $\gamma\delta$  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<sup>+</sup> T cells expressed a polyclonal population of TCR $\gamma\delta$  chains.





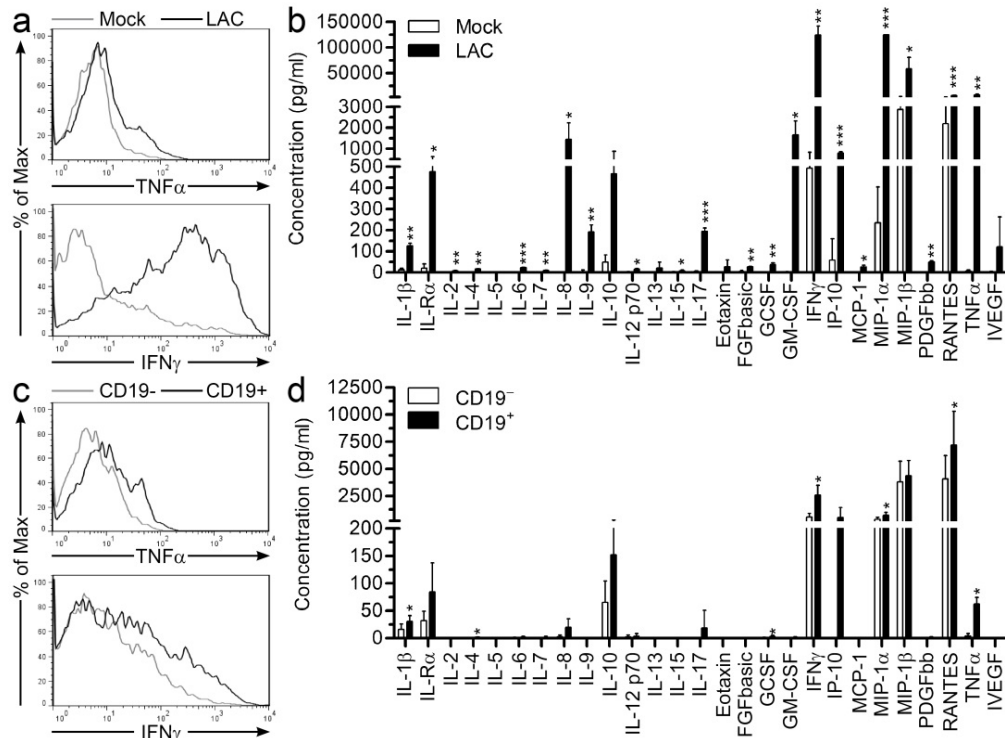
**Figure 21. Distribution of V $\delta$  and V $\gamma$  in  $\gamma\delta$  T cells Expanded on Aminobisphosphonate.** (a) Representative flow cytometry plot from T cells following 36 days of numeric expansion with Zol. (b) V $\delta$  and (c) V $\gamma$  allele mRNA expression in Zol-expanded T cells. Data are mean  $\pm$  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<sup>+</sup> γδ T cells.** (a) Representative FACS of Vδ populations (top) into Vδ1<sup>neg</sup>Vδ2<sup>neg</sup> (left), Vδ1<sup>+</sup>Vδ2<sup>neg</sup> (middle), and Vδ1<sup>neg</sup>Vδ2<sup>+</sup> (right) populations and (b) Vδ allele mRNA expression in sorted T cells. (c) Vδ1<sup>neg</sup>Vδ2<sup>neg</sup>, Vδ1<sup>+</sup>Vδ2<sup>neg</sup>, and Vδ1<sup>neg</sup>Vδ2<sup>+</sup> frequencies in gated CAR<sup>+</sup> γδ T-cell populations from four donors. (d) Vγ allele mRNA expression in CAR<sup>+</sup> 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 TCR $\gamma\delta$ and Introduced CAR

The functional activity of the CAR<sup>+</sup> 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<sup>2+</sup> 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  $\gamma\delta$  T cells, with the highest expression of IFN $\gamma$ , TNF $\alpha$ , and chemokines MIP-1 $\alpha$ , MIP-1 $\beta$ , and RANTES (**Figure 23b**). Interleukin-17 (IL17) has been shown to be important for anti-tumor efficacy of  $\gamma\delta$  T cells and this cytokine was secreted by CAR<sup>+</sup>  $\gamma\delta$  T cells. These results suggest that TCR $\gamma\delta$  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 TNF $\alpha$  and IFN $\gamma$  were produced by CAR<sup>+</sup>  $\gamma\delta$  T cells in response to CD19 (**Figure 23c**). A less diverse repertoire of cytokines was secreted following CAR stimulation when compared with stimulation of TCR $\gamma\delta$ , but IFN $\gamma$ , TNF $\alpha$ , MIP-1 $\alpha$ , MIP-1 $\beta$ , and RANTES were all increased in response to activation through CAR (**Figure 23d**). In aggregate, pro-inflammatory cytokines were upregulated by bi-specific CAR<sup>+</sup>  $\gamma\delta$  T cells through their TCR and CAR.

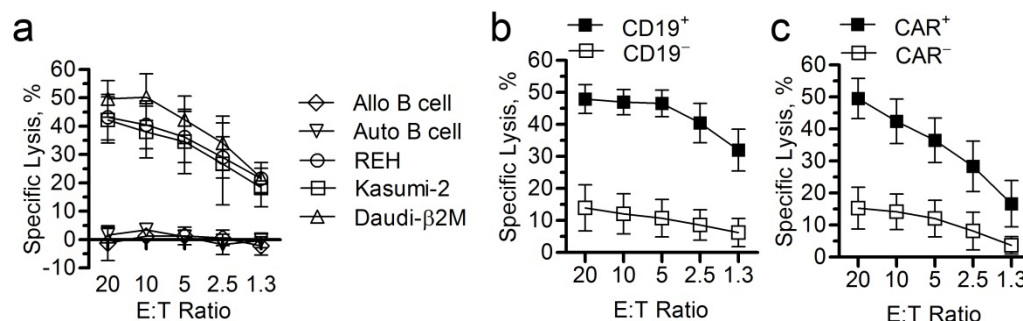


**Figure 23. Bi-specific  $\gamma\delta$  T cells Produce Pro-inflammatory Cytokines when Endogenous TCR and Introduced CAR are Stimulated.** (a)  $\text{CAR}^+$   $\gamma\delta$  T cells at Day 35 of co-culture on aAPC were stimulated for 4 hours with a mock cocktail (media alone) or Leukocyte Activation Cocktail (LAC, PMA/Ionomycin) to induce TCR stimulation and then analyzed by flow cytometry.  $\text{CAR}^+$  T cells were gated and tumor necrosis factor- $\alpha$  (TNF $\alpha$ , top) and interferon- $\gamma$  (IFN $\gamma$ , bottom) production is shown. (b) Luminex array (27-Plex) of cytokines secreted by  $\text{CAR}^+$   $\gamma\delta$  T cells in conditions described in (a). (c) Similar to (a) except that EL4-CD19<sup>neg</sup> and EL4-CD19<sup>+</sup> were used instead of Mock/LAC. (d) Same as (b) but with EL4-CD19<sup>neg</sup> and EL4-CD19<sup>+</sup> targets. Student's t-test for statistical analysis between (b) Mock and LAC and (d) EL4-CD19<sup>neg</sup> and EL4-CD19<sup>+</sup> where \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$ . Data are representative of four donors for (a) and (c) and mean  $\pm$  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<sup>+</sup> $\gamma\delta$ T cells Exhibit Enhanced Anti-tumor Effects against CD19<sup>+</sup>

#### Targets *in vitro*

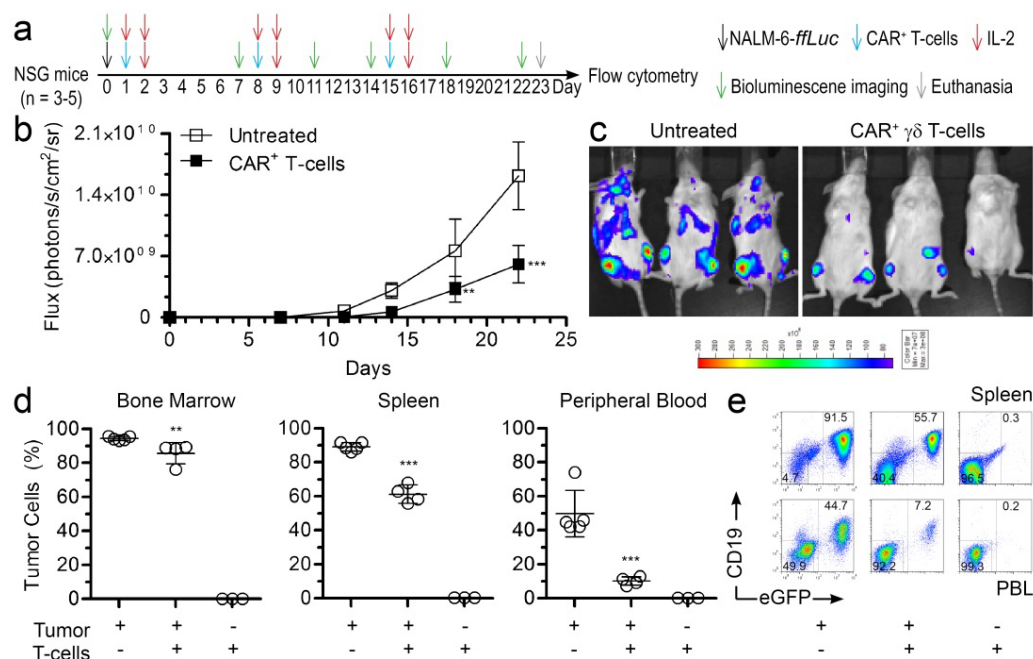
It was anticipated that  $\gamma\delta$  T cells would display endogenous cytotoxicity to leukemia cells. Therefore,  $\gamma\delta$  T cells without CAR were numerically expanded on aAPC in order to test their anti-leukemia activity. Human CD19<sup>+</sup> B-ALL cell lines (REH, Kasumi2, and Daudi genetically modified to express  $\beta$ 2M) were lysed by CAR<sup>neg</sup>  $\gamma\delta$  T cells while primary, healthy CD19<sup>+</sup> 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<sup>neg</sup>  $\gamma\delta$  T cells. In particular, EL4 and NALM6 cells were largely resistant to cytolysis by  $\gamma\delta$  T cells. Thus, the ability of the CD19-specific CAR to amplify the inherent anti-tumor activity of  $\gamma\delta$  T cells was investigated. Enforced expression of CD19 on the surface of EL4 cells improved targeting and killing of this cell line by CAR<sup>+</sup>  $\gamma\delta$  T cells at significantly higher ( $p = 0.0001$ ) levels compared with the parental CD19<sup>neg</sup> EL4 cell line (**Figure 24b**). Similarly, CAR<sup>+</sup>  $\gamma\delta$  T cells exhibited improved ability ( $p = 0.001$ ) to kill CD19<sup>+</sup> NALM6 cells compared with CAR<sup>neg</sup>  $\gamma\delta$  T cells (**Figure 24c**). In summary, the introduced CAR enhanced the specific killing capability of genetically modified  $\gamma\delta$  T cells.



**Figure 24. Specific lysis of CD19<sup>+</sup> Tumor Cell Lines by CAR<sup>+</sup> γδ T cells.** (a) Standard 4-hour CRA of (a) CAR<sup>neg</sup> γδ T cells against CD19<sup>+</sup> B-ALL cell lines (REH, Kasumi2, and Daudi-β2M) or primary CD19<sup>+</sup> B cells from autologous (Auto) or allogeneic (Allo) donors, (b) CAR<sup>+</sup> γδ T cells against EL4-CD19<sup>neg</sup> (open squares) and EL4-CD19<sup>+</sup> (closed squares) tumor cells, and (c) CAR<sup>neg</sup> γδ T cells (open squares) and CAR<sup>+</sup> γδ T cells (closed squares) against CD19<sup>+</sup> 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<sup>+</sup> $\gamma\delta$ T cells can Target CD19<sup>+</sup> Tumor *in vivo*

The ability of electroporated and propagated  $\gamma\delta$  T cells to target CD19<sup>+</sup> tumor was then investigated *in vivo*. NALM6 is an aggressive CD19<sup>+</sup> B-cell leukemia model and immunocompromised mice engrafted with 10<sup>5</sup> 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<sup>+</sup>  $\gamma\delta$  T cells (Donor#4 from **Figure 22c**) compared to untreated mice (**Figure 25**). Mice in treatment group receiving CAR<sup>+</sup> 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<sup>+</sup>  $\gamma\delta$  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<sup>+</sup>  $\gamma\delta$  T cells exhibited significant reductions in tumor burden (CD19<sup>+</sup>eGFP<sup>+</sup>) in the bone marrow, spleen, and peripheral blood (**Figure 25d and 25e**). These data reveal that polyclonal CAR<sup>+</sup>  $\gamma\delta$  T cells exhibit therapeutic activity *in vivo*.



**Figure 25. *In vivo* Anti-tumor Activity of CAR<sup>+</sup> γδ T cells.** (a) Schematic of experiment. (b) BLI derived from eGFP<sup>+</sup>ffLuc<sup>+</sup>CD19<sup>+</sup> NALM-6 tumor and (c) representative images of mice at day 22. (d) Post-mortem analysis of tissues and blood where tumor cells (CD19<sup>+</sup>eGFP<sup>+</sup>) 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<sup>+</sup>eGFP<sup>+</sup> 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 2<sup>nd</sup> generation CAR could (i) drive the numeric expansion of T cells independent of usage of TCR $\gamma\delta$  chains and (ii) augment the lytic potential of CD19<sup>+</sup> tumors by  $\gamma\delta$  T cells. Propagating bi-specific CAR<sup>+</sup> T cells with a broad diversity of TCR $\gamma\delta$  chains is desirable based on their therapeutic potential. Indeed,  $\gamma\delta$  T cells other than those expressing V $\gamma$ 9V $\delta$ 2 have been generated from PBMC using TCR $\gamma\delta$ -specific and CD3-specific mAbs.(313-315) These prior approaches did not comprehensively measure TCR $\gamma\delta$  isotype expression nor did they yield V $\delta$ 1 and V $\delta$ 3 at frequencies as high as seen in this study. The V $\gamma$ 2 TCR chain was detected on our T cells, which has been described to pair with V $\delta$ 2, and these T cells can have antigen presentation capabilities.(166) Our CAR<sup>+</sup>  $\gamma\delta$  T cells expressed molecules consistent with antigen presentation, *e.g.*, CD86, CD137L, and HLA-DR (data not shown), and V $\gamma$ 9V $\delta$ 2 cells have served as aAPC for  $\alpha\beta$  T cells.(298) Future experiments will investigate if our polyclonal CAR<sup>+</sup>  $\gamma\delta$  T cells also have an ability to serve as aAPC. Also present were T-cell sub-populations expressing V $\gamma$ 7, and V $\gamma$ 8, and V $\gamma$ 10, 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<sup>+</sup> T cells that maintained a polyclonal TCR $\gamma\delta$  expression.

### III.D.2. Changes Observed in V $\delta$ Populations Following Expansion on aAPC

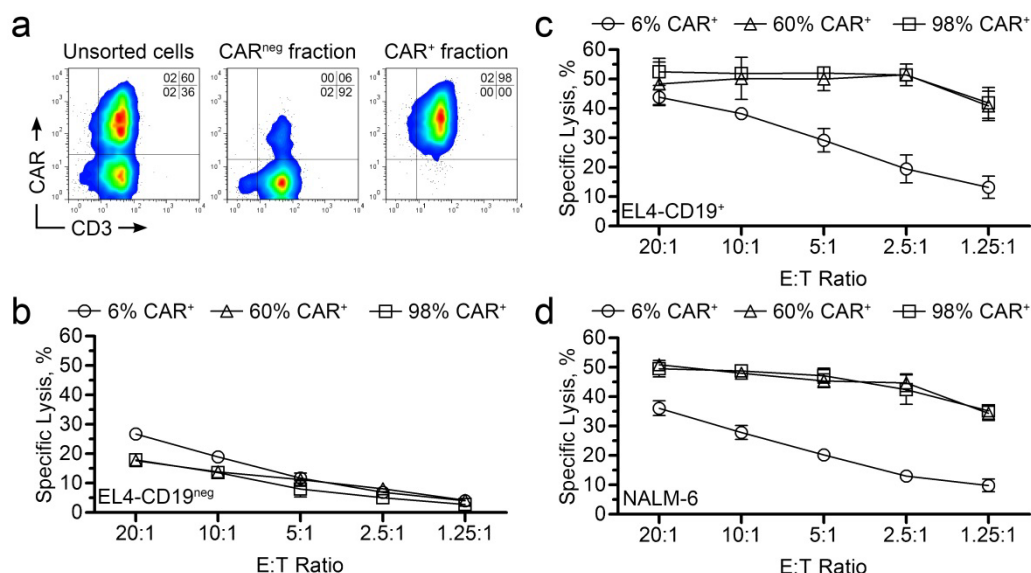
The repertoire of TCR $\gamma\delta$  chains employed by CAR<sup>+</sup> T cells was similar to the initial pool of  $\gamma\delta$  T cells in PBMC with two exceptions. We noted an increase in V $\delta$ 3 usage, but this may be advantageous as it is associated with specificity for viruses that could offer enhanced immune responses to viral infections in immunocompromised patients receiving therapy.(165) A decrease in V $\gamma$ 9V $\delta$ 2 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 V $\gamma$ 9V $\delta$ 2 ligand expression in the co-culture. Whether this loss of V $\gamma$ 9V $\delta$ 2 TCR expression was due to preferential activation induced cell death or selective out-growth of T cells expressing V $\delta$ 1 and V $\delta$ 3 TCR is not known. Nonetheless, V $\gamma$ 9V $\delta$ 2 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 $\gamma\delta$ T cells

Recombinant retroviruses have been previously employed to achieve stable expression of CARs in  $\gamma\delta$  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  $\gamma\delta$  T cells capable of CAR-mediated (i) production and secretion of pro-inflammatory cytokines in response to CD19, (ii) enhanced lysis of CD19<sup>+</sup> tumor targets, and (iii) *in vivo* anti-tumor activity against a CD19<sup>+</sup> tumor. The ability of these T cells to exhibit effector functions was not correlated to a particular V $\delta$  or V $\gamma$  usage as cells with different V $\delta$  TCR frequencies (**Figure 22c**) produced the same cytokines (**Figure 23**) and displayed similar cytotoxicity of CD19<sup>+</sup> targets (**Figure 24b**). We noted that frequency of CAR expression was more variable on  $\gamma\delta$  T cells compared with  $\alpha\beta$  T cells. This was likely due to an endogenous ability of K562 cells to sustain proliferation of  $\gamma\delta$  T cells independent of CAR. Nevertheless, adoptive transfer of  $\gamma\delta$  T cells of which 60% expressed CAR could still yield the same *in vitro* lytic ability as 98% CAR<sup>+</sup>  $\gamma\delta$  T cells (**Figure 26**). This indicated that (i) CAR<sup>+</sup>  $\gamma\delta$  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  $\gamma\delta$  T cells. Furthermore, the chimeric signaling molecules in the CAR endodomain could be specifically designed to enhance triggering of  $\gamma\delta$  T cells. For example,  $\gamma\delta$  T cells can be activated through Fc $\gamma$ RIIIA (CD16) in the TCR complex,(319) which raises the possibility that signaling through chimeric FcR $\gamma$  (as compared with CD3 $\zeta$  in our current design) in a CAR endodomain may improve activation. However, CD16 was not detected on CAR<sup>+</sup>  $\gamma\delta$  T cells in this study (data not shown). Since clinical responses against CD19<sup>+</sup> 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  $\gamma\delta$  T cells.



**Figure 26. Specific Lysis of CD19<sup>+</sup> Tumor Cell Lines by CAR<sup>+</sup>, CAR<sup>++</sup>, and CAR<sup>+++</sup>  $\gamma\delta$  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<sup>neg</sup> and CAR<sup>+</sup> fractions are displayed in the middle and right, respectively. Four-hour CRA (Day 19 of co-culture on aAPC) of  $\gamma\delta$  T cells genetically modified to enforce expression of CD19-specific CAR with 6% (CAR<sup>+</sup>, circles), 60% (CAR<sup>++</sup>, triangles), and 98% (CAR<sup>+++</sup>, squares) expression of CAR targeting (b) EL4-CD19<sup>neg</sup>, (c) EL4-CD19<sup>+</sup>, and (d) CD19<sup>+</sup> NALM-6 tumor cells. Data are mean  $\pm$  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 $\gamma\delta$ T cell used for CAR Immunotherapy

In addition to improving CAR expression on  $\gamma\delta$  T cells, the type of  $\gamma\delta$  T cell arising after electroporation with SB system and propagation on aAPC could be manipulated to further improve anti-tumor activity. For instance, some  $\gamma\delta$  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 IFN $\gamma$ .(325) Inducible co-stimulator of T cells (ICOS) leads to IL17 polarization in CD4<sup>+</sup> T cells and CD28 co-stimulation overcame this effect to dictate that CD4<sup>+</sup> T cells now produce IFN $\gamma$ .(326) CD86 is one of the co-stimulatory molecules on our aAPC and the majority of CAR<sup>+</sup>  $\gamma\delta$  T cells secrete IFN $\gamma$  in response to CD19 with diminished production of IL17. Furthermore, the CAR contains a chimeric CD28 endodomain which may contribute to IFN $\gamma$  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<sup>+</sup>  $\gamma\delta$  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<sup>+</sup>  $\gamma\delta$  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<sup>+</sup>  $\gamma\delta$  T cells. Our data provides a clinically-appealing approach to numerically expand and manipulate CAR<sup>+</sup> T cells with multiple V $\gamma$  and V $\delta$  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  $\gamma\delta$  T cells that will have broad anti-tumor immunity. The *rationale* for this chapter is that (i)  $\text{CAR}^{\text{neg}}$  polyclonal  $\gamma\delta$  T cells proliferated in parallel to  $\text{CAR}^+$   $\gamma\delta$  T cells described in **Chapter III** on aAPC, (ii) no current expansion protocols exist for polyclonal  $\gamma\delta$  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)  $\gamma\delta$  T cells expressing V $\delta$ 1 are correlated with long-term remissions in cancer therapy but have not been directly infused as an adoptive immunotherapy, (v)  $\gamma\delta$  T cells expressing V $\delta$ 2 have shown anti-tumor effects as direct adoptive immunotherapies, (vi)  $\gamma\delta$  T cells expressing V $\delta$ 3 have not been described to have direct anti-tumor immunity leaving a gap in the field of knowledge, and (vii) a polyclonal approach to  $\gamma\delta$  T cell immunotherapy could target multiple ligands on the tumor through a diverse repertoire of TCR $\gamma\delta$ . Therefore, development of an expansion protocol to generate clinically-relevant numbers of polyclonal  $\gamma\delta$  T cells would have implications as both cancer immunotherapies and for immunologists studying  $\gamma\delta$  T cells.



## IV.B. Introduction

Human  $\gamma\delta$  T cells exhibit inherent anti-tumor activity and hold promise for immunotherapy of cancer. They are distinguished by the heterodimeric pairing of  $\gamma$  and  $\delta$  T-cell receptor (TCR) chains from the more prevalent  $\alpha\beta$  T cell lineage (~95% of circulating T cells), which are defined by TCR $\alpha$ /TCR $\beta$  heterodimers.(327) TCR $\alpha\beta$  recognizes peptide complexed with MHC but TCR $\gamma\delta$  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  $\gamma\delta$  TCRs may have appeal for human application.

$\gamma\delta$  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  $\gamma\delta$  T cells.(314, 328) Proliferation of monoclonal  $\gamma\delta$  T cell populations (V $\gamma$ 9V $\delta$ 2) 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  $\gamma\delta$  T cells was not curative as a stand-alone therapy.(318) Novel polyclonal  $\gamma\delta$  T cell expansion protocols are needed to improve upon these findings, but are lacking in clinically-relevant methods to expand multiple  $\gamma\delta$  T cell subsets in one cellular therapy product.

Since many ligands that signal through  $\gamma\delta$  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<sup>+</sup> T cell growth *ex vivo*

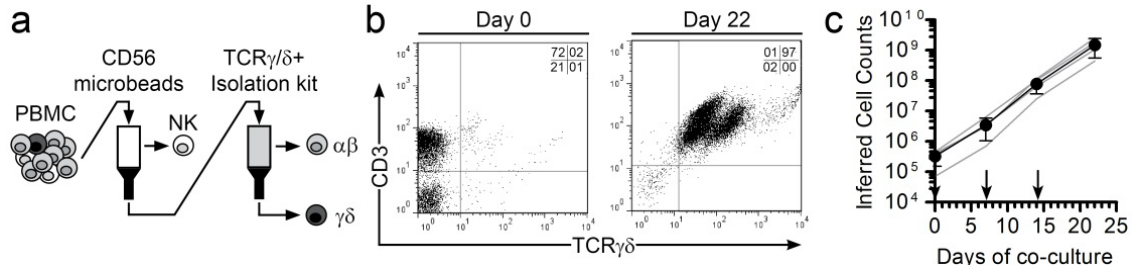
and are derived from K562 cells, a natural cytolytic target of  $\gamma\delta$  T cells.(57, 280, 310, 329) As seen in **Chapter III**, CAR-modified  $\gamma\delta$  T cells expanded on aAPC while expressing multiple TCR $\gamma\delta$  alleles and displayed enhanced cytolysis to antigen-positive tumors.(311) Moreover,  $\gamma\delta$  T cells not expressing CAR were present in CAR<sup>+</sup>  $\gamma\delta$  T cell cultures in high frequencies (**Figure 19a, bottom right panels**). Therefore, we hypothesized that  $\gamma\delta$  T cells could expand on aAPC independent of CAR<sup>+</sup> T cells and that these  $\gamma\delta$  T cells would maintain a polyclonal TCR $\gamma\delta$  repertoire. Given that the aAPC are available as a master-cell bank, these data provide a translational pathway for adapting  $\gamma\delta$  T cells for human application. Thus, this could be the first time that polyclonal  $\gamma\delta$  T cells could be used for cancer immunotherapy.

## IV.C. Results

### IV.C.1. Propagation of $\gamma\delta$ T cells on aAPC

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

TCR $\gamma\delta$  (**Figure 27b**). Cultures yielded  $>10^9$   $\gamma\delta$  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  $\gamma\delta$  T cells were rare in peripheral blood, they were readily sorted then expanded on aAPC to sufficient numbers for experiments and potential clinical application.

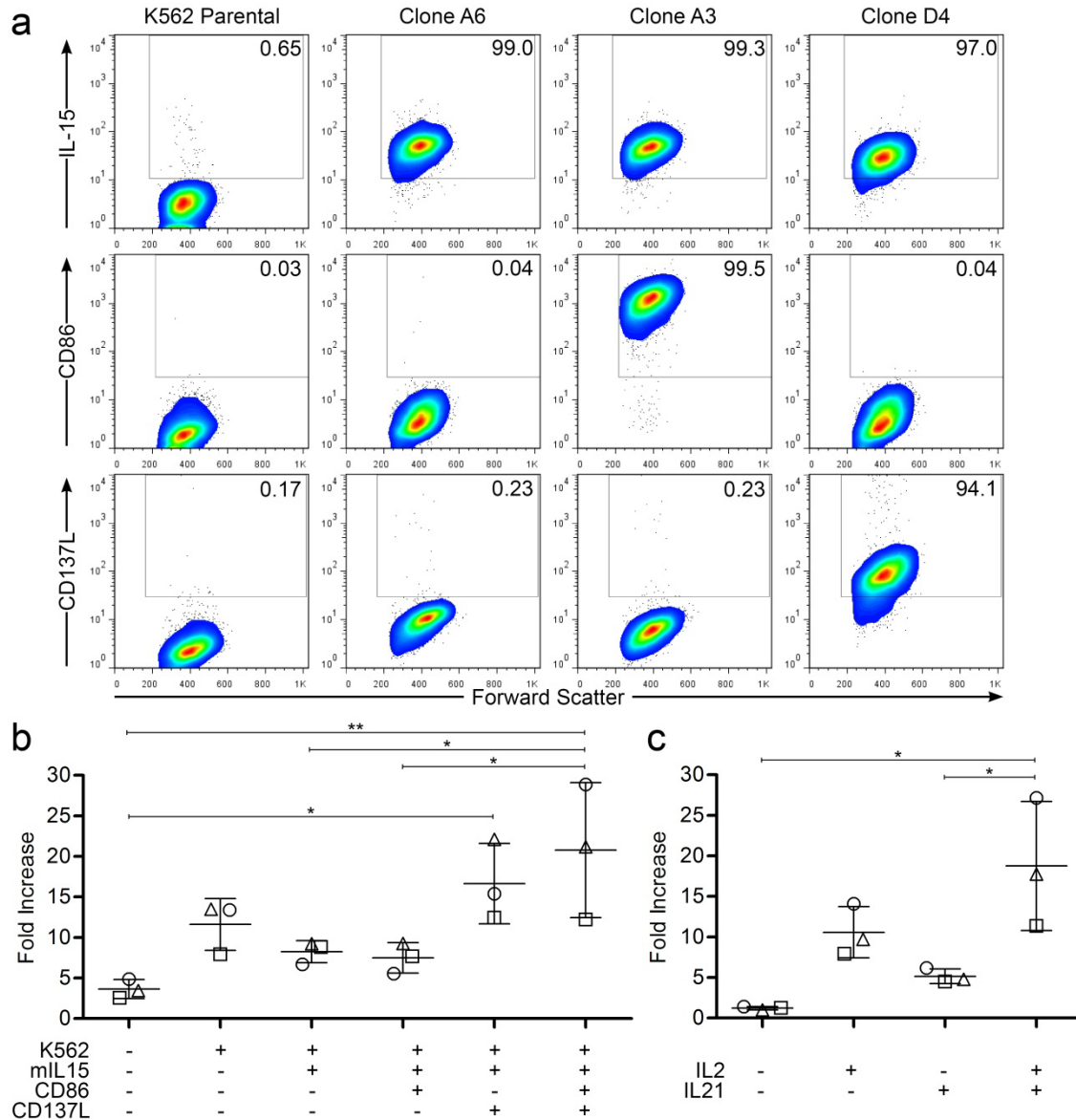


**Figure 27. Sustained Proliferation of  $\gamma\delta$  T cells on aAPC and IL2/21.** (a) Schematic of experimental design where NK cells are in open shapes,  $\alpha\beta$  T cells are in light gray shapes, and  $\gamma\delta$  T cells are in dark gray shapes. Columns represent paramagnetic isolation. (b) Expression by flow cytometry of CD3 (y-axis) and TCR $\gamma\delta$  (x-axis) in PBMC prior to isolation of  $\gamma\delta$  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  $\pm$  SD from 4 healthy donors, gray lines are individual donors, and arrows represent addition of  $\gamma$ -irradiated aAPC.

#### IV.C.2. Roles for Co-stimulation and Cytokine Support in $\gamma\delta$ T cell Proliferation on aAPC

The mechanism of  $\gamma\delta$  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  $\gamma\delta$  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  $\gamma\delta$  T cells and  $\gamma$ -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  $\gamma\delta$  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  $\gamma\delta$  T cell propagation was only observed with mIL15<sup>+</sup>CD137L<sup>+</sup> and mIL15<sup>+</sup>CD86<sup>+</sup>CD137L<sup>+</sup> aAPC over IL2 and IL21 alone. After establishing that co-stimulation on aAPC was necessary for  $\gamma\delta$  T cell proliferation, IL2 and IL21 were added separately or in combination to assess their contribution to growth on clone#4 aAPC. No  $\gamma\delta$  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  $\gamma\delta$  T cells (**Figure 28c**).

This validated our approach to use both IL2 and IL21 for maximum  $\gamma\delta$  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  $\gamma\delta$  T cell proliferation *ex vivo*.

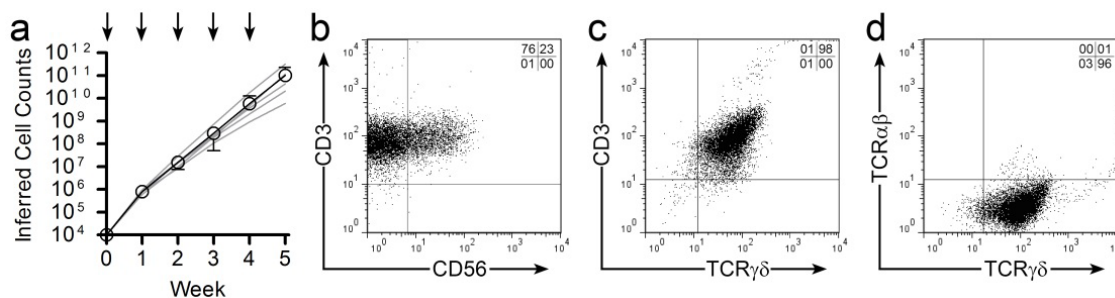


**Figure 28. Co-stimulation and Cytokine Requirements for  $\gamma\delta$  T cell Expansion on aAPC *ex vivo*.** (a) Surface phenotype of aAPC expressing single co-stimulatory molecules with membrane-bound IL15 (mIL15). (b)  $\gamma\delta$  T cell proliferation was measured after 10 days of growth with IL2 and IL21 on (i) no aAPC, (ii) parental K562 cells, (iii) mIL15<sup>+</sup> aAPC (clone A6), (iv) mIL15<sup>+</sup>CD86<sup>+</sup> aAPC (clone A3), (v) mIL15<sup>+</sup>CD137L<sup>+</sup> aAPC (clone D4), or (vi) clone#4 aAPC. All aAPC were  $\gamma$ -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 $\gamma\delta$ T cells Expansion on aAPC

Umbilical cord blood (UCB) is a source of  $\gamma\delta$  T cells with unique use for immunotherapy because they have limited immunological education and thus potential utility in allogeneic settings. Moreover, UCB-derived  $\gamma\delta$  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  $\gamma\delta$  T cells are a small fraction of an already limited resource. Fluorescence activated cell sorting (FACS) was used to isolate  $\gamma\delta$  T cells in order to maximize yields and purity of this valuable resource. Indeed, clone#4 aAPC induced substantial proliferation of  $\gamma\delta$  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  $\gamma\delta$  T cells (Range:  $6 \times 10^9 - 3 \times 10^{11}$ ;  $n = 5$ ) were propagated from just  $10^4$   $\gamma\delta$  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,  $\gamma\delta$  T cell cultures were pure as assessed by uniform expression of CD3 (**Figure 29b**) and TCR $\gamma\delta$  (**Figure 29c**) without expression of TCR $\alpha\beta$  (**Figure 29d**) or presence of CD3<sup>neg</sup>CD56<sup>+</sup> NK cells (**Figure 29b**). Collectively, these data demonstrate that aAPC clone#4 when used with IL2 and IL21 could sustain the proliferation of  $\gamma\delta$  T cells *ex vivo* from limited starting populations.





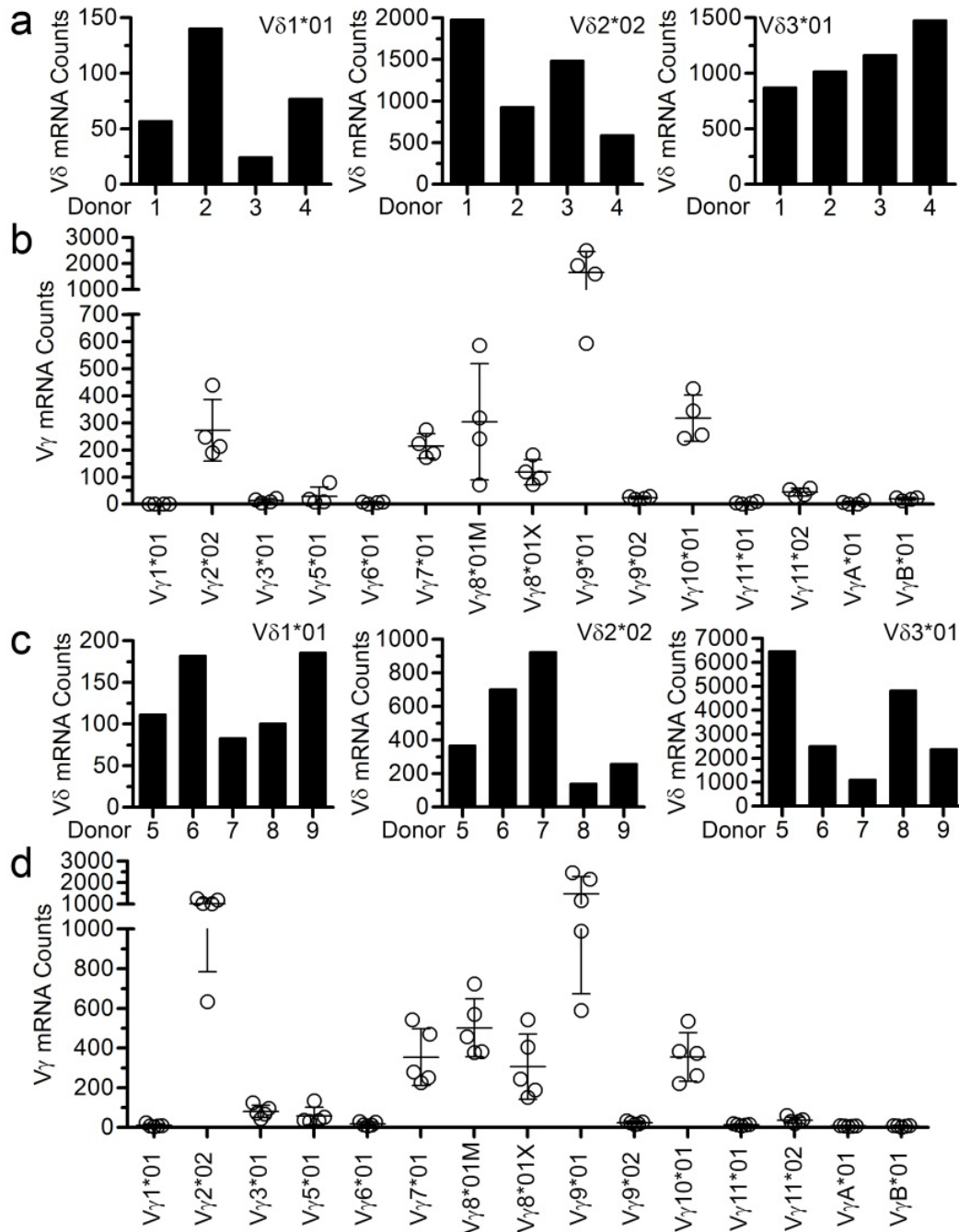
**Figure 29. Expansion of UCB-derived  $\gamma\delta$  T cells on aAPC.**  $\gamma\delta$  T cells were sorted by FACS following staining with CD3 and TCR $\gamma\delta$  and were stimulated weekly with clone#4 aAPC, IL2, and IL21 (a) Total inferred cell numbers from co-cultures where black line represents mean  $\pm$  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 TCR $\gamma\delta$  (x-axis), and (d) TCR $\alpha\beta$  (y-axis) and TCR $\gamma\delta$  (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 $\gamma$ and $\delta$ TCR Usage in aAPC-propagated $\gamma\delta$ T cells

Previously,  $CAR^+$   $\gamma\delta$  T cells expanded on clone#4 aAPC maintained polyclonal repertoire of  $TCR\gamma$  and  $TCR\delta$  chains, and  $\gamma\delta$  T cells proliferating in parallel to  $CAR^+$   $\gamma\delta$  T cells also maintained polyclonal  $TCR\gamma\delta$  distribution (**Chapter III**).<sup>(311)</sup> Whether the aAPC-expanded  $\gamma\delta$  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  $\gamma\delta$  T cells subsets in one cellular product for cancer therapy.

##### *IV.C.4.a. $V\delta$ and $V\gamma$ mRNA Expression*

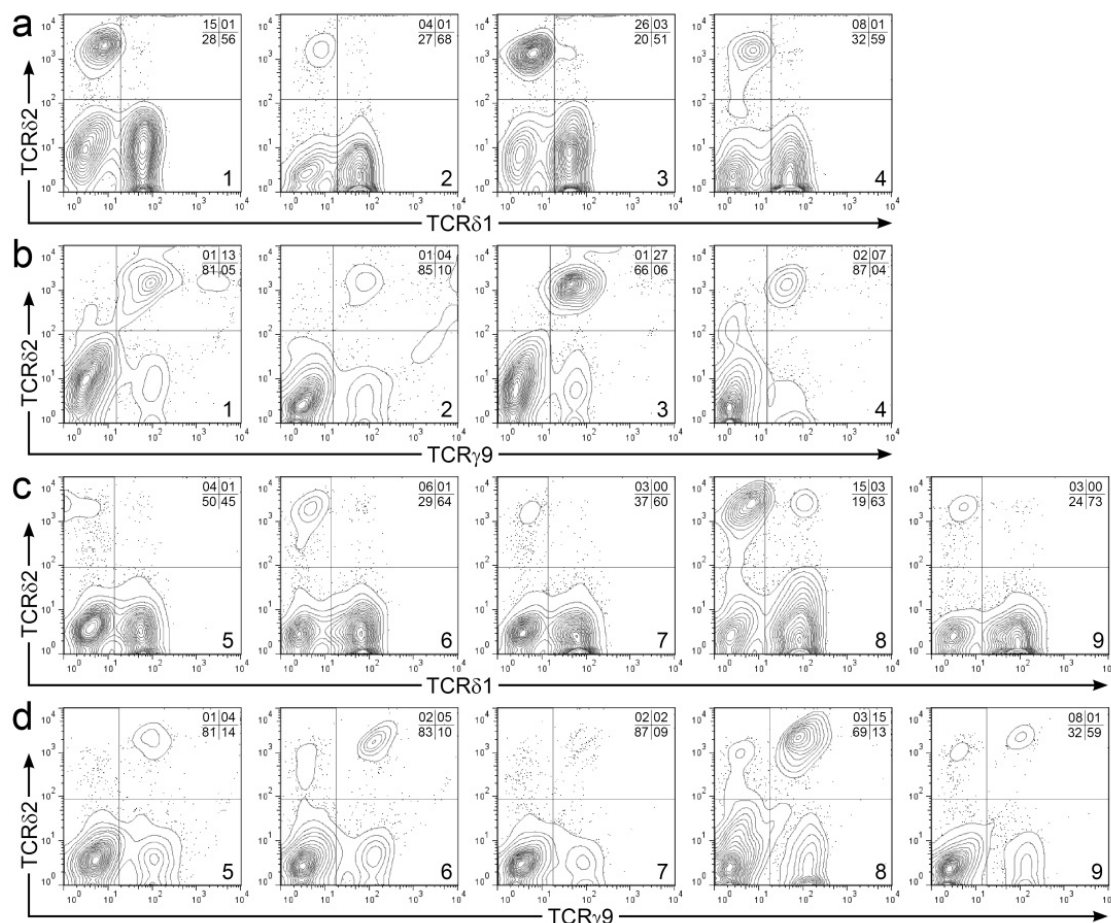
Now that it is established that  $\gamma\delta$  T cells can expand on aAPC independently of  $CAR^+$  T cells (**Figures 27, 28, and 29**), the TCR isotype variable (V) region repertoire was evaluated at the mRNA level by DTEA. As anticipated, mRNA species for all three  $V\delta$  alleles were identified (**Figure 30a**) and  $V\gamma 2$ ,  $V\gamma 5$ ,  $V\gamma 7$ ,  $V\gamma 8$  (two alleles),  $V\gamma 9$ ,  $V\gamma 10$ , and  $V\gamma 11$  mRNA species were co-expressed in the aAPC-expanded  $\gamma\delta$  T cells from PBMC (**Figure 30b**). Similar polyclonal TCR expression of  $V\delta$  (**Figure 30c**) and  $V\gamma$  (**Figure 30d**) was observed in  $\gamma\delta$  T cells expanded from UCB with fewer  $V\delta 2$  cells, more  $V\gamma 2$  and  $V\gamma 5$  cells, and presence of  $V\gamma 3$  cells not seen in PBMC. Thus, aAPC are able to repeatedly expand  $\gamma\delta$  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. TCR $\gamma\delta$ Surface Protein Expression*

After establishing V $\gamma$  and V $\delta$  mRNA expression from a number of different isotypes, surface expression of TCR $\gamma$  and TCR $\delta$  was investigated. However, there are only 3 commercially available antibodies specific for individual TCR $\gamma\delta$  isotypes, which are specific for TCR $\delta$ 1, TCR $\delta$ 2, and TCR $\gamma$ 9. As was seen in CAR<sup>+</sup>  $\gamma\delta$  T cells, aAPC-expanded  $\gamma\delta$  T cells from PBMC stained for all three V $\delta$  populations (TCR $\delta$ 1<sup>+</sup>TCR $\delta$ 2<sup>neg</sup>, TCR $\delta$ 1<sup>neg</sup>TCR $\delta$ 2<sup>+</sup>, and TCR $\delta$ 1<sup>neg</sup>TCR $\delta$ 2<sup>neg</sup>), corroborating DTEA detection of V $\delta$ 1, V $\delta$ 2, and V $\delta$ 3 populations of  $\gamma\delta$  T cells, respectively (**Figure 31a**). Moreover, TCR $\delta$  expression frequencies followed the trend of TCR $\delta$ 1>TCR $\delta$ 3>TCR $\delta$ 2, and most TCR $\delta$ 2 chains paired with TCR $\gamma$ 9 (**Figure 31b**). Fewer TCR $\delta$ 2 cells were seen in UCB-derived  $\gamma\delta$  T cells (**Figure 31c**) compared to PBMC-derived  $\gamma\delta$  T cells (**Figure 31a**), but UCB-derived  $\gamma\delta$  T cells followed the same TCR $\delta$ 1>TCR $\delta$ 3>TCR $\delta$ 2, trend and most V $\delta$ 2 paired with V $\gamma$ 9 as expected (**Figure 31d**). Analysis of other V $\gamma$  pairings with V $\delta$  could not be performed because there are no other V $\gamma$ -specific commercially antibodies available. Thus, aAPC-expanded  $\gamma\delta$  T cells were polyclonal at both mRNA and protein levels, and this protocol therefore represents the first clinically-relevant expansion approach of polyclonal  $\gamma\delta$  T cells.

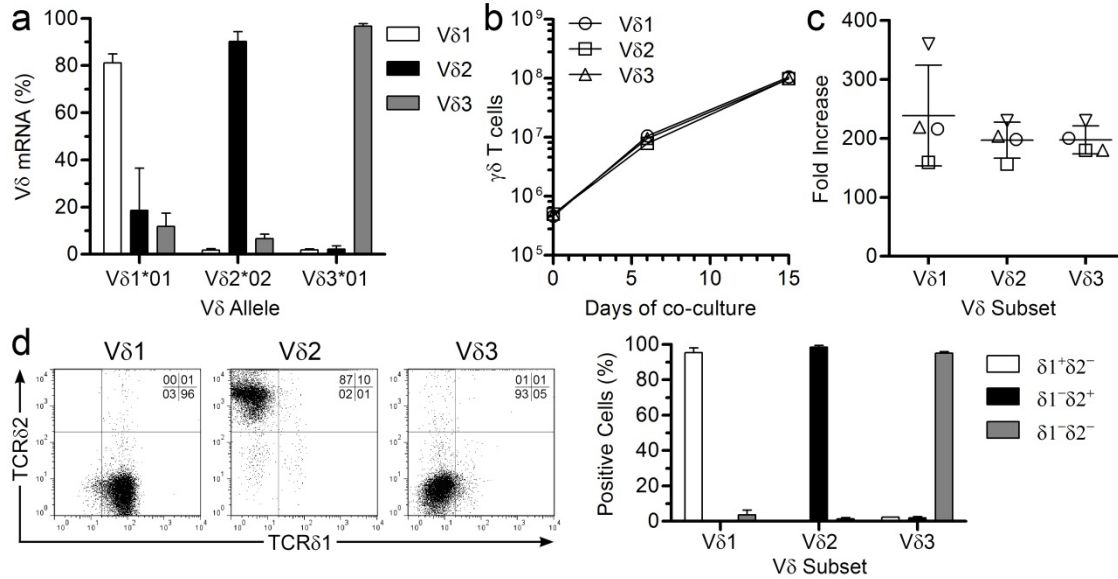


**Figure 31. TCRδ and TCRγ Isotype Surface Expression on aAPC-expanded γδ T cells.** Expression by flow cytometry of (a) TCRδ2 (y-axes) and TCRδ1 (x-axes) and (b) TCRδ2 (y-axes) and TCRγ9 (x-axes) in PBMC-derived γδ T cells at day 22 of co-culture on aAPC/IL2/IL21. Expression by flow cytometry of (c) 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 V $\delta$ 3 Subset and V $\delta$ Lineage Propagation*

Little is known about the V $\delta$ 3 lineage of  $\gamma\delta$  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 TCR $\delta$ 1<sup>neg</sup>TCR $\delta$ 2<sup>neg</sup> cells were, in fact, V $\delta$ 3 cells was warranted. Complicating this matter is the fact that no commercially available antibodies for TCR $\delta$ 3. However, an indirect means was successfully used by combining FACS and DTEA. As there are only three V $\delta$  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,  $\gamma\delta$  T cells expanded in the presence of CAR<sup>+</sup> T cells (**Chapter III**) were sorted for TCR $\delta$ 1<sup>+</sup>TCR $\delta$ 2<sup>neg</sup>, TCR $\delta$ 1<sup>neg</sup>TCR $\delta$ 2<sup>+</sup>, and TCR $\delta$ 1<sup>neg</sup>TCR $\delta$ 2<sup>neg</sup>  $\gamma\delta$  T cells populations by FACS and they expressed only V $\delta$ 1\*01, V $\delta$ 2\*02, and V $\delta$ 3\*01 mRNA, respectively (**Figure 22a and 22b**).<sup>(311)</sup> The second approach directly applied the same techniques to  $\gamma\delta$  T cells expanded on aAPC as described in **Chapter IV** without CAR<sup>+</sup> T cells. Again, TCR $\delta$ 1<sup>+</sup>TCR $\delta$ 2<sup>neg</sup>, TCR $\delta$ 1<sup>neg</sup>TCR $\delta$ 2<sup>+</sup>, and TCR $\delta$ 1<sup>neg</sup>TCR $\delta$ 2<sup>neg</sup> populations isolated by FACS consisted primarily of V $\delta$ 1\*01, V $\delta$ 2\*02, and V $\delta$ 3\*01 mRNA, respectively, and were therefore denoted V $\delta$ 1, V $\delta$ 2, and V $\delta$ 3, respectively (**Figure 32a**). It is important to note that V $\delta$ 1\*01 only resulted in ~150 mRNA counts whereas V $\delta$ 2\*02 and V $\delta$ 3\*01 ranged in the ~1000-2000 mRNA count range (**Figure 30**), so the purity as measured by mRNA counts appeared to have contaminating V $\delta$ 1 cells in V $\delta$ 2 and V $\delta$ 3 populations but these populations were minor contributors in the V $\delta$ 1 population as measured by flow

cytometry (**Figures 32d and 32e**). Furthermore, the FACS sorted V $\delta$ 1, V $\delta$ 2, and V $\delta$ 3 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 V $\delta$  mRNA signatures were observed suggesting the cells remained pure during propagation (data not shown). As expected, all three V $\delta$  populations proliferated well on aAPC as separate populations (**Figure 32b**), where fold increase capability was ranked as V $\delta$ 1>V $\delta$ 3=V $\delta$ 2 although there were no statistically different differences (**Figure 32c**). Indeed, there are more V $\delta$ 1 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  $\gamma\delta$  T cell surface where V $\delta$ 1, V $\delta$ 2, and V $\delta$ 3 subsets were pure for TCR $\delta$ 1<sup>+</sup>TCR $\delta$ 2<sup>neg</sup>, TCR $\delta$ 1<sup>neg</sup>TCR $\delta$ 2<sup>+</sup>, and TCR $\delta$ 1<sup>neg</sup>TCR $\delta$ 2<sup>neg</sup>, respectively, after 15 days of isolated expansion on aAPC (**Figure 32d and 32e**). All separated V $\delta$  subsets co-expressed CD3 and TCR $\gamma\delta$  verifying that they were, in fact,  $\gamma\delta$  T cells (**Figures 34a and 34b**). Collectively, these results showed that (i) TCR $\delta$ 1<sup>neg</sup>TCR $\delta$ 2<sup>neg</sup>  $\gamma\delta$  T cells contained the V $\delta$ 3 lineage, (ii) DTEA accurately measured V $\delta$  mRNA, (iii) V $\delta$ 1, V $\delta$ 2, and V $\delta$ 3 lineages are stimulated by aAPC leading to their proliferation, and (iv) aAPC-expanded  $\gamma\delta$  T cells are truly polyclonal.



**Figure 32. Vδ Subset Separation, Propagation, and Resultant TCR Expression on Sorted T cells.** PBMC were sorted for  $\gamma\delta$  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<sup>+</sup>TCRδ2<sup>neg</sup>, TCRδ1<sup>neg</sup>TCRδ2<sup>+</sup>, and TCRδ1<sup>neg</sup>TCRδ2<sup>neg</sup> cells in Vδ1, Vδ2, and Vδ3 subsets. Data are mean  $\pm$  SD (n = 3-4).



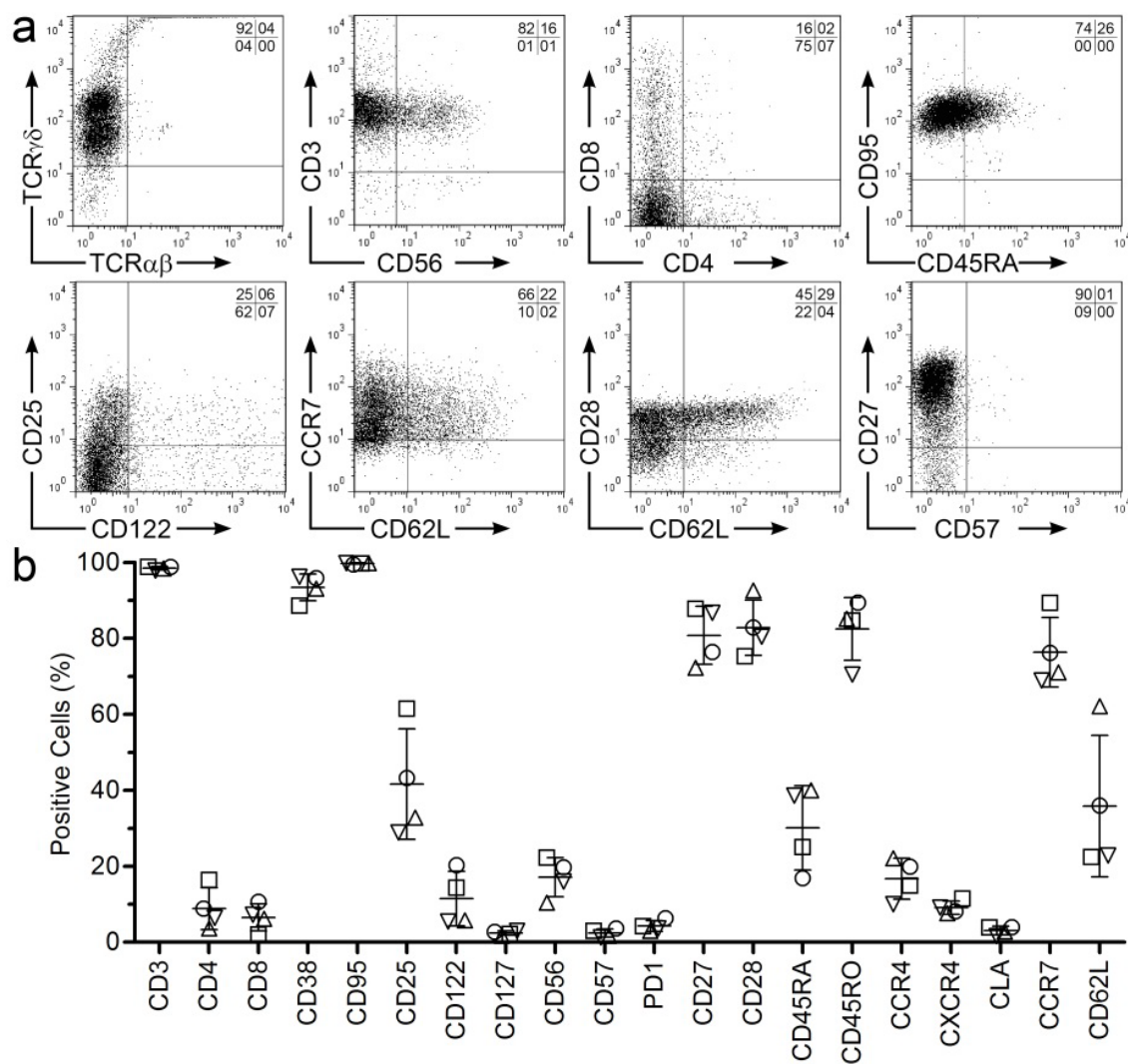
#### IV.C.5. Immunophenotype of $\gamma\delta$ T cells Expanded on aAPC

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  $\gamma\delta$  T cells cultures first as a polyclonal population to be used as therapy and then as sorted V $\delta$  populations to gain insight into lineage differences.

##### *IV.C.5.a. Immunophenotype of Polyclonal $\gamma\delta$ 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  $\gamma\delta$  T cell surfaces was performed as a mixed V $\delta$  population. After 22 days of co-culture on aAPC, few  $\alpha\beta$  T cells (TCR $\alpha\beta$ ) and NK cells (CD3<sup>neg</sup>CD56<sup>+</sup>) were detected in the cultures where strong staining for  $\gamma\delta$  T cells (TCR $\gamma\delta$ ) was observed (**Figure 33a**). Most  $\gamma\delta$  T cells were CD4<sup>neg</sup>CD8<sup>neg</sup>, 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; IL2R $\alpha$  and CD122; IL2R $\beta$ ) were detected, but limited surface expression of IL7R $\alpha$  (CD127) was identified.  $\gamma\delta$  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  $\gamma\delta$  T cells indicated that they were highly activated and antigen experienced with potential for memory formation and homing to tissues.

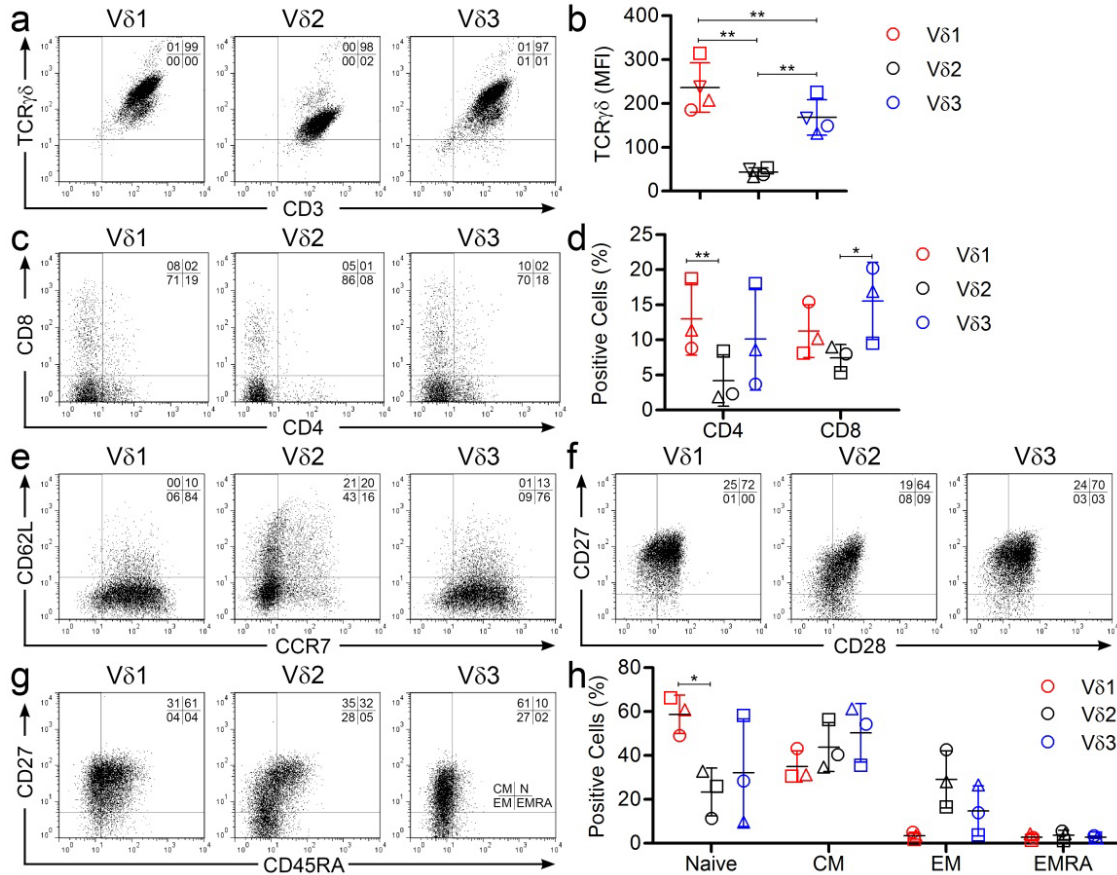


**Figure 33. Immunophenotype of Polyclonal  $\gamma\delta$  T cells Propagated on aAPC, IL2, and IL21.** (a) Gating (one representative of four donors is shown) and (b) frequency of T cell surface makers by flow cytometry of T cells at Day 22 of culture. Lines show mean (horizontal)  $\pm$  SD (vertical)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

#### *IV.C.5.b. Immunophenotype of V $\delta$ 1, V $\delta$ 2, and V $\delta$ 3 Subsets*

It is of interest to identify differences amongst V $\delta$ 1, V $\delta$ 2, and V $\delta$ 3 lineages that could enable us to predict functional responses and therapeutic efficacy. In particular, distinct differences were observed in TCR $\gamma\delta$  cell surface density and memory-associated markers. TCR $\gamma\delta$  often stained as a two populations with distinct MFI when co-stained with CD3 (**Figure 27b**). Separation of V $\delta$ 1, V $\delta$ 2, and V $\delta$ 3 subsets clearly identified V $\delta$ 2 as the low ( $43 \pm 9$ ; mean  $\pm$  SD;  $n = 4$ ), V $\delta$ 3 as the medium ( $168 \pm 40$ ), and V $\delta$ 1 as the high ( $236 \pm 56$ ) MFI populations in TCR $\gamma\delta$  staining (**Figure 34a**). CD4 and CD8 are not commonly expressed on  $\gamma\delta$  T cells, but there were differences detected in limited surface expression of both CD4 and CD8 between the separated subsets (**Figure 34b**). V $\delta$ 1 and V $\delta$ 3 cells consistently expressed more CD4 and CD8 than did V $\delta$ 2 cells ( $p = 0.001$ ; Two-way ANOVA), and there were significantly more CD4<sup>+</sup> V $\delta$ 1 and CD8<sup>+</sup> V $\delta$ 3 cells than CD4<sup>+</sup> V $\delta$ 2 and CD8<sup>+</sup> V $\delta$ 2 cells, respectively (**Figure 34c and 34d**). CCR7 and CD62L mediate homing to the lymph nodes and other secondary lymphoid organs. CD8<sup>+</sup> T cells expressing CCR7 and/or CD62L were described as T<sub>CM</sub> cells but CCR7<sup>neg</sup>CD62L<sup>neg</sup> were defined as T<sub>EM</sub> cells.(330, 331) Almost all V $\delta$ 1 and V $\delta$ 3 cells were CCR7<sup>+</sup>CD62L<sup>neg</sup>, but larger proportions of V $\delta$ 2 cells were CCR7<sup>neg</sup>CD62L<sup>neg</sup> with roughly equal remaining proportions staining as single or double positive for CCR7 and CD62L, suggesting V $\delta$ 1 and V $\delta$ 3 were T<sub>CM</sub> and V $\delta$ 2 cells were mostly T<sub>EM</sub> (**Figure 34e**). CD27 and CD28 are both memory markers for CD8<sup>+</sup> 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 V $\delta$ 1>V $\delta$ 3>V $\delta$ 2 but all were

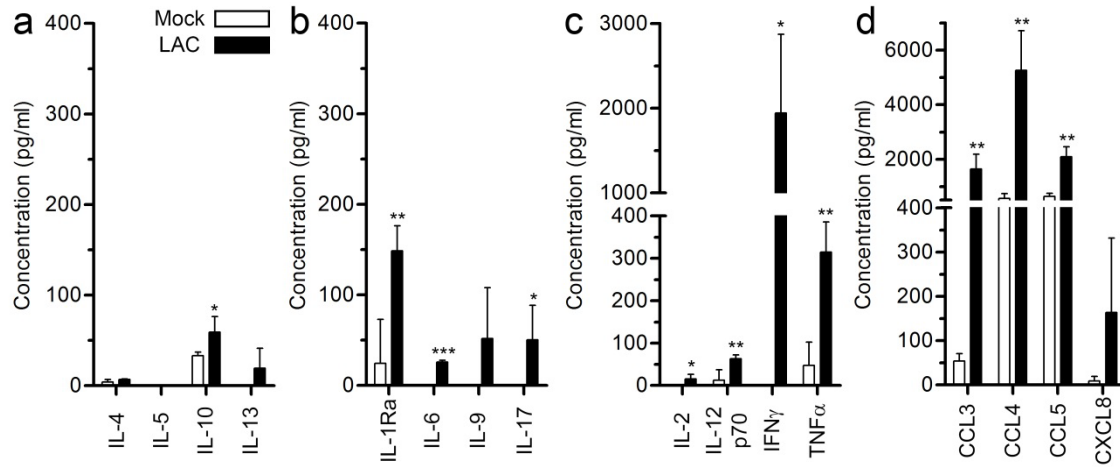
>80% CD27<sup>+</sup> (**Figure 34f y-axes**). In contrast, there was almost no difference between the three V $\delta$  populations in CD28 expression (**Figure 34f x-axes**). Human  $\gamma\delta$  T cell memory has been most extensively reported as combinations of CD27 and CD45RA expression where CD27<sup>+</sup>CD45RA<sup>+</sup>, CD27<sup>+</sup>CD45RA<sup>neg</sup>, CD27<sup>neg</sup>CD45RA<sup>neg</sup>, and CD27<sup>neg</sup>CD45RA<sup>+</sup> correspond to T<sub>N</sub>, T<sub>CM</sub>, T<sub>EM</sub>, and T<sub>EMRA</sub>, respectively (**Figure 34g**). (151, 333) Indeed, these were the markers that showed the most convincing differences between the V $\delta$  populations although all subsets contained at least some of each population. More specifically, the most T<sub>N</sub> cells were V $\delta$ 1, the most T<sub>CM</sub> were V $\delta$ 3, the most T<sub>EM</sub> cells were V $\delta$ 2, and virtually no T<sub>EMRA</sub> were detected (**Figure 34h**). Given these differences in surface memory phenotype, different functional abilities were expected from the  $\gamma\delta$  T cell subsets and a polyclonal approach to adoptive T cell therapy could utilize these different attributes as needed.



**Figure 34. Immunophenotype of V $\delta$  Lineages Propagated on aAPC, IL2, and IL21.** After 15 days of proliferation as separated populations, V $\delta$ 1, V $\delta$ 2, and V $\delta$ 3 subsets were stained for lymphocyte markers. (a) Representative flow cytometry plots of CD3 (x-axes) and TCR $\gamma\delta$  (y-axes) expression in V $\delta$ 1, V $\delta$ 2, and V $\delta$ 3 subsets (from left to right). (b) Mean fluorescence intensities (MFI) of TCR $\gamma\delta$  staining in V $\delta$ 1 (red), V $\delta$ 2 (black), and V $\delta$ 3 (blue) subsets where each shape represents a different donor and data are mean  $\pm$  SD (n = 4). (c) Representative flow cytometry plots of CD4 (x-axes) and CD8 (y-axes) expression in V $\delta$ 1, V $\delta$ 2, and V $\delta$ 3 subsets (from left to right) and (d) summary of frequencies in V $\delta$ 1 (red), V $\delta$ 2 (black), and V $\delta$ 3 (blue) cells where data are mean  $\pm$  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 V $\delta$ 1, V $\delta$ 2, and V $\delta$ 3 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 V $\delta$ 3 in (g) where each shape represents a different donor and data are mean  $\pm$  SD (n = 3).

#### IV.C.6. Polyclonal $\gamma\delta$ T cells Secrete Pro-inflammatory Cytokines and Chemokines

To determine whether  $\gamma\delta$  T cells would foster an inflammatory environment during therapy, a multiplex analysis (27-Plex Luminex) of cytokines and chemokines was performed on polyclonal  $\gamma\delta$  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  $\gamma\delta$  T cells and have roles together for  $T_H17$  inflammatory responses (**Figure 35b**). Moreover, pro-inflammatory  $T_H1$  cytokines IL2, IL12 (p70), IFN $\gamma$ , and TNF $\alpha$  were all significantly produced by  $\gamma\delta$  T cells when TCR was stimulated compared to mock stimulated controls (**Figure 35c**). High expression of chemokines CCL3 (macrophage inflammatory protein-1 $\alpha$ ; MIP1 $\alpha$ ), CCL4 (MIP1 $\beta$ ), 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%  $\pm$  2% (mean  $\pm$  SD; n = 4) of  $\gamma\delta$  T cells expressed this receptor. Nonetheless, recruitment of other immune cells expressing CCR5 is possible based on  $\gamma\delta$  T cell's production of CCL3, CCL4, and CCL5. In aggregate, TCR stimulation in  $\gamma\delta$  T cells led to a largely pro-inflammatory response desired for cell-based cancer therapies.

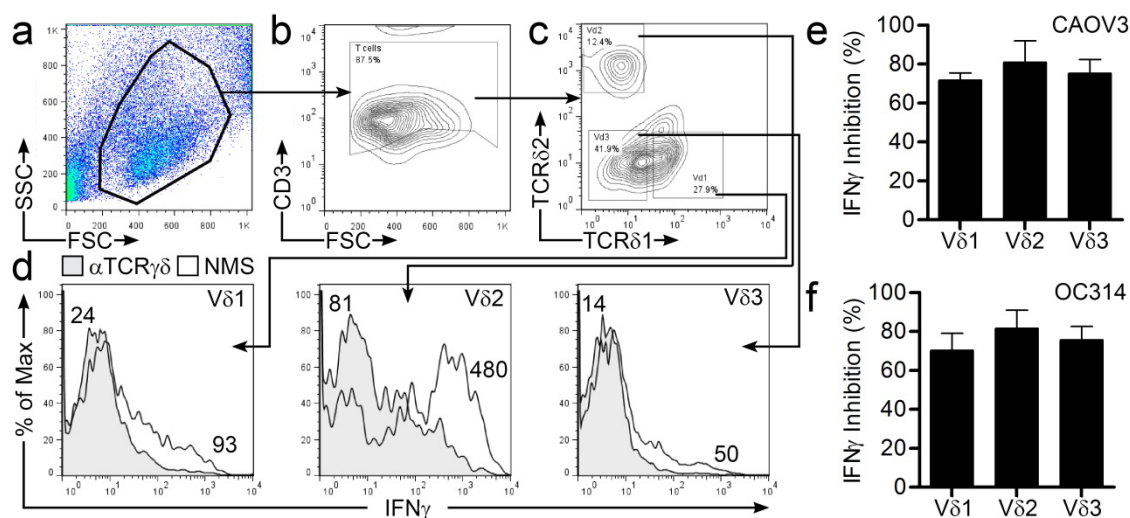


**Figure 35. Cytokines and Chemokines Secreted by Polyclonal  $\gamma\delta$  T cells.** At Day 22 of culture on aAPC/IL2/21, T cells were incubated with complete media (mock) or leukocyte activation cocktail (LAC; PMA/Ionomycin) for 6 hours at 37°C. Conditioned media was interrogated on 27-Plex Luminex array to detect cytokines and chemokines. (a) T<sub>H</sub>2 cytokines, (b) T<sub>H</sub>17 cytokines, (c) T<sub>H</sub>1 cytokines, and (d) Chemokines. Data are mean  $\pm$  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 TCR $\gamma\delta$ Involvement in V $\delta$ 1, V $\delta$ 2, and V $\delta$ 3 Production of IFN $\gamma$

After establishing that polyclonal  $\gamma\delta$  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 TCR $\gamma\delta$ . IFN $\gamma$  was produced most highly of all the cytokines interrogated by Luminex (**Figure 35c**), so it was chosen as a marker for  $\gamma\delta$  T cell response to OvCa in a classical intracellular cytokine staining (ICS) assay. Co-cultures with polyclonal  $\gamma\delta$  T cells and two different OvCa cell lines were incubated at 37°C for 6 hours in the presence of the secretory pathway inhibitor Brefeldin-A (GolgiPlug) in order to trap IFN $\gamma$  within the T cells. Parallel co-cultures were set up with (i) normal mouse serum (NMS) for negative control or (ii) neutralizing TCR $\gamma\delta$  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, TCR $\delta$ 1, and TCR $\delta$ 2 in order to separate V $\delta$ 1, V $\delta$ 2, and V $\delta$ 3 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 V $\delta$  subset produced IFN $\gamma$  in response to OvCa in the NMS (negative blocking control) treated cells (**Figure 36d**). Furthermore, the amount of IFN $\gamma$  produced followed the order V $\delta$ 2>V $\delta$ 1>V $\delta$ 3 as evidenced by IFN $\gamma$  MFI of  $855 \pm 475$ ,  $242 \pm 178$ , and  $194 \pm 182$  (mean  $\pm$  SD; n = 4), respectively. Addition of antibody neutralizing TCR $\gamma\delta$  significantly inhibited IFN $\gamma$  production by all three V $\delta$  subsets where V $\delta$ 2 was most affected (**Figure 36d, 36e, and 36f**). Therefore, polyclonal  $\gamma\delta$  T cells responded to tumor cells indicating that they have specific anti-tumor effects through their TCR $\gamma\delta$ .



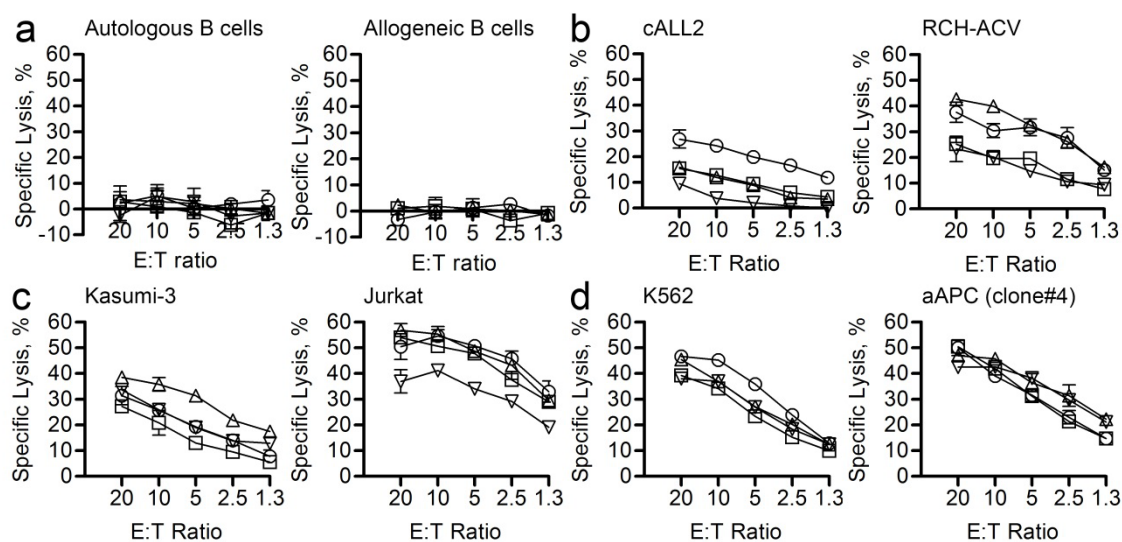
**Figure 36. TCR $\gamma\delta$ -specific IFN $\gamma$  Production by V $\delta$ 1, V $\delta$ 2, and V $\delta$ 3 Subsets.** Polyclonal  $\gamma\delta$  T cells were incubated for 1 hour prior to co-culture and during co-cultures with normal mouse serum (NMS; negative control) or neutralizing TCR $\gamma\delta$  antibody ( $\alpha$ TCR $\gamma\delta$ ; clone IMMU510). Co-cultures were initiated in the presence of the secretory inhibitor BrefeldinA (GolgiPlug) where polyclonal  $\gamma\delta$  T cells and one of two OvCa cell lines (CAOV3 or OC314) and were incubated at 37°C for 6 hours. Cells were stained for TCR $\delta$ 1, TCR $\delta$ 2, CD3, and IFN $\gamma$  in order to gate each T cell subset and assess IFN $\gamma$  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<sup>+</sup> T cells from contaminating tumor cells in T cell gate, and (c) separation into V $\delta$ 1, V $\delta$ 2, and V $\delta$ 3 based on TCR $\delta$ 1<sup>+</sup>TCR $\delta$ 2<sup>neg</sup>, TCR $\delta$ 1<sup>neg</sup>TCR $\delta$ 2<sup>+</sup>, and TCR $\delta$ 1<sup>neg</sup>TCR $\delta$ 2<sup>neg</sup>, respectively. (d) Histogram comparisons of V $\delta$ 1, V $\delta$ 2, and V $\delta$ 3 gates (from left to right) co-cultured with CAOV3 and treated with NMS (open) or  $\alpha$ TCR $\gamma\delta$  (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 V $\delta$  subset was calculated by the following equation: Inhibition (%) = 100 – 100 x [(MFI<sub>TUMOR + T CELL</sub> – MFI<sub>T CELL ONLY</sub>) $\alpha$ TCR $\gamma\delta$  / (MFI<sub>TUMOR + T CELL</sub> – MFI<sub>T CELL ONLY</sub>)<sub>NMS</sub>]. Data are mean  $\pm$  SD (n = 3).

#### IV.C.8. Broad Anti-tumor Cytolysis by Polyclonal $\gamma\delta$ T cells

After establishing that  $\gamma\delta$  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 $\gamma\delta$ T cells Lyse Hematological Tumors*

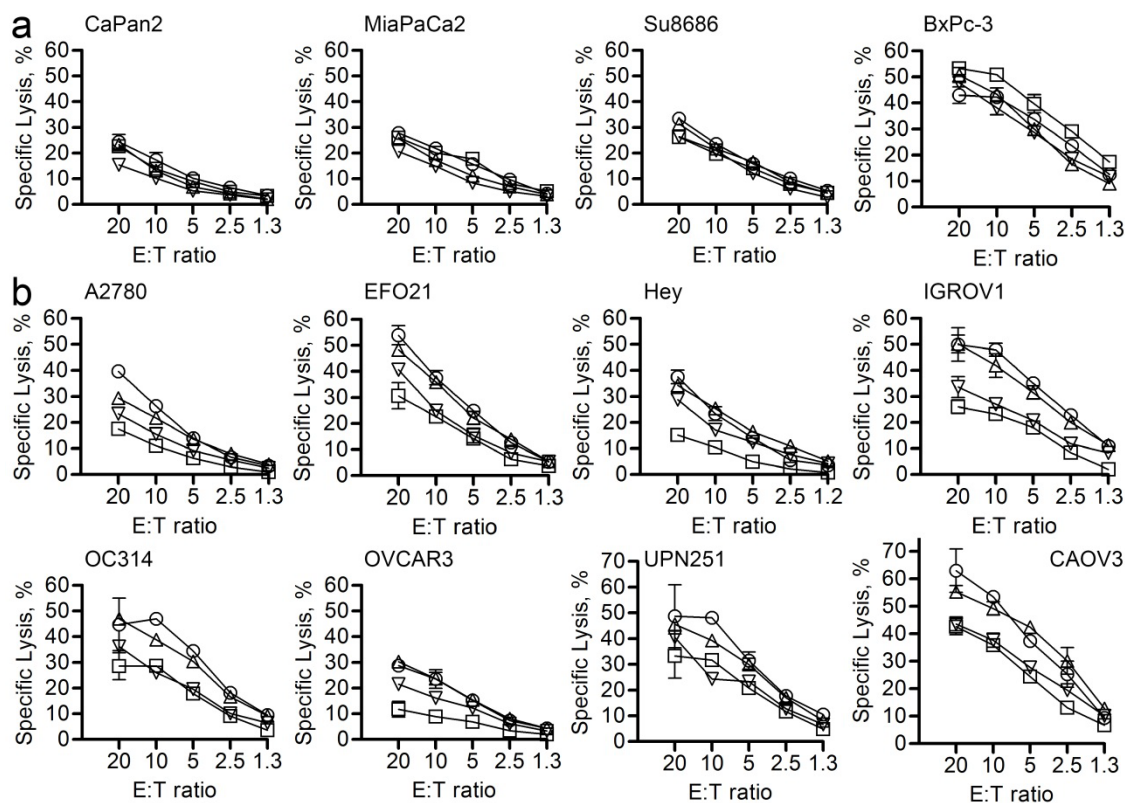
We previously established that  $\gamma\delta$  T cells could lyse B-ALL cell lines (Daudi- $\beta$ 2M, Kasumi2, and REH) but not healthy autologous or allogeneic B cells.(311) This observation was confirmed again with healthy autologous and allogeneic B cells, which were not lysed by polyclonal  $\gamma\delta$  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  $\gamma\delta$  T cell killing suggesting that  $\gamma\delta$  T cells could be used to kill T cell malignancies (**Figure 37c**). CML cell line K562 was also killed by  $\gamma\delta$  T cells and has been a well-known target for  $\gamma\delta$  T cell cytotoxicity.(303) Moreover, K562-derived clone#4 aAPC were lysed by  $\gamma\delta$  T cells, as expected (**Figure 37d**). Thus, polyclonal  $\gamma\delta$  T cells propagated on aAPC have anti-tumor immunity towards hematological malignancies.



**Figure 37. *In vitro* Cytolysis of Hematological Tumor Cells by  $\gamma\delta$  T cells.** Standard 4-hour CRA were performed with increasing effector ( $\gamma\delta$  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  $\pm$  SD (n = 3 wells per assay).

*IV.C.8.b. Polyclonal  $\gamma\delta$  T cells Lyse Solid Tumors*

After establishing that polyclonal  $\gamma\delta$  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  $\gamma\delta$  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  $\gamma\delta$  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  $\gamma\delta$  T cells were able to kill solid tumors *in vitro* and other solid tumor cell lines may also be sensitive to cytolysis by  $\gamma\delta$  T cells.



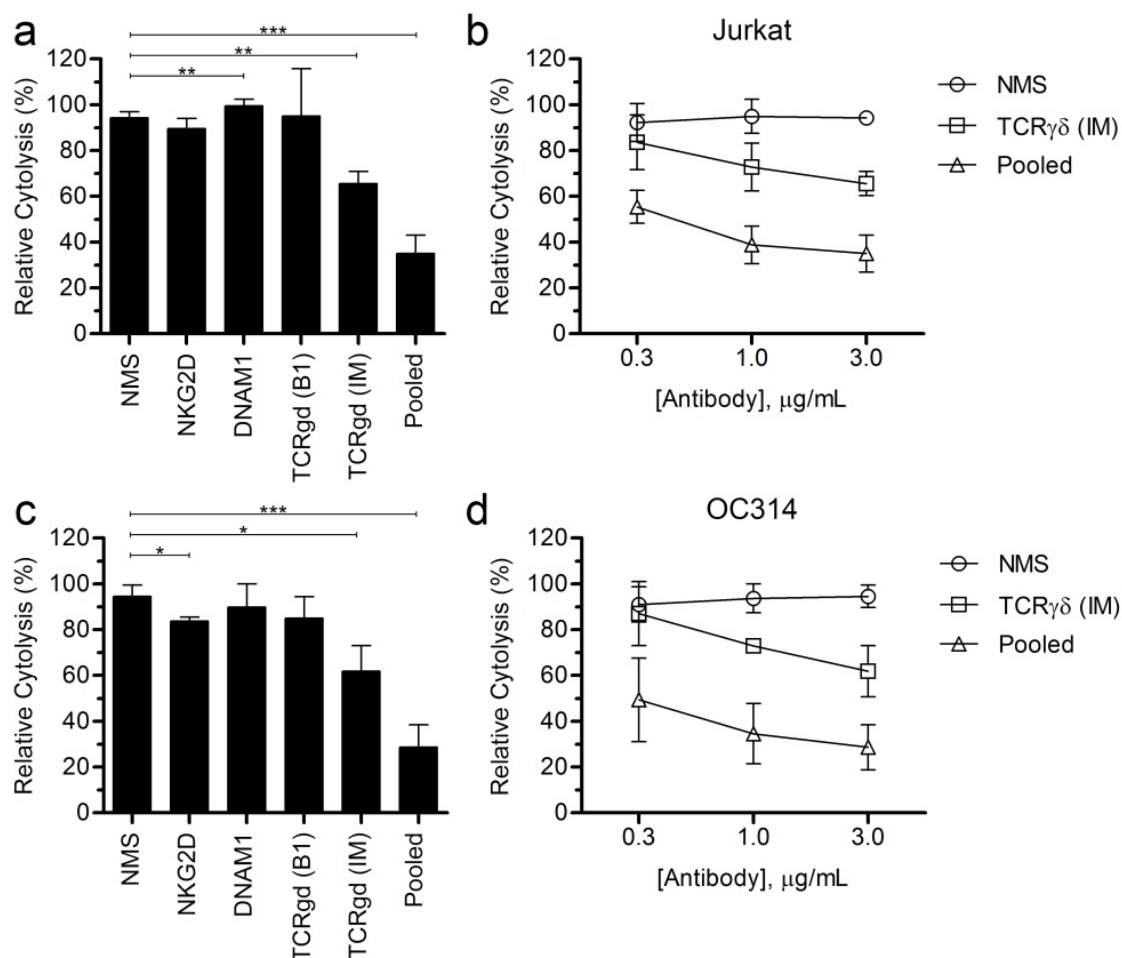
**Figure 38. *In vitro* Cytolysis of Solid Tumor Cells by  $\gamma\delta$  T cells.** Standard 4-hour CRA were performed with increasing effector ( $\gamma\delta$  T cells) to target (E:T) ratios against (a) PaCa cell lines CaPan2, MiaPaCa2, Su8686, and BxPc3 and (b) OvCa cell lines A2780, EFO21, Hey, IGROV1, OC314, OVCAR3, UPN251, and CAO3. Each line represents an individual effector where data are mean  $\pm$  SD (n = 3 wells per assay).

#### *IV.C.8.c. Mechanism of Tumor Cytolysis by $\gamma\delta$ T cells was Multi-factorial*

We sought to determine if polyclonal  $\gamma\delta$  T cell cytolysis was directly dependent upon the TCR $\gamma\delta$  by neutralizing killing with antibodies. Confounding these assays was the observation that  $\gamma\delta$  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 TCR $\gamma\delta$  neutralizing antibody since the company information for TCR $\gamma\delta$ -specific antibodies did not report on neutralization. In the end, the TCR $\gamma\delta$ -specific antibody used for staining in this study (clone B1, BD Biosciences) and clone IMMU510 TCR $\gamma\delta$ -specific antibody (IM) from Thermo Fisher were used for neutralization studies. Also, because there were many activating receptors (TCR $\gamma\delta$ , DNAM1, NKG2D) on the  $\gamma\delta$  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  $\gamma\delta$  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 TCR $\gamma\delta$  (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, TCR $\gamma\delta$  (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, TCR $\gamma\delta$  (B1), and TCR $\gamma\delta$  (IM)) resulted in synergistic inhibition of  $\gamma\delta$  T cell cytotoxicity of Jurkat ( $65\% \pm 8\%$ ) and OC314 ( $71\% \pm 10\%$ ) cells (**Figures 39a and 39c bars to far right**). Moreover, dose-dependent inhibition was observed by both TCR $\gamma\delta$  (IM) and pooled antibodies when concentrations were diluted from 3.0  $\mu\text{g/mL}$  (shown in **Figures 39a and 39c**) to 1.0  $\mu\text{g/mL}$  and 0.3  $\mu\text{g/mL}$  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  $\gamma\delta$  T cell killing (**Figure 38b**).<sup>(337, 339, 340)</sup>. In sum, these results suggested that killing by  $\gamma\delta$  T cells is multi-factorial with an emphasis on the TCR $\gamma\delta$  to mediate cytotoxicity.



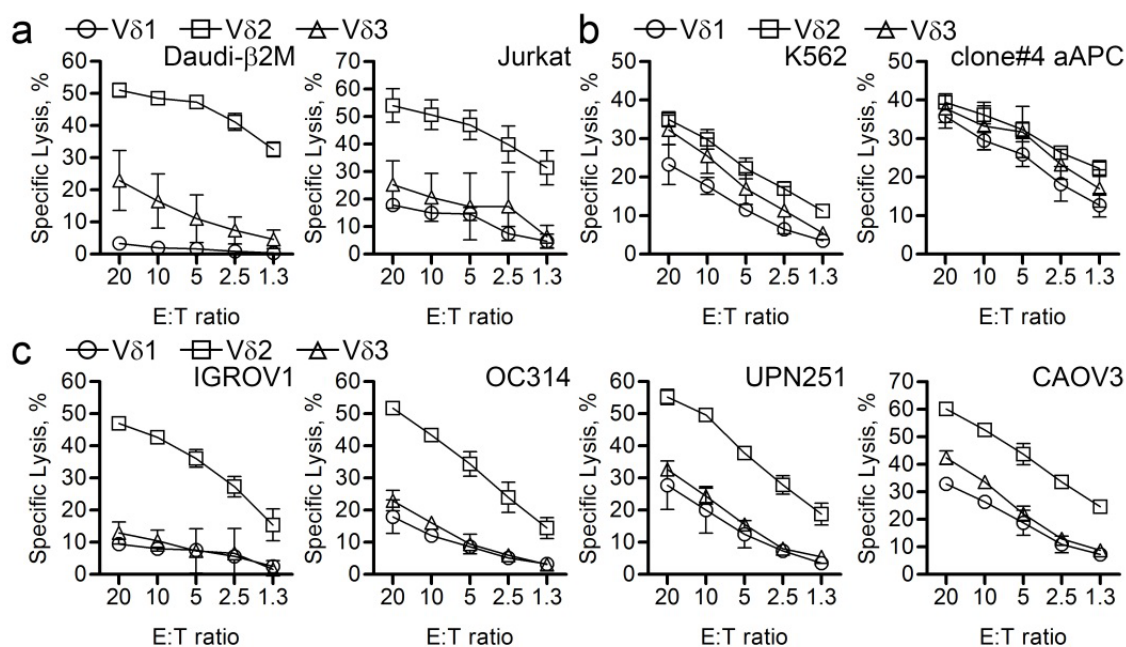


**Figure 39. Neutralization of Polyclonal  $\gamma\delta$  T cell Cytolysis.** Neutralizing antibodies to NKG2D, DNAM1, TCR $\gamma\delta$  (B1), TCR $\gamma\delta$  (IM) were used to block killing of Jurkat or OC314 tumor targets at an E:T ratio of 12:1 in standard 4-hour CRA. Antibodies were pre-incubated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

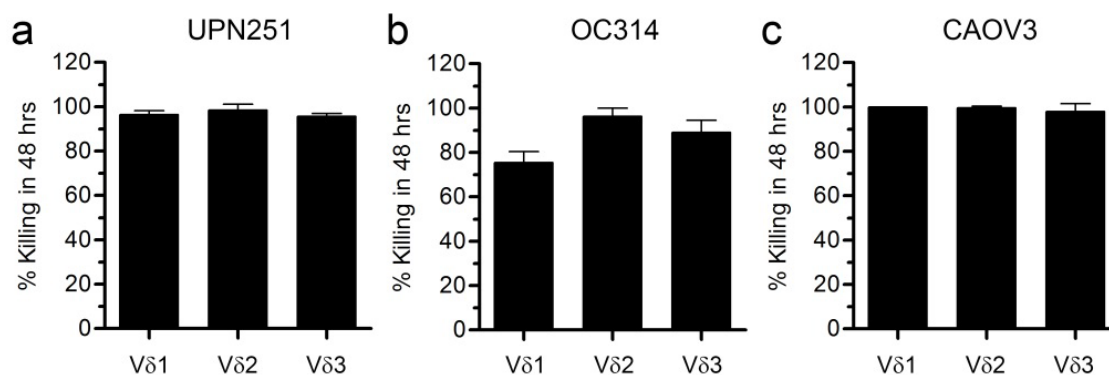
#### *IV.C.8.d. Importance for TCR $\delta$ in $\gamma\delta$ T cell Cytolysis*

Because the separated V $\delta$  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- $\beta$ 2M, 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  $\gamma\delta$  T cells (**Figures 37 and 38**). All eight tumor cell lines were lysed by the separated V $\delta$  lineages, but a distinct order of lysis was observed against all targets where V $\delta$ 2>>V $\delta$ 3>V $\delta$ 1 in killing capabilities (**Figure 40**). As the phenotype indicated that V $\delta$ 1 cells were mainly naïve, it was expected that they would have the most limited cytolytic ability, which is what was observed. Likewise, T<sub>CM</sub> have less immediate effector function relative to T<sub>EM</sub> cells, and these memory populations were dominated by V $\delta$ 3 and V $\delta$ 1, respectively. Importantly, this was the first report of anti-tumor activity by V $\delta$ 3 cells. It was interesting that all three V $\delta$  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 V $\delta$  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%  $\pm$  4% of OC314 cells were killed by V $\delta$ 2 cells, and V $\delta$ 1 and V $\delta$ 3 achieved 76%  $\pm$  5% and 89%  $\pm$  5% (mean  $\pm$  SD; n = 3) killing, respectively, in 48 hours of culture. Collectively, the V $\delta$  subset lineage was

important for cytolysis in both acute and long-term conditions, and established that each V $\delta$  lineage propagated on aAPC was capable of tumor killing.



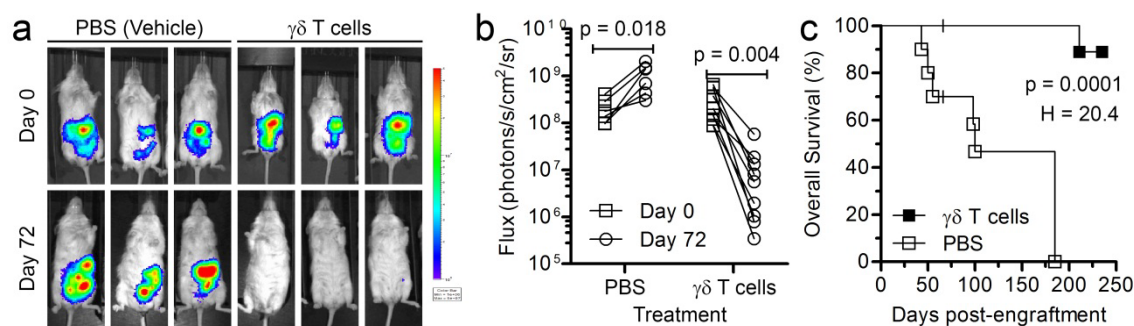
**Figure 40.  $\gamma\delta$  T cell Subset Acute Cytolysis.** V $\delta$  subsets were tested in 4-hour CRA against (a) ALL cell lines Daudi- $\beta$ 2M and Jurkat, (b) CML cell line K562 and its derivative clone#4 aAPC, and (c) OvCa cell lines IGROV1, OC314, UPN251, and CAOV3. V $\delta$ 1 (circles), V $\delta$ 2 (squares), and V $\delta$ 3 (triangles) are displayed as mean  $\pm$  SD from averaged triplicate measurements from four normal donors.



**Figure 41.  $\gamma\delta$  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 (%) =  $(\text{Viable cells})_{\text{Co-culture}} / (\text{Viable cells})_{\text{Tumor only}} \times 100$ . Data are mean  $\pm$  SD (n = 3).

#### IV.C.9. Clearance of Established Tumor Xenografts by Polyclonal $\gamma\delta$ T cells

As polyclonal  $\gamma\delta$  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  $\gamma\delta$  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  $\gamma\delta$  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  $\gamma\delta$  T cell-treated mice had significantly decreased ( $p = 0.004$ ) BLI flux (**Figure 42b**). Moreover, treatment with  $\gamma\delta$  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  $\gamma\delta$  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  $\gamma\delta$  T cells.** CAOV3-*ffLuc*-mKate tumor cells ( $3 \times 10^6$ ) 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  $\gamma\delta$  T cells. Four doses were given with  $3 \times 10^6$ ,  $6 \times 10^6$ ,  $10 \times 10^6$ , and  $15 \times 10^6$  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  $\gamma\delta$  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  $\gamma\delta$  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 $\gamma\delta$ T cells for Immunotherapy

This study establishes clone#4 aAPC as a cellular platform for the sustained proliferation of populations of  $\gamma\delta$  T cells that exhibit broad reactivity against hematologic malignancies and solid tumors. T cells expressing certain V $\delta$  TCR usage have been associated with clinical responses against cancer. For example, the V $\delta$ 1 TCR subset correlated with complete responses observed in patients with ALL and AML who underwent  $\alpha\beta$  T cell-depleted allogeneic HSCT.(302, 304, 305) However, V $\delta$ 1 cells have not been directly infused for therapy. This chapter established direct evidence that V $\delta$ 1 cells could mediate anti-tumor immunity and strengthens support for their use in adoptive T cell cancer treatments. In contrast to V $\delta$ 1 and V $\delta$ 3 cells, T cells expressing V $\delta$ 2 TCR have been directly infused and generated responses against solid and hematological tumors, but complete responses were unpredictable and sometimes not directly correlated to V $\delta$ 2 therapy (175, 318). Similarly, V $\delta$ 2 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 V $\delta$ 2 T cell infusions. A role for T cells expressing V $\delta$ 3 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 V $\delta$ 3 cells have anti-tumor activity and this study could, therefore, represent a significant contribution to both translational research strategies and to immunologists studying  $\gamma\delta$  T cell function. In



aggregate, the data herein lend impetus to adoptive transfer of  $\gamma\delta$  T cells that maintain expression of all V $\delta$  TCR types as investigational treatment for tumors and opportunistic viral infections.

#### IV.D.2. Potential Ligands for TCR $\gamma\delta$ on aAPC

The molecules on aAPC that stimulate TCR $\gamma\delta$  for their numeric expansion are not known. K562-derived aAPC express endogenous MICA and MICB molecules (329) which are ligands for both V $\delta$ 1 and NKG2D.(152) NKG2D was expressed (40%  $\pm$  16%; mean  $\pm$  SD, n = 4) on aAPC-expanded  $\gamma\delta$  T cells that were also predominantly V $\delta$ 1 cells (**Figure 31**). Polyclonal  $\gamma\delta$  T cells also demonstrate expression (26%  $\pm$  7%) for other activating NK receptors (NKp30, NKp44, and NKp46), which may contribute to  $\gamma\delta$  T cell function. Two ligands described for V $\delta$ 2 TCR are surface mitochondrial F<sub>1</sub>-ATPase and phosphoantigens, both of which are described in K562 cells.(171, 172, 297, 299) Indeed, enhanced responses of T cells expressing V $\gamma$ 9V $\delta$ 2 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 V $\delta$ 2 TCR usage.(173) Otherwise, patients receiving polyclonal  $\gamma\delta$  T cells could be primed to expand V $\delta$ 2 cells *in vivo* through administration of aminobisphosphonates. Now that aAPC have been established as a means to propagate polyclonal  $\gamma\delta$  T cells, these molecular questions can be answered and used for future therapies.

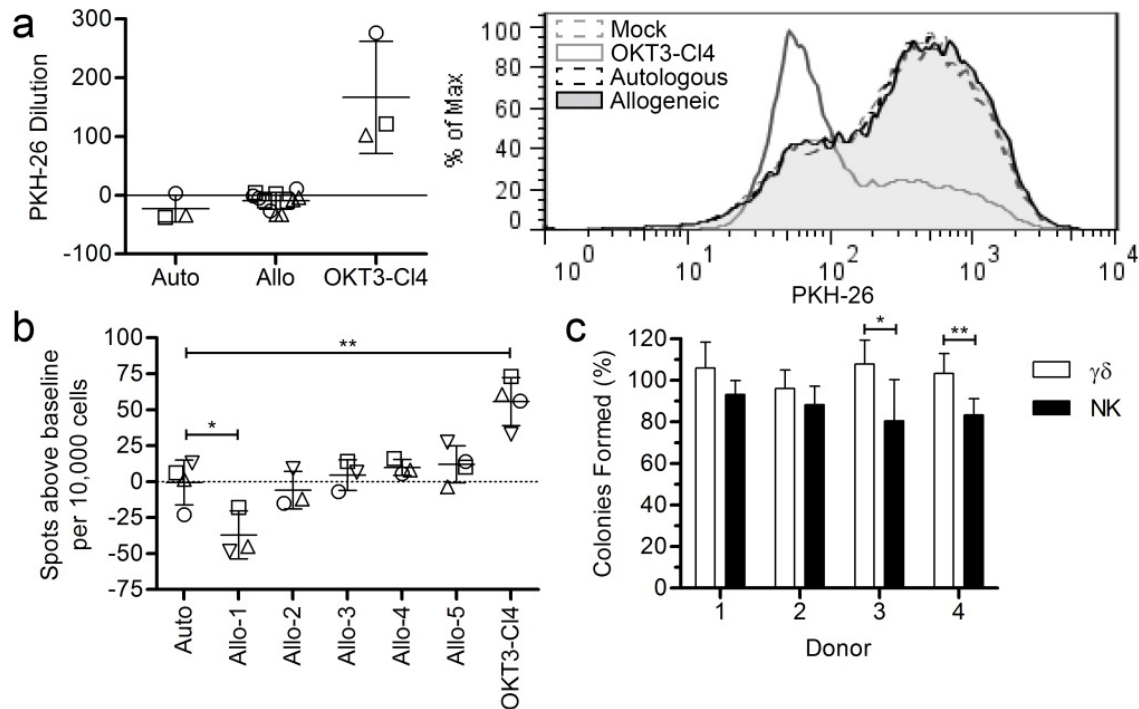
#### IV.D.3. Co-stimulation in Polyclonal $\gamma\delta$ T cell Expansion

We introduced co-stimulatory molecules to improve the ability of aAPC to propagate  $\gamma\delta$  T cells. CD28 and CD137 (4-1BB) expressed on  $\gamma\delta$  T cells bind CD86 and CD137L, respectively, expressed on aAPC. The absence of both CD86 and CD137L abrogated  $\gamma\delta$  T-cell proliferation and expression of single co-stimulatory molecules only partially restored the ability of  $\gamma\delta$  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  $\gamma\delta$  T cells ( $36\% \pm 15\%$ ) 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  $\gamma\delta$  T cells that produce IFN $\gamma$ , and CD27<sup>neg</sup>  $\gamma\delta$  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  $\gamma\delta$  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  $\gamma\delta$  T cells, which was required for expansion of CD4<sup>+</sup> T<sub>H</sub>17 cells *ex vivo* on stimulating beads.(326) Indeed, the addition of IL2 and IL21 was also crucial for the numeric expansion of  $\gamma\delta$  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  $\gamma\delta$  T cell produced for adoptive T cell therapy.

#### IV.D.4. Polyclonal $\gamma\delta$ T cells Apparently Lack Allogeneic Responses to Healthy Tissue

An attractive therapeutic strategy is to employ third party allogeneic  $\gamma\delta$  T cells as an “off-the-shelf” therapy. This may be feasible, as  $\gamma\delta$  T cells have reduced potential to cause graft-versus-host disease (GvHD) resulting from inappropriate TCR-mediated recognition of normal host tissue (305). Unlike TCR $\alpha\beta$  that recognizes peptides in the context of MHC, TCR $\gamma\delta$  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  $\gamma\delta$  T cells from one donor can be infused into multiple recipients. Autologous T cells expressing V $\gamma$ 9V $\delta$ 2 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  $\gamma\delta$  T cell has not been reported. We have evaluated aAPC-expanded  $\gamma\delta$  T cells for allogeneic responses and are not able to detect such reactivity. For example,  $\gamma\delta$  T cells proliferate (**Figure 43a**) and secrete IFN $\gamma$  (**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  $\gamma\delta$  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  $\gamma\delta$  T cells (**Figure 43c**). Autologous EBV-transformed LCL stimulated  $\gamma\delta$  T cells suggesting they may react with EBV antigens (data not shown) as indicated by previous studies.(342, 343) Bi-specific  $\alpha\beta$  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  $\gamma\delta$  T cell

population using aAPC.(236, 344) The ability to infuse donor-derived  $\gamma\delta$  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 TCR $\alpha\beta$  to help generate “universal” CAR<sup>+</sup> T cells.(345)



**Figure 43. Absence of Allogeneic Responses by Polyclonal  $\gamma\delta$  T cells to Partially Mis-matched Donors' Healthy Cells.** (a) Polyclonal  $\gamma\delta$  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°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<sup>+</sup>TCR $\gamma\delta$ <sup>+</sup> cells. Each shape represents a polyclonal  $\gamma\delta$  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 IFN $\gamma$  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  $\gamma\delta$  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 $\gamma\delta$ T cells for Immunotherapy

These data demonstrate that our aAPC can be used to generate large numbers of  $\gamma\delta$  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  $\gamma\delta$  T cells for human application. A polyclonal approach to  $\gamma\delta$  T cell immunotherapy is supported by the ability to of aAPC generate  $T_N$ ,  $T_{CM}$ , and  $T_{EM}$   $\gamma\delta$  T cells from V $\delta$ 1, V $\delta$ 2, and V $\delta$ 3 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  $T_{CM}$   $\gamma\delta$  T cells. Clinical trials can now, for the first time, test the efficacy of polyclonal  $\gamma\delta$  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  $\alpha\beta$  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<sup>+</sup> aAPC are currently in clinical trials at MD Anderson and were the fastest way to translate a strategy to use CAR<sup>+</sup>  $\gamma\delta$  T cells for immunotherapy. Therefore, the second approach used polyclonal  $\gamma\delta$  T cells expressing TCR $\gamma\delta$  with anti-tumor reactivity as sentinels of CD19-specific CAR anti-tumor immunity. These CAR<sup>+</sup>  $\gamma\delta$  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  $\gamma\delta$  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<sup>+</sup> T cells, CARs can be paired to other cellular products to increase anti-tumor efficacy. For instance, polyclonal  $\gamma\delta$  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<sup>+</sup> OvCa cells were lysed by ROR1-specific CAR<sup>+</sup> T cells (**Figure 16c**),(67) so a combinational immunotherapy of ROR1-specific  $\alpha\beta$  T cells and polyclonal  $\gamma\delta$  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  $\gamma\delta$  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  $\gamma\delta$  T cell-mediated cytotoxicity may be sensitive to synergistic killing by ROR1-specific CAR<sup>+</sup>  $\alpha\beta$  T cells and polyclonal  $\gamma\delta$  T cells. Also,  $\gamma\delta$  T cells are unlikely to participate in GvHD in allogeneic transplantation, so a universal bank of polyclonal  $\gamma\delta$  T cells could be established that was known to have high anti-tumor immunity or containing a particular set frequency of V $\delta$ 1, V $\delta$ 2, and V $\delta$ 3 populations with maximum efficacy.(305) Polyclonal  $\gamma\delta$  T cells could also be used as front-line therapy before addition of HSCT, CAR<sup>+</sup> 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 IFN $\gamma$ . Indeed, most of the T cells expanded on aAPC in this dissertation, with or without CARs, produced IFN $\gamma$ , and the expanded  $\gamma\delta$  T cells secreted IL17 in diminished quantities compared to IFN $\gamma$  (**Figures 23 and 35**). CD27 has been a marker for these cytokines in  $\gamma\delta$  T cells where CD27<sup>neg</sup> and CD27<sup>+</sup> are associated with IL17 and IFN $\gamma$ , respectively.(333, 351, 352) It holds then that ~80% of polyclonal  $\gamma\delta$  T cells stain positive for CD27 (**Figures 33 and 34**). CD28 co-stimulation was shown to inhibit T<sub>H</sub>17 polarization in CD4<sup>+</sup> 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 IFN $\gamma$  in polyclonal  $\gamma\delta$  T cells, CAR<sup>+</sup>  $\gamma\delta$  T cells, and CAR<sup>+</sup>  $\alpha\beta$  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<sup>+</sup> T cells signaling through CD137 produce less IFN $\gamma$  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<sup>+</sup> 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<sup>+</sup> T cells from T<sub>REG</sub> to T<sub>H</sub>17.(127) In regards to the latter, release of immunosuppression by T<sub>REG</sub> and production of IL17 could explain these impressive complete responses. **Chapter II** did not directly evaluate the influence of T<sub>REG</sub> cells on CAR<sup>+</sup> 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 IFN $\gamma$ -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 $\gamma\delta$ T cells to Immunology**

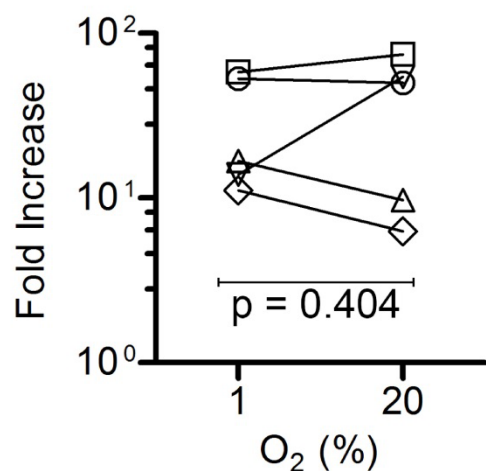
One of the major accomplishments of this dissertation was creating a method for expanding polyclonal  $\gamma\delta$  T cells (**Chapter IV**), which has broader applications outside of immunotherapy to the immunology and cancer biology fields. For example, few mAb exist that are specific for TCR $\gamma\delta$  isotypes, which limits their detection in correlative studies and other assays.(165) Given the ability of aAPC to expand large numbers of polyclonal  $\gamma\delta$  T cells, mice can be immunized to generate mAb specific for desired TCR $\gamma\delta$  isotypes, e.g. V $\delta$ 3 and V $\gamma$  isotypes outside of V $\gamma$ 9. Commercial and academic use of these detection antibodies have tangible outcomes, including diagnostic and/or prognostic profiling of  $\gamma\delta$  T cell TIL within tumors. Other major unknowns are the ligands for many TCR $\gamma\delta$  heterodimers. Generation of  $\gamma\delta$  T cell clones could be used to determine the specific ligands of V $\delta$ /V $\gamma$  combinations and therefore lead to future

studies on  $\gamma\delta$  T cell affinity towards a particular disease. Moreover, the ligands on the K562-derived aAPC that TCR $\gamma\delta$  binds are unknown. Likely candidates include IPP (V $\delta$ 2) and MICA/B (V $\delta$ 1) but their exact roles have not been determined.(155, 172) Elucidation of these interactions could assist attempts to tailor the aAPC for total  $\gamma\delta$  T cell expansion, expansion of a particular  $\gamma\delta$  T cell lineage, or polarization towards a certain  $\gamma\delta$  T cell phenotype. Thus, aAPC could be an excellent source for the study of fundamental  $\gamma\delta$  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  $\gamma\delta$  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<sup>+</sup> 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  $\gamma\delta$  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  $\gamma\delta$  T cell graft may be rejected, but there may still be a therapeutic window. Another unknown is whether  $\gamma\delta$  T cells will be

subjected to the same regulation by T<sub>REG</sub> cells or other immunosuppressive forces. Some  $\gamma\delta$  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  $\gamma\delta$  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<sub>2</sub>) or normoxia (20% O<sub>2</sub>) and stimulated with clone#4 aAPC, IL2, and IL21, indicating that  $\gamma\delta$  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  $\gamma\delta$  T cells in humans will test the ability of  $\gamma\delta$  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 outweigh the anti-tumor benefits to cancer patients.



**Figure 44. Proliferation of  $\gamma\delta$  T cells in Hypoxia Compared to Normoxia.** Co-cultures were initiated in parallel with  $\gamma\delta$  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 (Cytosan and Fludarabine) chemotherapy. Proof-of-principle studies have established protocols for expanding CAR<sup>+</sup> T cells from patient samples by using an “electroporation-then-sort” strategy used for growing CAR<sup>+</sup>  $\gamma\delta$  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<sup>+</sup> T cells. Co-culture on aAPC led to CAR<sup>+</sup> 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<sup>+</sup> 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.<sup>(13)</sup> 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

$\gamma\delta$  T cells in the case that the autologous  $\gamma\delta$  T cells do not proliferate or respond to the tumor. If allogeneic  $\gamma\delta$  T cells are infused into this patient, this will represent the first time that purified polyclonal  $\gamma\delta$  T cells from an allogeneic host were ever infused into a human. There is great optimism that the polyclonal  $\gamma\delta$  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<sup>+</sup>  $\gamma\delta$  T cells, and polyclonal  $\gamma\delta$  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*<sup>-/-</sup> 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-IL15R $\alpha$ Fusion Construct*

This construct will produce an IL15 that is membrane-bound, but also presented in the context of IL15R $\alpha$ . A fusion of IL15 (NM\_000585.4) to the full length IL15R $\alpha$  (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 IL15R $\alpha$  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 CD3 $\zeta$  (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 through CD137 and CD3 $\zeta$  (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 IgG $\kappa$  signal peptide, (ii) V<sub>L</sub>, (iii) Whitlow linker, (iv) V<sub>H</sub>, 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 plasmids 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<sub>2</sub> at 37°C 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 Pennsylvania courtesy of Dr. Carl June.<sup>(57, 275, 278, 279)</sup> 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 through enforced expression of CD19, CD64, CD86, and CD137L on K562 cells (June CH, UPenn). This aAPC was further modified to express IL15/IL15R $\alpha$  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<sup>-/-</sup> 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 LNJ). Clone#4 aAPC expresses IL15, CD86, and CD137L, so in order to investigate the roles of these molecules on  $\gamma\delta$  T cell proliferation new aAPC were constructed on K562 background (**Figure 28**). SB transposon containing IL15/IL15R $\alpha$  fusion protein and SB11 transposase were electro-transferred into K562 cells (CD86<sup>neg</sup> and CD137L<sup>neg</sup>) using Amaxa nucleofection and Kit V (cat#VCA-1003, Lonza, Basel, Switzerland). FACS was used to isolate IL15<sup>+</sup> cells, which were electroporated with SB11 and SB transposons containing either CD86 or CD137L. Cells were sorted again by FACS to

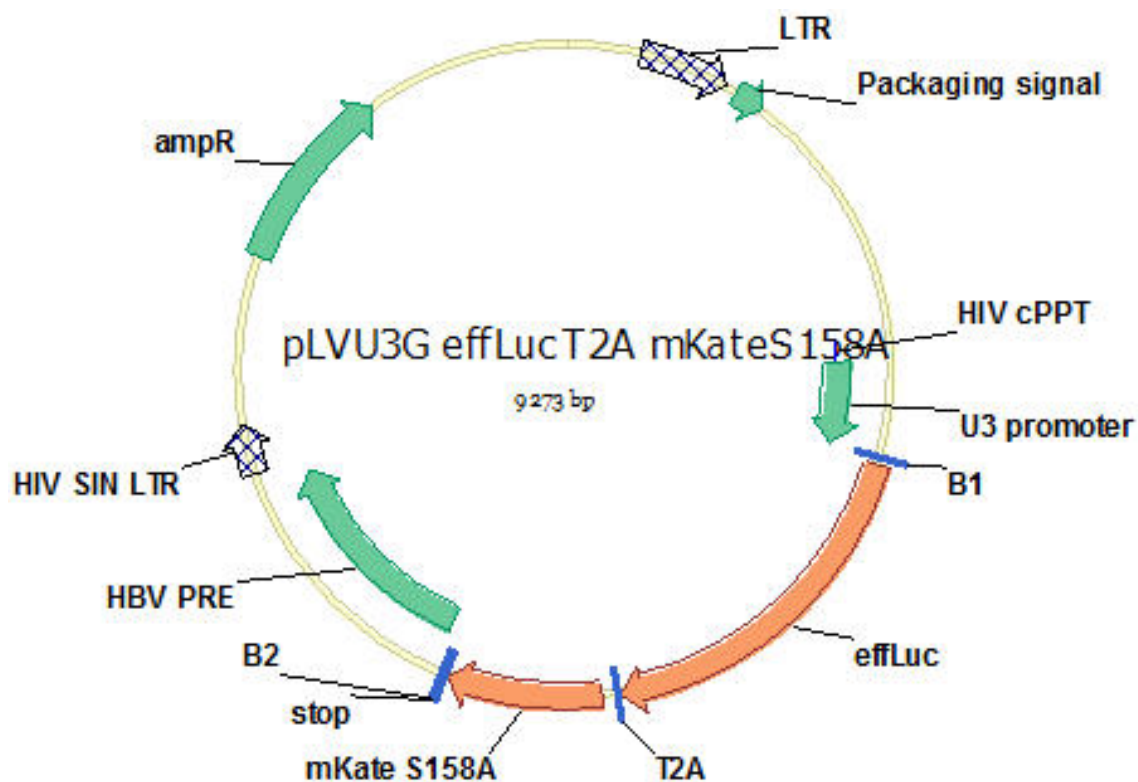
obtain IL15<sup>+</sup>CD86<sup>+</sup> or IL15<sup>+</sup>CD137L<sup>+</sup> as single cell clones A3 (IL15<sup>+</sup>CD86<sup>+</sup>CD137L<sup>neg</sup>) and D4 (IL15<sup>+</sup>CD86<sup>neg</sup>CD137L<sup>+</sup>), respectively. Single cell sorting FACS was also used to make a single cell clone (A6; IL15<sup>+</sup>CD86<sup>neg</sup>CD137L<sup>neg</sup>) of cells electroporated once. Each cell line was negative for mycoplasma and microbial pathogens.

#### *VI.B.2.c. Lentiviral 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<sup>6</sup> 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 ( $\beta$ -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  $\gamma$ -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. $CAR^{neg}$ $\alpha\beta$ T cells*

$\gamma$ -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<sup>+</sup> 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<sup>+</sup> $\alpha\beta$ T cells*

CAR<sup>+</sup> 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°C. Cells for electroporation were spun at 200g for 10 minutes, enumerated, and 2x10<sup>7</sup> 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<sup>+</sup> T cells was used and a 1:1 ratio of clone#1 to ROR1-specific CAR<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> T cell studies, 3 normal donors were tested in 3 independent experiments.

#### *VI.B.4.c. CAR<sup>+</sup> $\gamma\delta$ T cells*

CAR<sup>+</sup>  $\gamma\delta$  T cells were generated as previously described.<sup>(311)</sup> Briefly, 10<sup>8</sup> PBMC were electroporated as described above for CAR<sup>+</sup>  $\alpha\beta$  T cells (**Chapter VI.B.4.b.**), and were then sorted for  $\gamma\delta$  T cells using TCR $\gamma/\delta$ + 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<sup>+</sup>  $\gamma\delta$  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 TCR $\gamma\delta$  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 $\gamma\delta$ T cells*

Experiments were initiated to expand  $\gamma\delta$  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 TCR $\gamma/\delta$ + T-cell isolation kit and placed on LS columns to separate  $\gamma\delta$  T cells in the unlabeled fraction from other cells attached to magnet.  $\gamma\delta$  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 V $\delta$ 1 (TCR $\delta$ 1<sup>+</sup> TCR $\delta$ 2<sup>neg</sup>), V $\delta$ 2 (TCR $\delta$ 1<sup>neg</sup> TCR $\delta$ 2<sup>+</sup>), and V $\delta$ 3 (TCR $\delta$ 1<sup>neg</sup> TCR $\delta$ 2<sup>neg</sup>) 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 TCR $\gamma\delta$  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 V $\delta$  sorting assays.

*VI.B.4.e. NK cells*

As controls for killing and allogeneic reactivity, NK cells autologous to  $\gamma\delta$  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.  $\gamma\delta$  T cell Proliferation in Hypoxia*

A dedicated incubator set to 1% O<sub>2</sub>, 5% CO<sub>2</sub>, and 37°C under humidified conditions was used to assess proliferation in hypoxia in parallel to “normal” tissue culture incubators set at 20% O<sub>2</sub>, 5% CO<sub>2</sub>, and 37°C under humidified conditions. Parallel co-cultures were added to the incubators and were analyzed after the reported times.

VI.B.5  $\gamma\delta$  T cell Co-culture Deconstruction

Experiments were implemented to assess the relative contribution of co-culture molecules to  $\gamma\delta$  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 $\gamma\delta$ T cell Proliferation*

In order to assess the dependence of  $\gamma\delta$  T cells on cytokines for proliferation, co-cultures were initiated with  $10^5$   $\gamma\delta$  T cells and  $2 \times 10^5$  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 $\gamma\delta$ T cell Proliferation*

HLA<sup>-/-</sup> aAPC (**Chapter VI.B.2.b**) were used to assess effects of co-stimulation on  $\gamma\delta$  T cell growth. Co-cultures were then initiated with  $10^5$   $\gamma\delta$  T cells in CM, 100 U/mL IL2, and 60 ng/mL IL21 and were added to  $2 \times 10^5$   $\gamma$ -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  $\mu$ L RLT Buffer (Qiagen) per  $3 \times 10^4$  cells and frozen at  $-80^\circ\text{C}$  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 counts. 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 TCR $\alpha$  or TCR $\beta$  allele over the total sum of TCR $\alpha$  or TCR $\beta$  counts, respectively. Total counts for LCA genes described in ROR1-specific CAR<sup>+</sup> T cells (**Figures 12 and 13**) and for TCR $\delta$  and TCR $\gamma$  alleles in polyclonal  $\gamma\delta$  T cells were directly reported as normalized counts (**Figure 30**). For V $\delta$  sorted  $\gamma\delta$  T cells, the normalized counts were reported at frequencies of each V $\delta$  population per donor for each TCR $\delta$  or TCR $\gamma$  allele (**Figure 32**). For example,  $\%V\delta 1*01 = (V\delta 1*01)_{V\delta 1} / [(V\delta 1*01)_{V\delta 1} + (V\delta 1*01)_{V\delta 2} + (V\delta 1*01)_{V\delta 3}]$ .

#### 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  $\mu$ L 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 IIu 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°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. IFN $\gamma$  and TNF $\alpha$ , 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.<sup>(57)</sup> Purified antibodies specific for NKG2D (clone 1D11; BD Biosciences), DNAM1 (clone DX11; BD Biosciences), TCR $\gamma\delta$  (clone B1; BD Biosciences), and TCR $\gamma\delta$  (clone IMMU510; Thermo Fisher, Pittsburg, PA) were used for neutralization experiments at 0.3, 1.0, and 3.0  $\mu\text{g/mL}$  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  $4 \times 10^4$  cells/well. The following day,  $5 \times 10^5$   $\gamma\delta$  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, IFN $\gamma$  production (ELISpot), and cytotoxicity 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°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, IFN $\gamma$  ELISpot plate (Mabtech, Mariemont, OH) was set up with effector ( $\gamma\delta$  T cells) and target (B cells) at an E:T ratio of 0.3:1 and plate was incubated for 24 hours at 37°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<sup>+</sup> cells) at a 4:1 ratio of effectors to CD34<sup>+</sup> HSC were incubated at 37°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<sup>scid</sup> Il2rγ<sup>tm1Wjl</sup>/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 ( $4 \times 10^4$  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<sup>+</sup>  $\gamma\delta$  T cells were evaluated as previously described.(311)

### VI.H.3. $\gamma\delta$ T cells Clearance of Ovarian Cancer

CAOV3-*ffLuc*-mkate (clone 1C2;  $3 \times 10^6$  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  $\gamma\delta$  T cells administered i.p. and PBS administered i.p. as a negative control. T cell doses infused were  $3 \times 10^6$ ,  $6 \times 10^6$ ,  $10^7$ , and  $1.5 \times 10^7$  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	Accession	Target Region	Target Sequence
ABCB1	NM_000927.3	3910-4010	TATAGCACTAAAGTAGGAGACAAAGGAACTCAGCTCTCTGGTGGCCAGAAAC AACGCATTGCCATAGCTCGTGCCCTTGTTAGACAGCCTCATATTTTGC
ABCG2	NM_004827.2	285-385	AGGATTTAGGAACGCACCGTGACATGCTTGGTGGTCTTTGTTAAGTGGAACT GCTGCTTAGAGTTTGTGGGAAGGTCCGGGTGACTCATCCCAACAT
ACTB	NM_001101.2	1010-1110	TGCAGAAGGAGATCACTGCCCTGGCACCAGCACAATGAAGATCAAGATCAT TGCTCCTCCTGAGCGCAAGTACTCCGTGTGGATCGGCGGCTCCATCCT
ADAM19	NM_023038.3	1690-1790	GAGAAGGTGAATGTGGCAGGAGACACCTTTGGAACTGTGGAAAGGACATG AATGGTGAACACAGGAAGTGCAACATGAGAGATGCGAAGTGTGGGAAGA
AGER	NM_001136.3	340-440	GAAAGGAGACCAAGTCCAACCTACCGAGTCCGTGTCTACCAGATTCTGGGAA GCCAGAAATTGTAGATTCTGCCTCTGAACTCACGGCTGGTGTTCCTCAA
AHNAK	NM_001620.1	15420-15520	GGATTTGACCTGAATGTTCTGGGGGTGAAATTGATGCCAGCCTCAAGGCTCC GGATGTAGATGTCAACATCGCAGGGCCGGATGCTGCACTCAAAGTCG
AIF1	NM_032955.1	315-415	AAAAGCGAGAGAAAAGGAAAAGCCAACAGGCCCCAGCCAAGAAAGCTAT CTCTGAGTTGCCCTGATTTGAAGGGAAAAGGGATGATGGGATTGAAGGG
AIM2	NM_004833.1	607-707	ACGTGCTGCACCAAAAGTCTCTCTCATGTTAAGCCTGAACAGAAACAGATG GTGGCCAGCAGGAATCTATCAGAGAAGGGTTTCAGAAGCGCTGTTTG
AKT1	NM_005163.2	1772-1872	TTCTTTGCCGGTATCGTGTGGCAGCACGTGTACGAGAAGAAGCTCAGCCCACC CTTCAAGCCCCAGGTCACGTGCGGAGACTGACACCAGGTATTTTGATG
ALDH1A1	NM_000689.3	11-111	ATTGCTGAGCCAGTCACCTGTGTTCCAGGAGCCGAATCAGAAATGTCATCCTC AGGCACGCCAGACTTACCTGTCTACTACCGATTGGAAGATTCAAT
ANXA1	NM_000700.1	515-615	GAAATCAGAGACATTAACAGGGTCTACAGAGAGGAACTGAAGAGAGATCTG GCCAAAGACATAACCTCAGACACATCTGGAGATTTTCGGAACGCTTTGC
ANXA2P2	NR_003573.1	257-357	ATATTGTCTTCTCCTACCAGAGAAGGACCAAAAAGGAACTTGCATCAGCACT GAAGTCAGCCTTATCTGGCCACCTGGAGACGGTGATTTTGGGCCTATT
API	NM_002228.3	140-240	ACACAGCCAGCCAGCCAGGTCGGCAGTATAGTCCGAACCTGCAAATCTTATTTT CTTTTACCTTCTCTCTAACTGCCAGAGCTAGCGCCTGTGGCTCCC
Apaf1	NM_181869.1	1160-1260	TTCTGATGAAACTGCAGAATCTTTGCACACGGTTGGATCAGGATGAGAGTTTT TCCCAGAGGCTTCCACTTAATATTGAAGAGGCTAAAGACCGTCTCCG
ARG1	NM_000045.2	505-605	AAGGAACTAAAAGGAAAGATTCCCGATGTGCCAGGATTCTCCTGGGTGACTC CCTGTATATCTGCCAAGGATATTGTGTATATTGGCTTGAGAGACGTGG
ATM	NM_000051.3	30-130	ACGCTAAGTCGCTGGCCATTGGTGGACATGGCGCAGGCGGCTTTGCTCCGAC GGGCCGAATGTTTTGGGGCAGTGTTTTGAGCGCGGAGACCGCGTGATA
ATP2B4	NM_001684.3	7640-7740	CTTCCCATAGTATCATCTGTCTCTGGAATGACTCTCCTGTCCCTAAAGGGGTT AAGAGAGAGATCACCTAGAAATCCCCTGAGACACTTGTGGGTTCTT
B2M	NM_004048.2	25-125	CGGGCATTCTGAAGCTGACAGCATTCGGGGCCGAGATGTCTCGCTCCGTGGCC TTAGCTGTGCTCGCGCTACTCTCTCTTTCTGGCCTGGAGGCTATCCA
BACH2	NM_021813.2	3395-3495	TGTGGCACTGTTTCATCTGCTGTCCCGAAGAAACCGAGAACACATTTGGTGCAC ACTACAGCGGTCTTAGCAGCAATACTGTTCCGAAGTATCCTCTCCTC
BAD	NM_004195-295	195-295	CAGCTGTGCCTTGACTACGTAACATCTTGTCTCTACAGCCAGAGCATGTTCC

	322.2		AGATCCCAGAGTTTGTAGCCGAGTGAGCAGGAAGACTCCAGCTCTGCA
BATF	NM_006 399.3	825-925	CACTGTGGGTGTGAGGCCCAATGCAGAAGAGTATTAAGAAAAGATGCTCAAGT CCCATGGCACAGAGCAAGGCGGGCAGGGAACGGTTATTTTCTAAATA
BAX	NM_138 761.2	694-794	ATTTTTCTGGGAGGGGTGGGGATTGGGGGACATGGGCATTTTTCTTACTTTTG TAATTATTGGGGGGTGTGGGGAAGAGTGGTCTTGAGGGGGTAATAAA
BCL10	NM_003 921.2	1250- 1350	TGAAAATACCATCTTCTCTTCAACTACACTTCCCAGACCTGGGGACCCAGGGG CTCCTCCTTTGCCACCAGATCTACAGTTAGAAGAAGAAGGAAGTGT
Bcl2	NM_000 633.2	1525- 1625	CCAAGCACCGCTTCGTGTGGCTCCACCTGGATGTTCTGTGCCTGTAAACATAG ATTCGCTTTCCATGTTGTTGGCCGGATCACCATCTGAAGAGCAGACG
BCL2L1	NM_138 578.1	1560- 1660	CTAAGAGCCATTTAGGGGCCACTTTTGACTAGGGATTGAGGCTGCTTGGGATA AAGATGCAAGGACCAGGACTCCCTCCTCACCTCTGGACTGGCTAGAG
BCL2L11	NM_138 621.2	2825- 2925	TGTTGGCACCAGAAGCTTAAAGCGATGACTGGATGTCTCTGTACTGTATGTATC TGGTTATCAAGATGCCTCTGTGCAGAAAGTATGCCTCCCGTGGGTAT
Bcl6	NM_001 706.2	675-775	GTGTGGACACTTGCCGGAAGTTTATTAAGGCCAGTGAAGCAGAGATGGTTT CTGCCATCAAGCCTCCTCGTGAAGAGTTCCTCAACAGCCGGATGCTGA
Beta-arrestin (ARRB2 and ARRB2)	NM_004 313.3	1652- 1752	CATTAATTTTTTGACTGCAGCTCTGCTTCTCCAGCCCCGCCGTGGGTGGCAAG CTGTGTTTCATACCTAAATTTCTGGAAGGGGACAGTGAAAAGAGGAG
BHLHE41	NM_030 762.2	655-755	CGCCCATTCAGTCCGACTTGGATGCGTTCCTCCACTCGGGATTTCAAACATGCGCC AAAGAAGTCTTGCAATACCTCTCCCGGTTTGAGAGCTGGACACCCAG
BID	NM_197 966.1	2095- 2195	GCTTAGCTTTAGAAACAGTGCAACACTGGTCTGCTGTTCCAGTGGTAAGCTAT GTCCAGGAATCAGTTTAAAGCAGCAGAGTGGATGCTGGGTCCATA
BIRC2	NM_001 166.3	1760- 1860	TGGGATCCACCTCTAAGAATACGTCTCCAATGAGAAACAGTTTGCACATTCA TTATCTCCCACCTTGGAACATAGTAGCTTGTTTCAGTGGTTCCTACTC
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	TGCCCAATAGATTCAAGAGAAGCTAAGCGGAAATGGAGGGTGGAAAGTGTG ATCTGTGGGACTGTCTGGGCCGTGTTACTCATCTGCTATCAATTTCTTA
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	ACGATTACCGCAGCCAAGTGGCGCTGGCAAACTGTTGTAGGTGTTCTTCAG GTAAAGTGCTTCAAGTAGCAGCTCTTTGGGAAACAACTTCAGACC
CA9	NM_001 216.2	960- 1060	CAGGTCCCAGGACTGGACATATCTGCACTCTGCCCTCTGACTTCAGCCGCTA CTTCCAATATGAGGGGTCTCTGACTACACCGCCCTGTGCCAGGGTG
CASP1	NM_033 292.2	575-675	ACAGGCATGACAATGCTGCTACAAAATCTGGGGTACAGCGTAGATGTGAAAA AAAAATCTCACTGCTTCGGACATGACTACAGAGCTGGAGGCATTTGCAC
Caspase 9	NM_052 813.2	1850- 1950	CGCTGACTTGGCCTGGAACGAGGAATCTGGTGCCCTGAAAGGCCAGCCGGA CTGCCGGGCATTGGGGCCGTTTGTTAAGCGGCACTCATTTTGCAGAGG
CAT	NM_001 752.2	1130- 1230	ATGCTTCAGGGCCGCCTTTTTCCTATCTGCACTCACCGCCATCGCTGGG ACCCAATTATCTTCATATACCTGTGAACTGTCCCTACCGTGCTCGAG
CCL3	NM_002 983.2	681-781	CTGTGTAGGCGATCATGGCACCAAAGCCACCAGACTGACAAATGTGTATCGG ATGCTTTTGTTCAGGGCTGTGATCGGCCCTGGGGAAATAATAAAGATGC



CCL4	NM_002 984.2	35-135	TTCTGCAGCCTCACCTCTGAGAAAACCTCTTTGCCACCAATACCATGAAGCTC TGCGTGACTGTCTGTCTCTCCTCATGCTAGTAGCTGCCTTCTGCTC
CCL5	NM_002 985.2	280-380	AGTGTGTGCCAACCCAGAGAAGAAATGGGTTCGGGAGTACATCAACTCTTTG GAGATGAGCTAGGATGGAGAGTCCTTGAACCTGAACTTACACAAATTT
CCNB1	NM_031 966.2	715-815	AACTTGAGGAAGAGCAAGCAGTCAGACCAAAATACCTACTGGGTCGGGAAGT CACTGGAACATGAGAGCCATCCTAATTGACTGGCTAGTACAGGTTC
CCND1	NM_053 056.2	690-790	TTGAACACTTCTCTCCAAAATGCCAGAGGCGGAGGAGAACAAACAGATCAT CCGCAAACACGCGCAGACCTTCGTTGCCCTCTGTGCCACAGATGTGAA
CCR1	NM_001 295.2	535-635	CATCATTGGGGCCCTGGCCATCTTGGCTTCCATGCCAGGCTTATACTTTTCCAA GACCCAATGGGAATTCACCTACCACACCTGCAGCCTTCACTTTCTCT
CCR2	NM_001 123041.2	20-120	ACATTCTGTTGTGCTCATATCATGCAAATTATCACTAGTAGGAGAGCAGAGAG TGGAAATGTTCCAGGTATAAAGACCCACAAGATAAAGAAGCTCAGAG
CCR4	NM_005 508.4	35-135	GGTCCTTCTTAGCATCGTGCTTCCTGAGCAAGCCTGGCATTGCCTCACAGACC TTCCTCAGAGCCGCTTTCAGAAAAGCAAGCTGCTTCTGGTTGGGCCC
CCR5	NM_000 579.1	2730- 2830	TAGGAACATACTTCAGCTCACACATGAGATCTAGGTGAGGATTGATTACCTA GTAGTCATTTTCATGGGTTGTTGGGAGGATTCTATGAGGCAACCACAGG
CCR6	NM_031 409.2	935- 1035	CTTTAACTGCGGGATGCTGCTCCTGACTTGCATTAGCATGGACCGGTACATCG CCATTGTACAGGCGACTAAGTCATTCCGGCTCCGATCCAGAACACTA
CCR7	NM_001 838.2	1610- 1710	TTCCGAAAACCAGGCCTTATCTCCAAGACCAGAGATAGTGGGGAGACTTCTT GGCTTGGTGAGGAAAAGCGGACATCAGCTGGTCAAACAACTCTCTGA
CD11b	NM_000 632.3	515-615	GCCCTCCGAGGGTGTCTCAAGAGGATAGTGACATTGCCTTCTTGATTGATGG CTCTGGTAGCATCATCCACATGACTTTCGGCGGATGAAGGAGTTTG
CD16	NM_000 570.3	73-173	CCTATTCTGTCTTATGGTGGGGCTCCATTGCGAGACTTCAGATTGAGAAATC AGATGAAGTTTCAAGAAAAGGAAACTGGCAGGTGACAGAGATGGGTG
CD160	NM_007 053.2	500-600	TTGATGTTACCATTAAGCCAAGTCACACCGTTGCACAGTGGGACCTACCAGTG TTGTGCCAGAAGCCAGAAGTCAGGTATCCGCCTTCAGGGCCATTTTT
CD19	NM_001 770.4	1770- 1870	AGATTACACCTGACTCTGAAATCTGAAGACCTCGAGCAGATGATGCCAACCC TCTGGAGCAATGTTGCTTAGGATGTGTGCATGTGTGTAAGTGTGTGTG
CD19RCD2 8CAR	MDA_0 0002.1	2-102	CAGGTGTTCTGAAGATGAACAGCCTGCAGACCGACGACACCGCCATCTACT ACTGTGCCAAGCACTACTACTACGGCGGCAGCTACGCCATGGACTACT
CD2	NM_001 767.2	1400- 1500	TGGGTCTCACTACAAGCAGCCTATCTGCTTAAGAGACTCTGGAGTTCTTATG TGCCCTGGTGGACACTTGCCACCATCCTGTGAGTAAAAAGTGAAATA
CD244	NM_016 382.2	1150- 1250	AAGAGGAACCACAGCCCTTCCTTCAATAGCACTATCTATGAAGTGATTGGAA AGAGTCAACCTAAAGCCCAGAACCCTGCTCGATTGAGCCGCAAAGAGC
CD247	NM_198 053.1	1490- 1590	TGGCAGGACAGGAAAAACCCGTCAATGTACTAGGATACTGCTGCGTCATTAC AGGGCACAGGCCATGGATGAAAAACGCTCTCTGCTCTGCTTTTTTCT
CD274	NM_014 143.2	684-784	TAGGAGATTAGATCCTGAGGAAAACCATAACAGCTGAATTGGTCATCCAGAA CTACCTCTGGCACATCCTCCAAATGAAAGGACTCACTTGGTAATTCTG
CD276	NM_001 024736.1	2120- 2220	ACATTTCTTAGGGACACAGTACACTGACCACATCACCACCCTTCTTCCAGT GCTGCGTGGACCATCTGGCTGCCTTTTTTCTCCAAAAGATGCAATAT
CD28	NM_006 139.1	305-405	GCTTGTAGCGTACGACAATGCGGTCAACCTTAGCTGCAAGTATTCCTACAATC TCTTCTCAAGGGAGTTCCGGGCATCCCTTCACAAAGGACTGGATAGT
CD38	NM_001 775.2	1035- 1135	CCTTGACTCCTTGTGGTTTATGTCATCATACATGACTCAGCATACTGCTGGTG CAGAGCTGAAGATTTTGGAGGGTCTCCACAATAAGGTCAATGCCA
CD3D	NM_000 732.4	110-210	TATCTACTGGATGAGTTCCGCTGGGAGATGGAACATAGCACGTTTCTCTCTGG CCTGGTACTGGCTACCCCTTCTCTCGCAAGTGAGCCCTTCAAGATAC

CD3E	NM_000 733.2	75-175	AAGTAACAGTCCCATGAAACAAAGATGCAGTCGGGCACTCACTGGAGAGTTC TGGGCTCTGCCTCTTATCAGTTGGCGTTTGGGGGCAAGATGGTAATG
CD4	NM_000 616.3	835-935	AGACATCGTGGTGTAGCTTTCCAGAAGGCCTCCAGCATAGTCTATAAGAAA GAGGGGGAACAGGTGGAGTTCTCCTTCCCACTCGCCTTTACAGTTGAA
CD40LG	NM_000 074.2	1225- 1325	GCATTTGATTTATCAGTGAAGATGCAGAAGGGAAATGGGGAGCCTCAGCTCA CATTCAGTTATGGTTGACTCTGGGTTCCTATGGCCTTGTGGAGGGGG
CD44	NM_000 610.3	2460- 2560	GTGGGCAGAAGAAAAAGCTAGTGATCAACAGTGGCAATGGAGCTGTGGAGG ACAGAAAGCCAAGTGGACTCAACGGAGAGGCCAGCAAGTCTCAGGAAAT
CD58	NM_001 779.2	478-578	GTGCTTGAGTCTCTTCCATCTCCCACTAACTTGTGCATTGACTAATGGAAG CATTGAAGTCCAATGCATGATACCAGAGCATTACAACAGCCATCGAG
CD63	NM_001 780.4	350-450	GTCATCATCGCAGTGGGTGTCTTCCTTCTCCTGGTGGCTTTTGTGGGCTGTCTGC GGGGCCTGCAAGGAGAACTATTGTCTTATGATACGTTTGCCATCT
CD69	NM_001 781.1	460-560	AGGACATGAACTTTCTAAAACGATACGCAGGTAGAGAGGAACACTGGGTGG ACTGAAAAAGGAACCTGGTCACCCATGGAAGTGGTCAAATGGCAAAGA
CD80	NM_005 191.3	1288- 1388	AAAGATCTGAAGGTCCACCTCCATTTGCAATTGACCTCTTCTGGGAACCTCC TCAGATGGACAAGATTACCCACCTTGCCCTTTACGTATCTGCTCTT
CD86	NM_006 889.3	146-246	TATGGGACTGAGTAACATTCTCTTTGTGATGGCCTTCTGTCTCTGGTGTCTGC TCCTCTGAAGATTCAAGCTTATTTCAATGAGACTGCAGACCTGCCA
CD8A	NM_001 768.5	1320- 1420	GCTCAGGGCTCTTTCTCCACACCATTCAGGTCTTTCTTTCCGAGGCCCTGTCTC TCAGGGTGAGGTGCTTGAGTCTCCAACGGCAAGGGAACAAGTACTT
CDH1	NM_004 360.2	1230- 1330	CGATAATCCTCCGATCTTCAATCCCACCACGTACAAGGGTCAGGTGCCTGAGA ACGAGGCTAACGTCGTAATCACCACACTGAAAGTGACTGATGCTGAT
CDK2	NM_001 798.2	220-320	TCGCTGGCGCTTCATGGAGAACTTCCAAAAGGTGGAAAAGATCGGAGAGGGC ACGTACGGAGTTGTGTACAAAGCCAGAAACAAGTTGACGGGAGAGGTG
CDK4	NM_000 075.2	1055- 1155	ACTTTTAACCCACACAAGCGAATCTCTGCCTTTTCGAGCTCTGCAGCACTCTTA TCTACATAAGGATGAAGGTAATCCGGAGTGAGCAATGGAGTGGCTGC
CDKN1A	NM_000 389.2	1975- 2075	CATGTGTCCTGGTTCCCGTTTCTCCACCTAGACTGTAAACCTCTCGAGGGCAG GGACCACACCCTGTACTGTTCTGTGTCTTTCACAGCTCCTCCACAA
CDKN1B	NM_004 064.2	365-465	GCTTCCGAGAGGGGTTCCGGCCGCGTAGGGGCGCTTGTGTTTGTTCGGTTTGTG TTTTTTTGAGAGTGCGAGAGAGGCGTCTGTCAGACCCGGGAGAAAG
CDKN2C	NM_001 262.2	1295- 1395	ATAATGTAAACGTCAATGCACAAAATGGATTTGGAAGGACTGCGCTGCAGGT TATGAAACTTGAAATCCCGAGATTGCCAGGAGACTGCTACTTAGAGG
CEBPA	NM_004 364.2	1320- 1420	GAGCTGGGAGCCCGCAACTCTAGTATTTAGGATAACCTTGTGCCTTGGAAT GCAAACTCACCGTCCAATGCCTACTGAGTAGGGGGAGCAAATCGTG
CFLAR	NM_003 879.3	445-545	CAAGACCTTGTGAGCTTCCTAGTCTAAGAGTAGGATGTCTGCTGAAGTCAT CCATCAGGTTGAAGAAGCACTTGATACAGATGAGAAGGAGATGCTGC
CIITA	NM_000 246.3	470-570	GCCTGAGCAAGGACATTTTCAAGCACATAGGACCAGATGAAGTGATCGGTGA GAGTATGGAGATGCCAGCAGAAGTTGGGCAGAAAAGTCAGAAAAGACC
CITED2	NM_006 079.3	965- 1065	AGGAGCTGCCCCAACTCTGGCTGGGGCAAAACGAGTTTGATTTTATGACGGA CTTCGTGTGCAAACAGCAGCCCAGCAGAGTGAGCTGTTGACTCGATCG
CLA	NM_003 006.3	2297- 2397	CATGGGCTGTAGGTTGACTTCAGTTTTCCTCTTGGACAACAGGGGGTCTTG TACATCCTTGGGTGACCAGGAAAAGTTCAGGCTATGGGGGGCCAAAG
CLIC1	NM_001 288.4	310-410	GTGATGGGGCCAAGATTGGGAACTGCCATTCTCCAGAGACTGTTTCATGGT ACTGTGGCTCAAGGGAGTCACCTTCAATGTTACCACCGTTGACACCAA
CMRF-35H	NM_007 261.2	0-100	CGGGGAAGTGAGAGTCGGGGATCAGTCTGCAAGCTACGGAGTCACTACAGG GAGAGGTCTCATCACTAGAAATAGCCGAAGAACCTGCAGCCTCAACCA

CREB1	NM_004 379.3	4855- 4955	TTTGATGGTAGGTCAGCAGCAGTGCTAGTCTCTGAAAGCACAAATACCAGTCA GGCAGCCTATCCCATCAGATGTCATCTGGCTGAAGTTTATCTCTGTCT
CRIP1	NM_001 311.4	269-369	CAACCACCCCTGTACGCAGCCATGTTTGGGCCTAAAGGCTTTGGGCGGGGC GGAGCCGAGAGCCACACTTTCAAGTAAACCAGGTGGTGGAGACCCCAT
CSAD	NM_015 989.4	205-305	TCAAATTCTTCTGCCTAGCCTTAGCCATTAGAGAGAGGTCCTGCTAAAGATGG ACTGCAAAATGCGCTTGATGGAAGGAGATGTCAATTCCACTGAAGTCC
CSF2	NM_000 758.2	475-575	AGATGAGGCTGGCCAAGCCGGGAGTGCTCTCTCATGAAACAAGAGCTAGA AACTCAGGATGGTCATCTTGAGGGGACCAAGGGGTGGGCCACAGCCAT
CSNK2A1	NM_177 559.2	1930- 2030	CCATTCCCACCATTGTTCTCCACCGTCCCACACTTTAGGGGGTTGGTATCTCG TGCTCTTCTCCAGAGATTACAAAAATGTAGCTTCTCAGGGGAGGCA
CTGF	NM_001 901.2	1100- 1200	ACCACCCTGCCGGTGGAGTTCAAGTGCCCTGACGGCGAGGTCTGAAGAAGA ACATGATGTTTCATCAAGACCTGTGCCTGCCATTACAACCTGTCCCGGAG
CTLA4	NM_005 214.3	405-505	AGTCTGTGCGGCAACCTACATGATGGGGAATGAGTTGACCTTCTAGATGATT CCATCTGCACGGGCACCTCCAGTGGAATCAAGTGAACCTCACTATC
CTNNA1	NM_001 903.2	75-175	TCGCCCAGCTAGCCGCAGAAATGACTGCTGTCCATGCAGGCAACATAAACTT CAAGTGGGATCCTAAAAGTCTAGAGATCAGGACTCTGGCAGTTGAGAG
CTNNB1	NM_001 098210.1	1815- 1915	TCTTGCCCTTTGTCCCGCAAATCATGCACCTTTGCGTGAGCAGGGTGCCATTC CACGACTAGTTTCAGTTGCTTGTTTCGTGCACATCAGGATACCCAGCGC
CTNNBL1	NM_030 877.3	855-955	TGATGCCAACAACCTGTATTGCAGTGAAGTGCTGGCCATATTGTCCAGGAC AATGATGAAAAACAGGGAATTGCTTGGGGAGCTGGATGGAATCGATGTG
CX3C1	NM_002 996.3	140-240	AGCACCACGGTGTGACGAAATGCAACATCACGTGCAGCAAGATGACATCAAA GATACCTGTAGCTTTGCTCATCCACTATCAACAGAACCAGGCATCATG
CX3CR1	NM_001 337.3	1040- 1140	GGGCGCTCAGTCCACGTTGATTTCTCCTCATCTGAATCACAAAGGAGCAGGCA TGGAAGTGTTCTGAGCAGCAATTTTACTTACCACACGAGTGATGGAG
CXCCR1	NM_000 634.2	1950- 2050	GCAGCCACCAGTCCATTGGGCAGGCAGATGTTCTTAATAAAGCTTCTGTTCCG TGCTTGTCCCTGTGGAAGTATCTTGTTGTGACAGAGTCAAGGGTGT
CXCL10	NM_001 565.1	40-140	GCAGAGGAACCTCCAGTCTCAGCACCATGAATCAAACCTGCGATTCTGATTTC TGCCTTATCTTTCTGACTCTAAGTGGCATTCAAGGAGTACCTCTCTC
CXCL12	NM_199 168.2	505-605	GGGCCTGAGGTTTGCCAGCATTTAGACCCCTGCATTTATAGCATACGGTATGAT ATTGCAGCTTATATTCATCCATGCCCTGTACCTGTGCACGTTGGAAC
CXCL9	NM_002 416.1	1975- 2075	CACCATCTCCCATGAAGAAAGGGAACGGTGAAGTACTAAGCGCTAGAGGAA GCAGCCAAGTCGGTTAGTGAAGCATGATTGGTGCCAGTTAGCCTCTG
CXCR3	NM_001 504.1	80-180	GTGAGTGACCACCAAGTGCTAAATGACGCCGAGGTTGCCGCCCTCCTGGAGA ACTTCAGCTCTTCCTATGACTATGGAGAAAACGAGAGTGACTCGTGCT
CXCR4	NM_001 008540.1	135-235	GTCACTATGGGAAAAGATGGGGAGGAGATTGTAGGATTCTACATTAATTCT CTGTGCCCTTAGCCCACTACTTCAGAATTTCTGAAGAAAGCAAGCC
CYORF14	NR_001 544.2	143-243	GAGGCTGTCTGCCAACATCTTTTCATCACTCTGCCTGCAACTATGAAAAATTTA GTTCTAAAAAATGCAACCTTGCTAAATTGAGTACTAATAGGATTGGT
DAP10	NM_001 007469.1	132-232	ATCCTCTTCTGCTTTTGCTCCAGTGGCTGCAGCTCAGACGACTCCAGGAGA GAGATCATCACTCCCTGCCTTTTACCCTGGCACTTCAGGCTCTTGTT
DAP12	NM_003 332.2	457-557	CTGCACCTCATTCCAACCTCTACCGGATACAGACCCACAGAGTGCCATCCCT GAGAGACCAGACCGCTCCCAATACTCTCTCTAAATAAATGAAGC
DEC1	NM_017 418.2	190-290	AGGCCTTACTTTCCAGATCCAGATCCTTGTGCATACAACCTGACTTGTGTGGGT GAGGCTTGAGAAAAATCAGCTAGAACAGCCTTGGGGGTAGTGGCA
DNAM-1	NM_006 566.2	163-263	TAAACAGGATACGATAAAAGTCCTTAACCAAGACGCAGATGGGAAGAAGCG TTAGAGCGAGCAGCACTCACATCTCAAGAACCAGCCTTTCAAACAGTTT

DPP4	NM_001 935.3	2700- 2800	CAGCAGTCAGCTCAGATCTCCAAAGCCCTGGTCGATGTTGGAGTGGAATTTCCA GGCAATGTGGTATACTGATGAAGACCATGGAATAGCTAGCAGCACAG
EGLN1	NM_022 051.1	3975- 4075	AGCAGCATGGACGACCTGATACGCCACTGTAACGGGAAGCTGGGCAGCTACA AAATCAATGGCCGGACGAAAGCCATGGT
EGLN3	NM_022 073.3	800-900	AAGCTACATGGTGGGATCCTGCGGATATTTCCAGAGGGGAAATCATTTCATAG CAGATGTGGAGCCCCATTTTGGACAGACTCCTGTCTTCTGGTCAGATC
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	TTGAGTAACCTTTAGGAAAATGAGCTGCTGGACTCCTCAGTCAATCTGTCTT TCTAGTCAATGAAAAAGACAGGGTTTGAGGTTCCTTCCGAAACGGGG
Eomes	NM_005 442.2	1670- 1770	ATCCCATGCCCTGGGGTATTACCCAGACCCAACCTTTCTGCAATGGCAGGGT GGGGAGGTCGAGGTCTTACCAGAGGAAGATGGCAGCTGGACTACCA
EPHA4	NM_004 438.3	20-120	GCAGCGTTGGCACC GGCGAACCATGGCTGGGATTTTCTATTTCCGCCCTATTTT CGTGTCTCTTCGGGATTTGCGACGCTGTCACAGGTTCAGGGTATAC
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	TCCATGCCTCTGGAATGGGAAGACACCTATGGAATTGTCTGCTTTCTGGAGT GAAGTATAAGAAGGGTGGCCTTGTGATCAATGAAACTGGGCTGTACT
FLT1	NM_002 019.2	5615- 5715	TTCAACTGCTTTGAACTTGCCCTGGGGTCTGAGCATGATGGGAATAGGGAGA CAGGGTAGGAAAGGGCGCCTACTCTTCAGGGTCTAAAGATCAAGTGGG
FLT3LG	NM_001 459.2	927- 1027	CCTCCCCAGAAATGGAGGCAACGCCAGAATCCAGCACC GGCCCCATTACCCA ACTCTGTACAAAGCCCTTGTCCTCATGAAATTGTATATAAATCATCCT
FOS	NM_005 252.2	1475- 1575	ACTCAAGTCCTTACCTCTTCCGGAGATGTAGCAAAACGCATGGAGTGTGTATT GTTCCCACTGACACTTCAGAGAGCTGGTAGTTAGTAGCATGTTGAGC
FOXP3	NM_014 009.3	1230- 1330	GGGCCATCCTGGAGGCTCCAGAGAAGCAGCGGACACTCAATGAGATCTACCA CTGGTTCACACGCATGTTTGCCCTTCTTCAGAAACCATCCTGCCACCTG
FYN	NM_002 037.3	765-865	GTCTTTGGAGGTGTGAACTCTTCGTCTCATAACGGGGACCTTGCGTACGAGAGG AGGAACAGGAGTGACACTCTTTGTGGCCCTTTATGACTATGAAGCAC
FZD1	NM_003 505.1	2430- 2530	GTGCCAATCCTGACATCTCGAGGTTTCCTCACTAGACAACTCTTTTCGCAGG CTCCTTTGAACAACCTCAGCTCCTGCAAAAAGCTTCCGTCCCTGAGGCA
GAL3ST4	NM_024 637.4	1140- 1240	CGAGCCCCAAACCCTCAATCCCAATGCCCTCATCCATCCTGTTTCCACTGTTAC TGATCATCGCAGCCAGATATCAAGCCCTGCCTCTTTTCGATTTGGGGT
GARNL4	NM_015 085.4	4140- 4240	CCCACGGCTGGAAAGAGGCCTGTACGTTCTGGACGCGTTTGTGGCTGGGCT TCTGGAGGCACTGGCAAGGTCAAACCTGCATTTCTTTAAGAACAGTTG
GAS2	NM_005 256.3	915- 1015	GATCTCCCGTGTGGATGGCAAAACATCCCCTATCCAAAGCAAATCTCCAATC TAAAGGACATGAATCCAGATAACTACTTGGTGGTCTCTGCCAGTTAT
GATA2	NM_032 638.3	1495- 1595	GAAGAAGGAAGGGATCCAGACTCGGAACCGGAAGATGTCCAACAAGTCCAA GAAGAGCAAGAAAGGGGCGGAGTGCTTCGAGGAGCTGTCAAAGTGCATG

GATA3	NM_001 002295.1	2835- 2935	AAGAGTCCGGCGGCATCTGTCTTGTCCCTATTCTGCAGCCTGTGCTGAGGGT AGCAGTGTATGAGCTACCAGCGTGCATGTCAGCGACCCTGGCCCGAC
Gfi1	NM_005 263.2	2235- 2335	TCATCACTGGAGGTAAAAGCACAAAGCAATGCCTGTGGACAAGATGTCATTCA TTCACCTCAGCAAATGTTTCATGGATCACCGGCTACCAAGGTACCAGGCA
GILZ	NM_198 057.2	1400- 1500	TTAAGCAGAGGGCAACCTCTCTCTTCTCCTCTGTTTCGTGAAGGCAGGGGACAC AGATGGGAGAGATTGAGCCAAGTCAGCCTTCTGTTGGTTAATATGGT
GLIPR1	NM_006 851.2	255-355	CTGCGTTCGAATCCATAACAAGTTCCGATCAGAGGTGAAACCAACAGCCAGT GATATGCTATACATGACTTGGGACCCAGCACTAGCCCCAAATTGCAAAA
GLO1	NM_006 708.1	1240- 1340	GGAAATGATATGGTACCCAGACACTGGGCTAGGCTGCAACTTTATCTCATTTA ATACTCCCAGCTGTCATGTGAGAAAGAAAGCAGGCTAGGCATGTGAA
GSK3B	NM_002 093.2	925- 1025	ACTGATTATACCTCTAGTATAGATGTATGGTCTGCTGGCTGTGTGTTGGCTGA GCTGTTACTAGGACAACCAATATTTCAGGGGATAGTGGTGTGGATC
GZMA	NM_006 144.2	155-255	AGACCCTACATGGTCCTACTTAGTCTTGACAGAAAAACCATCTGTGCTGGGGC TTTGATTGCAAAAGACTGGGTGTTGACTGCAGCTCACTGTAACCTGA
GZMB	NM_004 131.3	540-640	ACACTACAAGAGGTGAAGATGACAGTGCAGGAAGATCGAAAGTGCGAATCT GACTTACGCCATTATTACGACAGTACCATTGAGTTGTGCGTGGGGGACC
GzmH	NM_033 423.3	705-805	AAAAAAGGGACACCTCCAGGAGTCTACATCAAGGTCTCACACTTCCTGCCCT GGATAAAGAGAACCAATGAAGCGCCTCTAACAGCAGGCATGAGACTAAC
HDAC1	NM_004 964.2	785-885	CAAGCCGGTCACTGTCCAAAGTAATGGAGATGTTCCAGCCTAGTGGGTGGTC TTACAGTGTGGCTCAGACTCCCTATCTGGGGATCGGTTAGGTTGCTTC
HDAC2	NM_001 527.1	930- 1030	AAGCCTATTATCTCAAAGGTGATGGAGATGTATCAACCTAGTGTGTGGTATT ACAGTGTGGTGCAGACTCATTATCTGGTGATAGACTGGGTTGTTTCA
HES1	NM_004 649.5	1340- 1440	TTGAGTTAATCAGCGTAAGGGGATTTCTAAAGCAGGCAATCCCTGTAGCCGC AGAGAATAAACGCCTTCCCAAAATGGCAACTTCCACAGCCACATTTC
HLA-A	NM_002 116.5	1000- 1100	GGAAGAGCTCAGATAGAAAAGGAGGGAGTTACACTCAGGCTGCAAGCAGTG ACAGTGCCAGGGCTCTGATGTGTCCCTCACAGCTTGTAAGTGTGAGA
HOXA10	NM_018 951.3	1503- 1603	TTCTATAGAGATAGATATTGTCCTAAGTGTCAAGTCCTGACTGGGCTGGGTTT GCTGTCTTGGGGTCCCACTGCTCGAAATGGCCCTGTCTTCGGCCGA
HOXA9	NM_152 739.3	1015- 1115	GGCTCTAAACCTCAGGCCACATCTTTTCCAAGGCAAACCTGTTAGGCTGGC TCGTAGGCCTGCCGCTTTGATGGAGGAGGTATTGTAAGCTTTCCATT
HOXB3	NM_002 146.4	60-160	TGTCCGTTTTAAATGCTGCTGGGAGACTCGTAAAAAAATCATCGTGACCTGG AGGATGAGAGGGGCGAGCTTTATTTCGGTCGGATTGCGGTGTGGTGGT
HOXB4	NM_024 015.4	1340- 1440	CCTTTCTTTGTCCCCACTCCCAGTACCCAGCGAAAGCACCTCTGACTGCCA GATAGTGCAGTGTGTTGGTACGGTAACACACACACTCTCCCTCA
HPRT1	NM_000 194.1	240-340	TGTGATGAAGGAGATGGGAGGCCATCACATTGTAGCCCTCTGTGTGCTCAAG GGGGGCTATAAAATCTTTGCTGACCTGCTGGATTACATCAAAGCACTG
HRH1	NM_000 861.2	3055- 3155	GTGGCAGCTCAAAATGATATGTTTGTAGTAGACGAACAGCTGACATGGAGTTC CCGTGCACCTACGGAAGGGGACGCTTTGAAGGAACCAAGTGCAATTTT
HRH2	NM_022 304.1	600-700	GCGTCTCATCTCATCACCGTTGCTGGCAATGTGGTCGTCTGTCTGGCCGT GGGCTTGAACCGCCGGCTCCGCAACCTGACCAATTGTTTCATCGTGT
IAP	NM_001 777.3	897-997	GCCATATTGGTTATTTCAGGTGATAGCCTATATCCTCGTGTGGTTGGACTGAG TCTCTGTATTGCGGCGTGTATACCAATGCATGGCCCTCTTCTGATT
ICOS	NM_012 092.2	640-740	AACTCTGGCACCCAGGCATGAAGCACGTTGGCCAGTTTCTCAACTTGAAGT GCAAGATTCTCTTATTTCCGGGACCACGGAGAGTCTGACTTAACTAC
ICOSLG	NM_015 259.4	1190- 1290	CTGCTGGCGTTGGCTGTGATCCTGGAATGAGGCCCTTTCAAAAGCGTCATCCA CACCAAAGGCAAATGTCCCAAGTGAGTGGGCTCCCCGCTGTCACTG

ID2	NM_002 166.4	505-605	CGGATATCAGCATCTGTCTTGCAGGCTTCTGAATCCCTTCTGAGTTAATGT CAAATGACAGCAAAGCACTGTGTGGCTGAATAAGCGGTGTTTCATGA
IFNa1	NM_024 013.1	585-685	ATCCCTCTCTTTATCAACAACTTGCAAGAAAGATTAAGGAGGAAGGAATAA CATCTGGTCCAACATGAAAACAATTCTTATTGACTCATAACCAGGTC
IFNG	NM_000 619.2	970- 1070	ATACTATCCAGTTACTGCCGGTTTGAAAAATATGCCTGCAATCTGAGCCAGTGC TTTAATGGCATGTCAGACAGAACTTGAATGTGTCAGGTGACCCTGAT
IFNGR1	NM_000 416.1	1140- 1240	CCCGGGCAGCCATCTGACTCCAATAGAGAGAGAGAGTTCTTACCTTTAAGT 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 AGGGTTACCTGGGTGCCAAGCCTTGCTGAGATGATCCAGTTTACC
IL10RA	NM_001 558.2	150-250	TGCCCAGCCCTCCGTCTGTGTGGTTTGAAGCAGAATTTTCCACCACATCCTC CACTGGACACCCATCCCAAATCAGTCTGAAAGTACCTGCTATGAAGT
IL12A	NM_000 882.2	775-875	CTTTCTAGATCAAAACATGCTGGCAGTTATTGATGAGCTGATGCAGGCCCTGA ATTCAACAGTGAGACTGTGCCACAAAAATCCTCCCTGAAGAACCG
IL12RB1	NM_005 535.1	1292- 1392	AGGAAAAGTGTTACTACATTACCATCTTTGCCTCTGCGCACCCCGAGAAGCTC ACCTTGTGGTCTACGGTCTGTCCACCTACCCTTTGGGGGCAATGC
IL12RB2	NM_001 559.2	1315- 1415	CCTCCGTGGGACATTAGAATCAAATTTCAAAGGCTTCTGTGAGCAGATGTAC CCTTTATTGGAGAGATGAGGGACTGGTACTGCTTAATCGACTCAGAT
IL13	NM_002 188.2	516-616	TTTCTTCTGATGTCAAAAATGTCTTGGGTAGGCGGGAAGGAGGGTTAGGGA GGGGTAAAATTCCTTAGCTTAGACCTCAGCCTGTGCTGCCCGCTCTTCA
IL15	NM_172 174.1	1685- 1785	AGGGTGATAGTCAAATTATGTATTGGTGGGGCTGGGTACCAATGCTGCAGGT CAACAGCTATGCTGGTAGGCTCCTGCCAGTGTGGAACCACTGACTACT
IL15Ra	NM_002 189.2	39-139	CGCTCGCCCGGGAGTCCAGCGGTGCTCTGTGGAGCTGCCGCCATGGCCCCG CGCGGGGCGCGCGGTGCCGGACCCTCGGTCTCCCGGCGCTGCTACTG
IL17A	NM_002 190.2	240-340	TACTACAACCGATCCACCTACCTTGGAATCTCCACCGAATGAGGACCCTGA GAGATATCCCTCTGTGATCTGGGAGGCAAAGTGCCGCCACTTGGGCT
IL17F	NM_052 872.3	210-310	GCCCGCCTGTGCCAGGAGGTAGTATGAAGCTTGACATTGGCATCATCAATGA AAACCAGCGCGTTTCATGTCACGTAACATCGAGAGCCGCTCCACCTC
IL17RA	NM_014 339.4	3020- 3120	CTACTATGTGGCGGGCATTGTTGGATACCAAGATAAATTGCATGCGGCATGGC CCCAGCCATGAAGGAACCTAACCCTAGTGCCGAGGACACGTAAACG
IL18	NM_001 562.2	48-148	GACAGTCAGCAAGGAATTGTCTCCAGTGCATTTTGCCCTCCTGGCTGCCAAC TCTGGCTGCTAAAGCGGCTGCCACCTGCTGCAGTCTACACAGCTTCG
IL18R1	NM_003 855.2	2025- 2125	GAATGAGGGGATTTTAAAGTGTCTGAAGAGGCATTTTCTAGGGACCAGTGGGT GACTGAGTAACCTGAAATGCTGCTTCACTCCCTAACACCATGGATCTG
IL18RAP	NM_003 853.2	2412- 2512	GCTTGATGGACAATGGAGTGGGATTGAGACTGTGGTTTAGAGCCTTTGATTTC CTGGACTGGACTGACGGCGAGTGAATTCTCTAGACCTTGGGTACTTT
IL2	NM_000 586.2	300-400	AGGATGCAACTCTGTCTTGCAATTGCACTAAGTCTTGCACTTGTCACAAACAG TGCACCTACTTCAAGTTCTACAAAGAAAACACAGCTACAACTGGAGC
IL21R	NM_021 798.2	2080- 2180	CGTGTGTTGGTCAACAGATGACAACAGCCGCTCCTCCCTCCTAGGGTCTTGTG TTGCAAGTTGGTCCACAGCATCTCCGGGGCTTTGTGGGATCAGGGCA
IL22	NM_020 525.4	319-419	CTATCTGATGAAGCAGGTGCTGAACTTCACCCTTGAAGAAGTGTGTTCCCTC AATCTGATAGGTTCCAGCCTTATATGCAGGAGGTGGTGCCCTTCCTG

IL23R	NM_144 701.2	710-810	AACTGCAAATTCACCTGGATGATATAGTGATACCTTCTGCAGCCGTCATTTCC AGGGCTGAGACTATAAATGCTACAGTGCCCAAGACCATAATTTATTG
IL2RA	NM_000 417.1	1000- 1100	CTTGGAAGAAGCCGGGAACAGACAACAGAAGTCATGAAGCCCAAGTGAAA TCAAAGGTGCTAAATGGTCGCCCAGGAGACATCCGTTGTGCTTGCCTGC
IL2RB	NM_000 878.2	1980- 2080	GTCCTGCTGCCCAGCCAGGAAGTGTGTGTTGCAGGGGGGCAGTAACTCC CCAACTCCCTCGTTAATCACAGGATCCCACGAATTTAGGCTCAGAAGC
IL2RG	NM_000 206.1	595-695	CCACAGCTGGACTGAACAATCAGTGGATTATAGACATAAGTTCTCCTTGCCTA GTGTGGATGGGCAGAAACGCTACACGTTTCGTGTTCCGAGCCGCTTT
IL4	NM_000 589.2	625-725	GACACTCGTGCCTGGGTGCGACTGCACAGCAGTTCCACAGGCACAAGCAGC TGATCCGATTCTGAAACGGCTCGACAGGAACCTCTGGGGCCTGGCGG
IL4R	NM_000 418.2	705-805	ATCATCTCACCTATGCAGTCAACATTTGGAGTGAAAACGACCCGGCAGATTTC AGAATCTATAACGTGACCTACCTAGAACCTCCCTCCGCATCGCAGC
IL5	NM_000 879.2	105-205	CCACAGAAATTCACAAAGTGCATTGGTGAAAGAGACCTTGGCACTGCTTTCT ACTCATCGAACTCTGCTGATAGCCAATGAGACTCTGAGGATTCTGT
IL6	NM_000 600.1	220-320	TGACAAACAAATTCGGTACATCCTCGACGGCATCTCAGCCCTGAGAAAGGAG ACATGTAACAAGAGTAACATGTGTGAAAGCAGCAAAGAGGCACTGGCA
IL6R	NM_000 565.2	993- 1093	CTTTCTACATAGTGTCCATGTGCGTCGCCAGTAGTGTGCGGAGCAAGTTCAGC AAAACCTCAAACCTTTCAGGGTTGTGGAATCTTGCAGCCTGATCCGCC
IL7R	NM_002 185.2	1610- 1710	TTGCTTTGACCACTCTTCTGAGTTCAGTGGCACTCAACATGAGTCAAGAGCA TCCTGCTTCTACCATGTGGATTGGTCAAGGTTAAGGTGACCCA
IL9	NM_000 590.1	300-400	AAGTACTAAAGAACAACAAGTGTCCATATTTTTCTGTGAACAGCCATGCAA CCAAACCACGGCAGGCAACGCGCTGACATTTCTGAAGAGTCTTCTGGA
INDO	NM_002 164.3	50-150	CTATTATAAGATGCTCTGAAAACCTCTTCAGACACTGAGGGGCACCAGAGGAG CAGACTACAAGAATGGCACACGCTATGGAAAACCTCTGGACAATCAGT
IRF1	NM_002 198.1	510-610	CTGTGCGAGTGTACCGGATGCTTCCACCTCTCACCAGAACCAGAGAAAAAGA AAGAAAGTCGAAGTCCAGCCGAGATGCTAAGAGCAAGGCCAAGAGGAA
IRF2	NM_002 199.2	1375- 1475	CAGTACCTGGAGCTTCTCTTAACTCAGGACTCCAGCCATTGGTAGACGTGT GTTTCTAGAGCCTGCTGGATCTCCAGGGCTACTCACTCAAGTTCAA
IRF4	NM_002 460.1	325-425	GGGCACTGTTAAAGGAAAGTTCGGAGAAGGCATCGACAAGCCGGACCTCC CACCTGGAAGACGCGCCTGCGGTGCGCTTTGAACAAGAGCAATGACTT
ITGA1	NM_181 501.1	1875- 1975	AAGTGGCAAGACTATAAGGAAAGAGTATGCACAACGTATTCCATCAGGTGGG GATGGTAAGACACTGAAATTTTGGCCAGTCTATCCACGGAGAAATG
ITGA4	NM_000 885.4	975- 1075	GCCCACTGCCAACTGGCTCGCCAACGCTTCAGTGATCAATCCCGGGGCGATTT ACAGATGCAGGATCGGAAAGAATCCCGGCCAGACGTGCGAACAGCTC
ITGA5	NM_002 205.2	925- 1025	AGAAGACTTTGTTGCTGGTGTGCCAAAGGGAACCTCACTTACGGCTATGTCA CCATCCTTAATGGCTCAGACATTCGATCCCTCTACAACCTTCTCAGGG
ITGAL	NM_002 209.2	3905- 4005	GTGAGGGCTTGTCAATTACCAGACGGTTCACCAGCCTCTCTGGTTTCTCCTT GGAAGAGAATGTCTGATCTAAATGTGGAGAACTGTAGTCTCAGGA
ITGB1	NM_033 666.2	2000- 2100	TTTTAACATTACCAAGGTAGAAAGTCGGGACAAATTACCCAGCCGGTCCAA CCTGATCCTGTGTCCATTGTAAGGAGAAGGATGTTGACGACTGTTGG
ITK	NM_005 546.3	3430- 3530	GCCAGTAAAGAAGTCAGTATAGAACCACTAGCGAATAGTGTGCTCTGGCAC AGACCACTGTGGTTGATGGCATGGCCCTCCAACCTTGAATAGGATTTT
JAK1	NM_002 227.1	285-385	GAGAACACCAAGCTCTGGTATGCTCCAAATCGCACCATCACCGTTGATGACA AGATGTCCCTCCGGCTCCACTACCGGATGAGGTTCTATTTACCAATT
JAK2	NM_004 972.2	455-555	CTCCTCCCGCGACGGCAAATGTTCTGAAAAAGACTCTGCATGGGAATGGCCT GCCTTACGATGACAGAAATGGAGGGAACATCCACCTCTTCTATATATC

JAK3	NM_000 215.2	1715- 1815	GTGCTGCTGAAGGTCATGGATGCCAAGCACAGAAGTGCATGGAGTCATTCC TGGAAGCAGCGAGCTTGATGAGCCAAGTGTCGTACCGGCATCTCGTGC
JunB	NM_002 229.2	1155- 1255	GCGCGCCTGGAGGACAAGGTGAAGACGCTCAAGGCCGAGAACGCGGGGCTG TCGAGTACCGCCGGCCTCCTCCGGGAGCAGGTGGCCCAGCTCAAACAGA
KIR2DL1 (NKAT1)/C D158a	NM_014 218.2	881-981	GCAGGAAACAGAACAGCGAATAGCGAGGACTCTGATGAACAAGACCCCTCAG GAGGTGACATACACACAGTTGAATCACTGCGTTTTACACAGAGAAAAA
KIR2DL2 (NKAT6)/C D158b	NM_014 219.2	814-914	TCTCCTTCATCGCTGGTGCTCCAACAAAAAAATGCTGCGGTAATGGACCAA GAGTCTGCAGGGAACAGAACAGCGAATAGCGAGGACTCTGATGAACAA
KIR2DL3 (NKAT2)/C D158b	NM_015 868.2	741-841	CTCCGAAACCGGTAACCCAGACACCTGCATGTTCTGATTGGGACCTCAGTGG TCATCATCCTCTTCATCCTCCTCCTCTTCTTCTCCTTCATCGCTGG
KIR2DL4 (p 49 CD158d)	NM_002 255.5	15-115	GCGTCTTGCGAGCAGAAGCTGCACCATGTCCATGTCAACCCACGGTCATCATCC TGGCATGTCTTGGGTCTTCTTGGACCAGAGTGTGTGGGCACACGTG
KIR2DL5A	NM_020 535.3	1451- 1551	GACACGTGCTGTTCCACCTTCCCTCATGCTGTTTCACCTTTCCTCAGACTATTT TCCAGCCTTCTGTCACTCAGCAGTGAAACTTATAAAATTTTTGTG
KIR2DS1	NM_014 512.1	698-798	CTTCAACCACTGAACCAAGCTCCGAAACCGGTAACCCAGACACCTACATGT TCTGATTGGGACCTCAGTGGTCAAAATCCCTTTCACCATCCTCCTCTT
KIR2DS2 (NKAT5)/C D158b	NM_012 312.2	856-956	CAAGAGCCTGCAGGGAACAGAACAGTGAACAGCGAGGATTCTGATGAACAA GACCATCAGGAGGTGTCATACGCATAATTGGATCACTGTGTTTTACAC
KIR2DS3 (NKAT7)	NM_012 313.1	693-793	GGCCTTCACCACTGAACCAAGCTCCAAAACCGGTAACCCAGACACCTACA CGTCTGATTGGGACCTCAGTGGTCAAACTCCCTTTCACCATCCTCCT
KIR2DS4 (NKAT8)	NM_012 314.3	1427- 1527	ACATACAAGAGGCTGCCTCTTAACACAGCACTTAGACACGTGCTGTTCCACCT CCCTTCAGACTATCTTTCAGCCTTCTGCCAGCAGTAAACTTATAAA
KIR2DS5 (NKAT9)	NM_014 513.2	204-304	CTTCCTTCTGCACAGAGAGGGGACGTTTAAACACACTTTGCGCCTCATTGGAG AGCACATTGATGGGGTCTCCAAGGGCAACTTCTCCATCGGTGCGATG
KIR3DL1 (NKAT3/NK B1)	NM_013 289.2	1054- 1154	CCAAATCTGGTAACCCAGACACCTGCACATTCTGATTGGGACCTCAGTGGTC ATCATCCTCTTCATCCTCCTCCTCTTCTTCTCCTTCATCTCTGGTG
KIR3DL2 (NKAT4)	NM_006 737.2	884-984	TGCCACCCACGGAGGGACCTACAGATGCTTCGGCTCTTTCCGTGCCCTGCCCT GCGTGTGGTCAAACCTCAAGTGACCCACTGCTTGTTCTGTACAGGA
KIR3DL3 (KIRC1 CD158z)	NM_153 443.3	508-608	CCTTGCGCCTCGTTGGACAGCTCCACGATGCGGGTTCCAGGTCAACTATTCC ATGGGTCCCATGACACCTGCCCTTGACAGGGACCTACAGATGCTTTGG
KIR3DS1 (NKAT10)	NM_001 083539.1	1000- 1100	CTCCAAATCTGGTAACCTCAGACACCTGCACATTCTGATTGGGACCTCAGTGG TCAAAATCCCTTTCACCATCCTCCTCTTCTTCTCCTTCATCGCTGG
KIT	NM_000 222.1	5-105	CATCGCAGTACCGCGATGAGAGGCGCTCGCGGCGCTGGGATTTCTCTGCG TTCTGCTCCTACTGCTTCGCGTCCAGACAGGCTCTTCTCAACCATCT
KLF10	NM_005 655.1	570-670	GCTCAGGCAACAAGTGTGATTCTGCATACAGCTGATGCCAGCTATGTAACC ACCAGACCTGCCCAATGAAAGCAGCCAGCATCCTCAACTATCAGAACAA
Klf2	NM_016 270.2	1015- 1115	GGAAGTTTTCGCGCTCAGACGAGCTCACGCGCCACTACCGAAAGCACACGGG CCACCGGCCATTCCAGTGCCATCTGTGCGATCGTGCTTCTCGCGCTC
KLF4	NM_004 235.4	1980- 2080	CGAGCATTTTCAGGTGCGACCACTCGCCTTACACATGAAGAGGCATTTTAA AATCCCAGACAGTGGATATGACCCACACTGCCAGAAGAGAATTCAGT



KLF6	NM_001 008490.1	1165- 1265	GGGATGCGTGTTCAGCCAAAGCATGCCGTTCTGCACCCTACCCAGTTGCCTC CAGGGCCTCTCCTTGGAAGGTCTTTTGAGGGCTAAAAAGTCTCTGTA
KLRB1	NM_002 258.2	85-185	TGAGTTAAACTTACCCACAGACTCAGGCCAGAAAAGTTCTTCACCTTCATCTC 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 CTCTCCAGGCCTTCTTGTCTTGCCTTGTGGCAATAGCTTTGGGGCT
LAIR1	NM_002 287.3	1195- 1295	GCACCTGAGGGTAGAAAAGTCACTCTAGGAAAAGCCTGAAGCAGCCATTGGA AGGCTTCCTGTGGATTCTCTTCATCTAGAAAGCCAGCCAGGCAGCT
LCK	NM_005 356.2	1260- 1360	ATTAAGTGGACAGCGCCAGAAGCCATTAACCTACGGGACATTCACCATCAAGT CAGATGTGTGGTCTTTTGGGATCCTGCTGACGGAAATTGTCACCCACG
LDHA	NM_005 566.1	985- 1085	CAGAATGGAATCTCAGACCTTGTGAAGGTGACTCTGACTTCTGAGGAAGAGG CCCGTTTGAAGAAGAGTGCAGATACACTTTGGGGGATCCAAAAGGAGC
Lef1	NM_016 269.3	1165- 1265	CCGTCAACATCCCATCAGATGTCAACTCCAAACAAGGCATGTCCAGACATC CTCCAGCTCCTGATATCCCTACTTTTTATCCCTTGTCTCCGGGTGGTG
LGALS3	NM_002 306.2	120-220	CAGCCGTCCGGAGCCAGCCAACGAGCGGAAAATGGCAGACAATTTTCGCTC CATGATGCGTTATCTGGGTCTGGAAACCCAAACCCTCAAGGATGGCCT
LNK	NM_005 475.2	4285- 4385	CCTCCAGCCAGAAGTTAAACATCTGGGATATGACGTCTTCATGCCAGGGGCA CTCATTTCTTAGCAGCCTCTCTACATACATCTCTCAGGTGGTGCCAAG
LOC282997	NR_026 932.1	665-765	TGATCACATTCTACCTGGCATTATTTTCATCTGAGTCCCTGTCTAGCCCTTCTG CCCATTAGACTGTAACCTTGTTTAGGGAAAGACCTGTGTCTTACTC
LRP5	NM_002 335.1	2515- 2615	TGGACACCAACATGATCGAGTCGTCCAACATGCTGGGTGAGGAGCGGGTCGT GATTGCCGACGATCTCCCGCACCCGTTCCGGTCTGACGCAGTACAGCGA
LRP6	NM_002 336.1	2185- 2285	CTTAGATTATCCAGAAGGCATGGCAGTAGACTGGCTTGGGAAGAAGTTGTAC TGGGCAGACACAGGAACGAATCGAATTGAGGTGTCAAAGTTGGATGGG
LRRC32	NM_005 512.2	3470- 3570	CACCCTGGTGTGGGTTCTCCTGTCTCTCTGTGCTCTTGCAATTCTCTATTCCCT TTCTCTATTGAGCAGAGCCTGGAGTTTGAGACTATGGAATCCA
MAD1L1	NM_003 550.2	306-406	GAAGACCTGGGGGAAAACACCATGGTTTTATCCACCCTGAGATCTTTGAACA ACTTCATCTCTCAGCGTGTGGAGGGAGGCTCTGGACTGGATATTCTA
MAP2K1	NM_002 755.2	970- 1070	ACGGAATGGACAGCCGACCTCCCATGGCAATTTTGTAGTTGTTGGATTACATA GTCAACGAGCCTCTCCAAAAGTCCCGAGTGGAGTGTTCAGTCTGGA
MAPK14	NM_001 315.1	450-550	TGGGCTCTGGCGCCTATGGCTCTGTGTGTGCTGCTTTTGACACAAAAACGGGG TTACGTGTGGCAGTGAAGAAGCTCTCCAGACCATTTCAGTCCATCAT
MAPK3	NM_002 746.2	580-680	AACGTGCTCCACCGAGATCTAAAGCCCTCCAACCTGCTCATCAACACCACCTG CGACCTTAAGATTTGTGATTTCCGGCTGGCCCGGATTGCCGATCCTG
MAPK8	NM_139 049.1	945- 1045	TCTCTGTAGATGAAGCTCTCCAACACCCGTACATCAATGTCTGGTATGATCCT TCTGAAGCAGAAGCTCCACCACCAAAGATCCCTGACAAGCAGTTAGA
MCL1	NM_021 960.3	1260- 1360	GCTGTAACCTCCTAGAGTTGACCCTAGCAACCTAGCCAGAAAAGCAAGTGG 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	AACTGTTGCATGGATCCTGTGTTTGCAACTGGGGAGACAGAACTGTGGTTG ATAGCCAGTCACTGCCTTAAGAACATTTGATGCAAGATGGCCAGCACT
Myc	NM_002 467.3	1610- 1710	TCGGACACCGAGGAGAATGTCAAGAGGCGAACACACAACGTCTTGGAGCGCC AGAGGAGGAACGAGCTAAAACGGAGCTTTTTTGCCTGCGTGACCAGA
MYO6	NM_004 999.3	6655- 6755	AAGTTGGGGAGATGGCACCTTCTCAGAGGATTGTGAAAATATGAGGAAGAAA CAAAACAGTGCATGTAGGAGCACAGGGCCACACAAAGGCATTCTATTG
NBEA	NM_015 678.3	8645- 8745	CTGAGAGCCCTTGAAGGACCAGAAAAGTCTTATTTCCACGCTTGATATCTGT CTCCAGCGAAGGCCACTGTATCATATACTATGAACGAGGGCGATTCA
NCAM1	NM_000 615.5	1620- 1720	GGTATTTGCCTATCCCACTGCCACGATCTCATGGTTTCGGGATGGCCAGCTGC TGCCAAGCTCCAATTACAGCAATATCAAGATCTACAACACCCCCTCT
NCL	NM_005 381.2	1492- 1592	GAACAGAGATCGATGGGCGATCTATTTCCCTGTACTATACTGGAGAGAAAGG TCAAAATCAAGACTATAGAGGTGGAAAGAATAGCACTTGAGTGGTGA
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 ATCCAGAGTCCAGTGGCAGAATCGTCTCTTTACAGACTGCATCTAAC
NFATC3	NM_004 555.2	2190- 2290	GTCCTTGAAGTTCCTCCATATCATAACCCAGCAGTTACAGCTGCAGTGCAGGT GCACTTTTATCTTTGCAATGGCAAGAGGAAAAAAGCCAGTCTCAAC
NKG2C	NM_002 260.3	942- 1042	TATGTGAGTCAGCTTATAGGAAGTACCAAGAACAGTCAAACCCATGGAGACA GAAAGTAGAATAGTGGTTGCCAATGTCTCAGGGAGGTTGAAATAGGAG
NKG2D	NM_007 360.1	760-860	GGACCAGGATTTACTTAACTGGTGAAGTCATATCATTGGATGGGACTAGTA CACATTCCAACAAATGGATCTTGGCAGTGGGAAGATGGCTCCATTCTC
NKG2E	NM_002 261.2	760-860	ACTCCTGAGCTCAAGAAATCAACACATCTTGGCCTCCCAAGTTGCTGGGATTA CTGACACAAGCCACCGCCCTGAGTGCTCATGTACCATTTAGCTTGT
NKG2F	NM_013 431.2	29-129	TTATATTGGTCAACAGCAAAATGAACATTACTACTAGCCTCCAACACATGCA GTTTGCCTATACCAGGGATCCTGTCAAAATATACACCATTATAGCT
NKp30 (CD337)	NM_147 130.1	50-150	GCATCTGTCTCTCTCTCAGGGAGGCAAGCATTTGATGCTCGAGGTCCCTGG CAGTTGTGGTCTTTGGCAAGTGATGTGTGAGTCCCGTGTGTCATAGG
NKp44 (CD336)	NM_004 828.3	798-898	CTTCAACAGGTCACGGACCTTCCCTGGACCTCAGTTTCTCACCTGTAGAGAG 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	CTGCCAGGCTTCAACGGCCAGAACTGTGAGGAAAATATCGACGATTGTCCAG GAAACAACCTGCAAGAACGGGGTGCTGTGTGGACGGCGTGAACACCT
NR3C1	NM_001 018077.1	1665- 1765	GCTTTCTCTCTGGCGGGAGAAGACGATTTCCTTTTGGAAGGAAACTCGA ATGAGGACTGCAAGCCTCTCATTTTACCGGACACTAAACCCAAAATT
NR4A1	NM_002 135.3	155-255	CGGCCGGGTAGGGTGCAGCCTGAGGCTTGTTTCAGCAGAACAGGTGCAAGCCA CATTGTTGCCAAGACCTGCCTGAAGCCGGATTCTCCCCACTGCCTCCT

NRIP1	NM_003 489.2	335-435	TGACTCATGGAGAAGAGCTTGGCTCTGATGTGCACCAGGATTCTATTGTTTAA ACTTACCTAGAAAGGATTACTAATGCATCAGGCAGCAGGGGGATCAGG
NT5E	NM_002 526.2	1214- 1314	ATTCGGGTTTTGAAATGGATAAACTCATCGCTCAGAAAGTGAGGGGTGTGGA CGTCGTGGTGGGAGGACACTCCAACACATTTCTTTACACAGGCAATCC
OPTN	NM_001 008211.1	625-725	TGAAGCTAAATAATCAAGCCATGAAAGGGAGATTTGAGGAGCTTTCGGCCTG GACAGAGAAACAGAAGGAAGAACGCCAGTTTTTGTAGATACAGAGCAA
P2RX7	NM_002 562.4	340-440	AGTTGGTGCACAGTGTCTTTGACACCGCAGACTACACCTTCCCTTTGCAGGGG AACTCTTTCTTCGTGATGACAAACTTTCTCAAAACAGAAGGCCAAGA
p38	NM_006 303.3	507-607	CCCTCTCCCTGCTTGTGCTGCACAGGCTGCTCTGTGAGCACTTCAGGGTCTG TCCACGGTGCACACGCACTCCTCGGTCAAGAGCGTGCCTGAAAACCT
Pax5	NM_016 734.1	2288- 2388	CTCCAAGAGGAGCACACTTTGGGGAGATGTCTCTGGTTTCTGCCTCCATTCT CTGGGACCGATGCAGTATCAGCAGCTCTTTCCAGATCAAAGAAGCTC
PDCD1	NM_005 018.1	175-275	CTTCTTCCCAGCCCTGCTCGTGGTGACCGAAGGGGACAACGCCACCTTCACCT GCAGCTTCTCCAACACATCGGAGAGCTTCGTGCTAAACTGGTACCGC
PDCD1LG2	NM_025 239.3	235-335	TGTGGAGCTGTGGCAAGTCCTCATATCAAATACAGAACATGATCTTCTCCTG CTAATGTTGAGCCTGGAATTGCAGCTTACCAGATAGCAGCTTTATT
PDE3	NM_000 921.3	3010- 3110	CTGGCCAACCTTCAGGAATCCTTCATCTCTCACATTGTGGGGCCTCTGTGCAA CTCCTATGATTCAGCAGGACTAATGCCTGGAAGTGGGTGGAAGACA
PDE4	NM_001 111307.1	3855- 3955	AATAATGGTGTATACCCTCATTCTCATTCCTGGGCAGCCCTTCTCTCCACCCTG GCACCAAAATAATTCTCCTCCATCCGTACCTTGCTAGCCTCTCC
PDE7	NM_002 604.2	2210- 2310	GTAGCTCAACAAGGAATAGAGGGAGGAGTGAATTTTGGTAGCTGGTGTGA ATAGGGCCTTTGAGAATCAGACTGAACACAGTGAAATATGTGCCAAA
PDK1	NM_002 610.3	1170- 1270	TGGATTGCCCATATCACGTCTTTACGCACAATACTTCCAAGGAGACCTGAAGC TGTATTCCCTAGAGGGTTACGGGACAGATGCAGTTATCTACATTAAG
PECAM1	NM_000 442.3	1365- 1465	ATCTGCACTGCAGGTATTGACAAAGTGGTCAAGAAAAGCAACACAGTCCAGA TAGTCGTATGTGAAATGCTCTCCCAGCCAGGATTCTTATGATGCCC
PHACTR2	NM_001 100164.1	8350- 8450	GGCAGAATGCCACTCTACCCTCAGGTCAATTTTATGGTATATGAAAATGCCAG TAATATTTGTGCCACTTGCCAACTCGGGGAGGAGGGGCTTTTCCCT
PHC1	NM_004 426.2	2905- 3005	ATACAGCTCCACCTACACCGGAATTACATGGCATCAACCCTGTGTTCCTGTCC AGTAATCCCAGCCGTTGGAGTGTAGAGGAGGTGTACGAGTTTATTGC
POP5	NM_015 918.3	560-660	GCTTCAGGCCCACTTGTGTAACAGAACAATCTGGGTAGCAACAGCATCTTCCA CAGTTTTCCAAACTGGATAGCTGCCAACCAGCAGACATTACCCACTT
PPARA	NM_001 001928.2	5220- 5320	GGGTGTGTTTGCTATACGAACATAATGGACGTGAAGTGGGGCAGAAACCCAG AACTCAGCATTCAAGGATGCCCAGGAGAGCTGTCCCTGTTTTAAAGAG
PPP2R1A	NM_014 225.3	1440- 1540	AACTTAACCTTGTGCATGGCCTGGCTTGTGGATCATGTATATGCCATCCGC GAGGCAGCCACCAGCAACCTGAAGAAGCTAGTGGAAGGTTTGGGAA
PRDM1	NM_182 907.1	310-410	CATCCCTGCCAACCAGGAACCTTCTTGTGTGGTATTGTGGGACTTTGCAGAAA GGCTTCACTACCCTTATCCCGGAGAGCTGACAATGATGAATCTCACA
PRF1	NM_005 041.3	2120- 2220	ACTGTTTTTCAGGGAGGTGGCTGGGTTTACACGCTAATCCCGATTACCCCTGT CCAAACTGCCTAAGCCCTCCGCCATTCTCAAGCCCTGCAGTCACAGC
PROM1	NM_006 017.1	925- 1025	AGCCTGCGGTATCTCTCAATGACCCTCTGTGCTTGGTGCATCCATCAAGTGA AACCTGCAACAGCATCAGATTGTCTCTAAGCCAGCTGAATAGCAACC
PTGER2	NM_000 956.2	1410- 1510	GTCAGAAGGAGCTACAAAACCTACCCTCAGTGAGCATGGTACTTGGCCTTTG GAGGAACAATCGGCTGCATTGAAGATCCAGCTGCCTATTGATTAAAGC
PTK2	NM_005 607.3	1005- 1105	GGTTCAAGCTGGATTATTTCAAGTGGAACTGGCAATCGGCCCAGAAGAAGGAA TCAGTTACCTAACGGACAAGGGCTGCAATCCCACACATCTTGCTGACT

PTPRK	NM_001 135648.1	4315- 4415	GTGATCAACCGGATTTTTAGGATATGCAATCTAACAAGACCACAGGAAGGTT ATCTGATGGTGCAACAGTTTCAGTACCTAGGATGGGCTTCTCATCGAG
RAC1	NM_198 829.1	1250- 1350	AAAGACCTTCGCTTTTGAGAAGACGGTAGCTTCTGCAGTTAGGAGGTGCAGA CACTTGCTCTCCTATGTAGTTCTCAGATGCGTAAAGCAGAACAGCCTC
RAC2	NM_002 872.3	1069- 1169	GCTGCCACAACCTTGTGTACCTTCAGGGATGGGGCTCTTACTCCCTCCTGAGGC CAGCTGCTCTAATATCGATGGTCCTGCTTGCCAGAGAGTTCTCTCTAC
RAP46	NM_004 323.3	1490- 1590	CTCTTGATCGTGTAGTCCCATAGCTGTAAAACCAGAATCACCAGGAGGTTG CACCTAGTCAGGAATATTGGGAATGGCCTAGAACAAGGTGTTTGGCA
RARA	NM_000 964.2	115-215	AGCCACCTAGCTGGGGCCCATCTAGGAGTGGCATCTTTTTTGGTGCCCTGAAG GCCAGCTCTGGACCTTCCCAGGAAAAGTGCCAGCTCACAGAACTGCT
RHOA	NM_001 664.2	1230- 1330	GGTACTCTGGTGAGTCACTTCAGGGCTTACTCCGTAACAGATTTTGTG GCATAGCTCTGGGGTGGGCAGTTTTTTGAAAATGGGCTCAACCAGAA
RORA	NM_134 261.2	1715- 1815	AAAATTAACCGAGACACTTTATATGGCCCTGCACAGACCTGGAGCGCCACAC ACTGCACATCTTTTGGTGATCGGGGTCAGGCAAAGGAGGGGAAACAAT
RORC	NM_001 001523.1	1350- 1450	CTCATCAATGCCCATCGGCCAGGGCTCCAAGAGAAAAGGAAAGTAGAACAGC TGCAGTACAATCTGGAGCTGGCCTTCATCATCATCTCTGCAAGACTC
RUNX1	NM_001 754.4	635-735	CAGCCATGAAGAACCAGGTTGCAAGATTTAATGACCTCAGGTTTGTGCGGTCG AAGTGAAGAGGGGAAAAGCTTCACTCTGACCATCACTGTCTTCACAAA
RUNX2	NM_004 348.3	1850- 1950	GAAGCCACAGCAGTTCCCCAACTGTTTTGAATTCTAGTGGCAGAATGGATGA ATCTGTTTTGGCGACCATATTGAAATTCCTCAGCAGTGGCCCACTGGTA
S100A4	NM_002 961.2	263-363	CAGGGACAACGAGGTGGACTTCCAAGAGTACTGTGTCTTCTGTCTGCATCG CCATGATGTGTAACGAATTCTTTGAAGGCTTCCAGATAAGCAGCCC
SATB1	NM_001 131010.1	1335- 1435	TTCCGAAATCTACCAGTGGGTACGCGATGAAGTGAACGAGCAGGAATCTCC CAGGCGGTATTTGCACGTGTGGCTTTTAACAGAACTCAGGGCTTGCTT
SCAP2	NM_003 930.3	3374- 3474	TTTTACAGTTAATCCAGGAGAGGGAGTCCTTTGCCAACTGATGACCAACAGTT CCAAGCCAGATAGTCTCGTGAACAGTGACAATACAGAAATAAGGTGT
SCML1	NM_001 037540.1	925- 1025	GCAACGTATGGTTCTTCTTCAGGGCTCTGCCTTGGAACCCCTCGGGCTGACAG CATCCACAACACTTACTCAACTGACCATGCTTCTGCAGCACCACCTT
SCML2	NM_006 089.2	360-460	ATTGGAAGCCCGTGACCCTCGCAATGCCACTTCAGTATGTATTGCTACGGTTA TTGGAATTACTGGGGCCAGGTTACGTTTACGACTGGATGGTAGTGAC
SEL1L	NM_005 065.4	980- 1080	GGGCAATCTAATAGCCCACATGGTTTTTGGGTTACAGATACTGGGCTGGCATCG GCGTCCTCCAGAGTTGTGAATCTGCCCTGACTCACTATCGTCTTGTT
SELL	NM_000 655.3	110-210	CTCCCTTTGGGCAAGGACCTGAGACCCTTGCTGCTAAGTCAAGAGGCTCAATG GGCTGCAGAAGAACTAGAGAAGGACCAAGCAAAGCCATGATATTTCCA
SERPINE2	NM_006 216.2	240-340	CGCTGCCTTCCATCTGCTCCCACTTCAATCCTCTGTCTCTCGAGGAACTAGGCT CCAACACGGGGATCCAGGTTTTCAATCAGATTGTGAAGTCGAGGCC
SHP-1	NM_002 831.5	1734- 1834	TGGTGCAGACGGAGGCGCAGTACAAGTTCATCTACGTGGCCATCGCCAGTT CATTGAAACCACTAAGAAGAAGCTGGAGGTCCTGCAGTCGCAGAAGGG
SIT1	NM_014 450.2	720-820	GCCCCAGCCCCCGTAGCAGGGGCATGACTGTTTCCCAACCAGCACCCAAAG ACGGGCGCCATTGCCAAGTCACAGGATGTGATCTACCCCGGACTTCCT
SLA2	NM_032 214.2	1640- 1740	AAAGGAAAGCTGAGATGATGTCTTACCGTAGCAGCAGATCTGGATGGTCCA GGCTCTATGTGACCTCCAGAGCAAAGAGAAAGACTTCGGACAGTCTAG
SLAMF1	NM_003 037.2	580-680	GTGTCTCTTGATCCATCCGAAGCAGGCCCTCCACGTTATCTAGGAGATCGCTA CAAGTTTTATCTGGAGAATCTCACCTGGGGATACGGGAAAGCAGGA
SLAMF7	NM_021 181.3	215-315	GGGCACTATCATAGTGACCCAAAATCGTAATAGGGAGAGAGTAGACTTCCCA GATGGAGGCTACTCCCTGAAGCTCAGCAAACCTGAAGAAGAATGACTCA

SLC2A1	NM_006 516.2	2500- 2600	AGGCTCCATTAGGATTTGCCCTTCCCATCTCTTCTACCCAACCACTCAAATT AATCTTTCTTTACCTGAGACCAGTTGGGAGCACTGGAGTGCAGGGA
SMAD3	NM_005 902.3	4220- 4320	TTAAAGGACAGTTGAAAAGGGCAAGAGGAAACCAGGGCAGTTCTAGAGGAG TGCTGGTGACTGGATAGCAGTTTTAAGTGGCGTTCACCTAGTCAACACG
SNAI1	NM_005 985.2	63-163	GACCACTATGCCGCGCTCTTTCCTCGTCAGGAAGCCCTCCGACCCCAATCGGA AGCCTAACTACAGCGAGCTGCAGGACTCTAATCCAGAGTTTACCTTC
SOD1	NM_000 454.4	35-135	GCCTATAAAGTAGTCGCGGAGACGGGGTGCTGGTTTGGCTCGTAGTCTCCTGC AGCGTCTGGGGTTTCCGTTGCAGTCTCGGAACCAGGACCTCGGCGT
SPI1	NM_003 120.1	730-830	CTCCGCAGCGCGACATGAAGGACAGCATCTGGTGGGTGGACAAGGACAAG GGCACCTTCCAGTTCTCGTCCAAGCACAAGGAGGCGCTGGCGCACCGCT
STAT1	NM_007 315.2	205-305	TTTGCTGTATGCCATCCTCGAGAGCTGTCTAGGTAAACGTTTCGCACTCTGTGT ATATAACCTCGACAGTCTTGGCACCTAACGTGCTGTGCGTAGCTGCT
STAT3	NM_139 276.2	4535- 4635	AGACTTGGGCTTACCATTGGGTTTAAATCATAGGGACCTAGGGCGAGGGTTC AGGGCTTCTCTGGAGCAGATATTGTCAAGTTCATGGCCTTAGGTAGCA
STAT4	NM_003 151.2	789-889	AGACAATGGATCAGAGTGACAAGAATAGTGCCATGGTGAATCAGGAAGTTTT GACACTGCAGGAAATGCTTAACAGCCTCGATTTCAGAGAAAGGAGGC
STAT5A	NM_003 152.2	3460- 3560	GAGACAGAGAGAGAGAAAGAGAGAGTGTGTGGGTCTATGTAATGCATCTGT CCTCATGTGTGTATGTAACCGATTCTCTCTCAGAAGGGAGGCTGGGG
STAT5B	NM_012 448.3	200-300	AAGGAGAAGCCCTTCATCAGATGCAAGCGTTATATGGCCAGCATTTTCCATT GAGGTGCGGCATTATTTATCCAGTGGAATGAAAGCCAAGCATGGGA
Stat6	NM_003 153.3	2030- 2130	AGAACATCCAGCCATTCTCTGCCAAAGACCTGTCCATTGCTCACTGGGGGAC CGAATCCGGGATCTTGCTCAGCTCAAAAATCTCTATCCCAAGAAGCC
STMN1	NM_203 401.1	287-387	CGTGGGTGGCGGCAGGACTTTCCTTATCCAGTTGATTGTGCAGAATACACTG CCTGTCTGCTTGTCTTCTATTACCATGGCTTCTTCTGATATCCAGGT
TBX21	NM_013 351.1	890-990	ACACAGGAGCGCACTGGATGCGCCAGGAAGTTTCATTGGGAAACTAAAGCT CACAAACAACAAGGGGGCGTCCAACAATGTGACCCAGATGATTGTGCT
TBXA2R	NM_001 060.3	385-485	CACACGCGCTCCTCCTCCTCACCTTCTCTGCGGCCTCGTCTCACCAGCTTC CTGGGGCTGCTGGTGACCGGTACCATCGTGGTGTCCAGCACGCCG
Tcf7	NM_003 202.2	2420- 2520	ATTCCATTTCAGTTCATCTATGGCAGTCCAGCCAGCTCCTGGGCAGCTTGAG AGGGCAAACCCAAAACCTCATGACAGCCAGAGCCTGTCTTTCAGCAT
TDGF1	NM_003 212.2	1567- 1667	AAGGAAAGAAAACATCTTTAAGGGGAGGAACCAGAGTGCTGAAGGAATGGA AGTCCATCTGCGTGTGTGCAGGGAGACTGGGTAGGAAAGAGGAAGCAAA
TDO2	NM_005 651.1	0-100	AAGGTCAATGATAGCATCTGCCTAGAGTCAAACCTCCGTGCTTCTCAGACAGT GCCTTTTACCATGAGTGGGTGCCCATTTTTAGGAAACAACCTTTGGA
TEK	NM_000 459.2	615-715	CGAGTTCGAGGAGAGGCAATCAGGATACGAACCATGAAGATGCGTCAACAA GCTTCTTCTACCAGCTACTTTAACTATGACTGTGGACAAGGGAGATA
TERT	NM_198 253.1	2570- 2670	GGCTTCAAGGCTGGGAGGAACATGCGTCGAAACTCTTTGGGGTCTTGCGGC TGAAGTGTACAGCCTGTTTCTGGATTTGCAGGTGAACAGCCTCCAGA
TF	NM_001 063.2	640-740	CTGCTCCACCCTTAACCAATACTTCGGCTACTCGGGAGCCTTCAAGTGTCTGA AGGATGGTGTCTGGGGATGTGGCCTTTGTCAAGCACTCGACTATATTT
TFRC	NM_003 234.1	1220- 1320	CAGTTTCCACCATCTCGTTCATCAGGATTGCCTAATATACCTGTCCAGACAAT CTCCAGAGCTGCTGCAGAAAAGCTGTTTGGGAATATGGAAGGAGACT
TGFA	NM_003 236.2	780-880	TGCCACAGACCTTCTACTTGGCCTGTAATCACCTGTGCAGCCTTTTGTGGGC CTTCAAAACTCTGTCAAGAACTCCGTCTGCTTGGGGTTATTCAAGTGT
TGFB1	NM_000 660.3	1260- 1360	TATATGTTCTTCAACACATCAGAGCTCCGAGAAGCGGTACCTGAACCCGTGTT GCTCTCCCGGGCAGAGCTGCGTCTGCTGAGGCTCAAGTTAAAGTGG

TGFB2	NM_003 238.2	1125- 1225	AAGCCAGAGTGCCTGAACAACGGATTGAGCTATATCAGATTCTCAAGTCCAA AGATTTAACATCTCCAACCCAGCGCTACATCGACAGCAAAGTTGTGAA
TGFBR1	NM_004 612.2	4280- 4380	GGGGAAATACGACTTAGTGAGGCATAGACATCCCTGGTCCATCCTTTCTGTCT CCAGCTGTTTCTTGGAACCTGCTCTCCTGCTTGCTGGTCCCTGACGC
TIE1	NM_005 424.2	2610- 2710	CATCGGGGAGGGGAACCTTCGGCCAGGTCATCCGGGCCATGATCAAGAAGGAC GGGCTGAAGATGAACGCAGCCATCAAAATGCTGAAAGAGTATGCCTCT
TLR2	NM_003 264.3	180-280	CTGCTTTCAACTGGTAGTTGTGGGTTGAAGCACTGGACAATGCCACATACTTT GTGGATGGTGTGGGTCTTGGGGGTCATCATCAGCCTCTCCAAGGAAG
TLR8	NM_138 636.3	2795- 2895	GACAAAAACGTTCTCCTTTGTCTAGAGGAGAGGGATTGGGATCCGGGATTGG CCATCATCGACAACCTCATGCAGAGCATCAACCAAAGCAAGAAAACAG
TNF	NM_000 594.2	1010- 1110	AGCAACAAGACCACCACTTCGAAACCTGGGATTAGGAATGTGTGGCCTGCA CAGTGAAGTGCTGGCAACCACTAAGAATTCAAACCTGGGGCCTCCAGAA
TNFRSF18	NM_004 195.2	445-545	AGGGGAAATTCAGTTTGGCTTCCAGTGTATCGACTGTGCCTCGGGGACCTTC TCCGGGGGCCACGAAGGCCACTGCAAACCTTGACAGACTGCACCCA
TNFRSF1B	NM_001 066.2	835-935	CCCAGCTGAAGGGAGCACTGGCGACTTCGCTCTTCCAGTTGGACTGATTGTGG GTGTGACAGCCTTGGGTCTACTAATAATAGGAGTGGTGAACCTGTGTC
TNFRSF4	NM_003 327.2	200-300	CCGTGCGGGCCGGGCTTCTACAACGACGTGGTCAGCTCCAAGCCGTGCAAGC CCTGCACGTGGTGTAACCTCAGAAGTGGGAGTGAGCGGAAGCAGCTGT
TNFRSF7	NM_001 242.4	330-430	CCAGATGTGTGAGCCAGGAACATTCTCGTGAAGGACTGTGACCAGCATAGA AAGGCTGCTCAGTGTGATCCTTGACATACCGGGGTCTCCTTCTCTCCT
TNFRSF9	NM_001 561.4	255-355	AGATTTGCAGTCCCTGTCTCCAAATAGTTTCTCCAGCGCAGGTGGACAAAGG ACCTGTGACATATGCAGGCAGTGTAAGGTGTTTTACGACCAGGAA
TNFSF10	NM_003 810.2	115-215	GGGGGGACCCAGCCTGGGACAGACCTGCGTGCTGATCGTGATCTTCACAGTG CTCTGCAGTCTCTCTGTGTGGCTGTAACCTACGTGTACTTTACCAAC
TNFSF14	NM_003 807.2	270-370	ATTTTCAGAAGCCTCTGGAAAGTCGTGCACAGCCCAGGAGTGTGAGCAATTT CGGTTTCTCTGAGGTTGAAGGACCCAGGCGTGTCAGCCTGTCTCCA
TOX	NM_014 729.2	3950- 4050	AATGAGCAGCTTTGACTTTGACAGGCGGTTTGTGCAGGAAAGCACAGTGCCG TGTTGTTTACAGCTTTTCTAGAGCAGCTGTGCGACCAGGGTAGAGAGT
TP53	NM_000 546.2	1330- 1430	GGGGAGCAGGGCTCACTCCAGCCACCTGAAGTCCAAAAAGGGTCAGTCTACC TCCC GCCATAAAAACTCATGTTCAAGACAGAAGGGCCTGACTCAGAC
TRAF1	NM_005 658.3	3735- 3835	CGAGTGATGGGTCTAGGCCCTGAACTGATGTCCTAGCAATAACCTCTTGATC CCTACTCACCGAGTGTGAGCCCAAGGGGGGATTTGTAGAACAAGCC
TRAF2	NM_021 138.3	1325- 1425	GTGGCCCTTCAACCAGAAGGTGACCTTAATGCTGCTCGACCAGAATAACCGG GAGCACGTGATTGACGCCTTCAGGCCCGACGTGACTTCATCCTCTTTT
TRAF3	NM_145 725.1	1795- 1895	ATATGATGCCTGCTTCTTGCCGTTTAAGCAGAAAGTGACACTCATGCTGA TGGATCAGGGGTCTCTCGACGTCATTGGGAGATGCATTCAAGCCC
TSLP	NM_033 035.3	395-495	CCGTCTCTGTAGCAATCGGCCACATTGCCTTACTGAAATCCAGAGCCTAACC TTCAATCCACCGCCGGCTGCGCGTCGCTCGCCAAAGAAATGTTTCGC
TYK2	NM_003 331.3	485-585	TCATCGCTGACAGCTGAGGAAGTCTGCATCCACATTGCACATAAAGTTGGTAT CACTCCTCCTTGCTTCAATCTCTTGGCCCTCTTCGATGCTCAGGCCC
VEGFA	NM_001 025366.1	1325- 1425	GAGTCCAACATCACCATGCAGATTATGCGGATCAAACCTCACCAAGGCCAGC ACATAGGAGAGATGAGCTTCTACAGCACAACAAATGTGAATGCAGAC
WEE1	NM_003 390.2	5-105	TGCGTTTGAGTTTGCCGCGAGCCGGGCCAATCGGTTTTGCCAACGCATGCCA CGTGCTGGCGAACAAATGTAAACACGGAGATCGTGTCGCGGCACTT
ZAP70	NM_001 079.3	1175- 1275	GGAGCTCAAGGACAAGAAGCTCTTCTGAAGCGCGATAACCTCCTCATAGCT GACATTGAACCTGGCTGCGGCAACTTTGGCTCAGTGCGCCAGGGCGTG

ZNF516	NM_014 643.2	4830- 4930	GGTGGGGGACGGCTTCATATACCTCTTCCTCAGTAATGCAAATGCGAGTTTTT GTGGTGGGGGTAAAGGCCCATAAACAAAGGATCTTAAACCATGCAGTG
p16	NM_000 077.3	975- 1075	AAGCGCACATTTCATGTGGGCATTTCTTGCGAGCCTCGCAGCCTCCGGAAGCTG TCGACTTCATGACAAGCATTTTGTGAAGTAGGGAAGCTCAGGGGGGT
SHP2	NM_002 834.3	4650- 4750	TAGTCCCTAGGTTGCTACGGCTTATCATGTGCTTGGTAAAAAGGTGATCGCAGG TTCTCAGACGAGTTTACTTTACATGAGATGGAATCAGGCAGAGAGGC
CD57/B3GA T1	NM_018 644.3	145-245	CTGGACAGCGACCCCTTCTCAGACTCCAGTTGGGCCGAGCTCTCCAAACCTGC TTCCGCAATGGGTGGGTGTGAGTGCTGGTAATGAGGAGCCGTGGGT
CD85/LILR B1	NM_001 081637.1	2332- 2432	AGCTGAGAAAACTAAGTCAGAAAGTGCATTAAACTGAATCACAATGTAAATA TTACACATCAAGCGATGAAACTGGAAAACTACAAGCCACGAATGAATG
Neil1	NM_024 608.2	1675- 1775	TTAGCAGGAGGCTCTCCTTGCTTGCACTCACCTTTCTTATTGTCTTGCCCTGC ATCTGGGGGTCTGAATTTTGGGAGCAGGCAATATCTGAAGGTGCA
Neil2	NM_145 043.2	2570- 2670	GCCCGGTGGTGTGTAGAGAAAAGCTGCTTGTTTACTCCTTAAGTCAATGTATT GGTGACTGTTGATTTGTTGAACAATTCAGGAATCAAGGGCTGTGGAG
PNK	NM_003 681.3	580-680	TCCCGGAGGACCTCCTTCCCGTCTACAAAGAAAAAGTGGTGCCGCTTGCAGA CATTATCACGCCCAACAGTTTGAGGCCGAGTTACTGAGTGGCCGGAA
POLR2A	NM_000 937.2	3775- 3875	TTCCAAGAAGCCAAAAGACTCCTTCGCTTACTGTCTTCTGTGGGCCAGTCCG CTCGAGATGCTGAGAGAGCCAAGGATATTCTGTGCCGTCTGGAGCAT
POLR1B	NM_019 014.3	3320- 3420	GGAGAACTCGGCCTTAGAATACTTTGGTGAGATGTTAAAGGCTGCTGGCTAC AATTTCTATGGCACCGAGAGGTTATATAGTGGCATCAGTGGGCTAGAA
IL-1alpha	NM_000 575.3	1085- 1185	ACTCCATGAAGGCTGCATGGATCAATCTGTGTCTCTGAGTATCTCTGAAACCT CTAAACATCCAAGCTTACCTTCAAGGAGAGCATGGTGGTAGTAGCA
IL-1beta	NM_000 576.2	840-940	GGGACCAAAGGCGGCCAGGATATAACTGACTTCACCATGCAATTTGTGTCTTC CTAAAGAGAGCTGTACCCAGAGAGTCTGTGTGAATGTGGACTCAA
IL-12p40	NM_002 187.2	1435- 1535	GCAAGGCTGCAAGTACATCAGTTTTATGACAATCAGGAAGAATGCAGTGTTC TGATACCAGTGCCATCATACACTTGTGATGGATGGGAACGCAAGAGAT
Raf-1	NM_002 880.2	1990- 2090	CCTATGGCATCGTATTGTATGAACTGATGACGGGGGAGCTTCCTTATTCTCAC ATCAACAACCGAGATCAGATCATCTTCATGGTGGGCCGAGGATATGC
IL-23p19	NM_016 584.2	411-511	CAGGGACAACAGTCAGTTCTGCTTGCAAAGGATCCACCAGGGTCTGATTTTTT ATGAGAAGCTGCTAGGATCGGATATTTTCACAGGGGAGCCTTCTCTG
gBAD- 1R_scfv	SCFV00 1.1	1-101	AGACAGACACCCTGCTCCTCTGGGTGTCCGGCACCTGTGGCGACATCGTGATG AGCAGAAGCCCCAGCAGCCTGGCCGTGTCCGTGGGCGAGAAAGTGAC
CD20_scfv_r utuximab	SCFV00 2.1	8-108	GCTGTCCCAGAGCCCCGCCATCCTGAGCGCCAGCCCTGGCGAGAAGGTGACC ATGACCTGCCGGGCCAGCAGCTCTGTGAGCTACATGCACTGGTATCAG
c-MET_scfv	SCFV00 4.1	138-238	CTGATCTACGCCGCCAGCAGCCTGAAGAGCGGCGTGCCAGCCGGTTAGCG GCTCTGGCTCTGGCGCCGACTTCACCCTGACCATCAGCAGCCTGCAGC
CD45R_scfv	SCFV00 6.1	222-322	TTCACCCTGAACATCCACCCGTGGAGGAAGAGGACGCCGCCACCTACTACT GCCAGCACAGCAGAGAGCTGCCCTTACCTTCGGCTCCGGCACCAAGC
Thymidine_k inase	SCFV00 7.1	100-200	TCTACGTACCCGAGCCGATGACTTACTGGCAGGTGCTGGGGGCTTCGAGAC AATCGCGAACATCTACACCACACAACACCGCCTCGACCAGGGTGAGAT
CD56R_scfv	SCFV00 8.1	197-297	ATTCAGCGGCTCTGGCTCCGGCACCGACTTCACTCTGATGATCTCTCGGTGG AGGCCGAGGACCTGGGCGTGTACTACTGCTTTCAGGGCAGCCACGTG
Human_CD1 9R_scfv	SCFV00 9.1	215-315	CTTACCATCAGCAGCCTGCAGCCCAGGACATCGCCACCTACTACTGCCAG CAGTACCAGAGCCTGCCCTACACCTTCGGCCAGGGCACCAAGCTGCAG
DECTIN-1R	SCFV01 0.1	270-370	CTGAAGATCGACAGCAGCAACGAGCTGGGCTTCATCGTGAAGCAGGTGTCCA GCCAGCCCCGACAACTCCTTCTGGATCGGCCTGAGCAGGCCCCAGACCG

HERV-K_6H5_scfv	SCFV01 2.1	137-237	CGGCGGCACCAGCTACAACCAGAAGTTCAAGGACAAGGCCATCCTGACCGTG GACAAGAGCAGCAGCACC GCCTACATGGAAGTGC GGAGCCTGACCAGC
CD19R_scfv	SCFV01 3.1	204-304	GGCACCGACTACAGCCTGACCATCTCCAACCTGGAGCAGGAGGACATCGCCA CCTACTTTTGCCAGCAGGGCAACACACTGCCCTACACCTTTGGCGGCG
HER2_scfv	SCFV01 4.1	64-164	CCTGCAGCGCCAGCAGCAGCGTGTCTACATGCACTGGTATCAGCAGAAAGTC CGGCACTAGCCCCAAGCGGTGGATCTACGACACCTACAAGCTCGCCAG
EGFR_scfv_NIMO_CAR	SCFV01 5.1	7-107	AGATGACCCAGAGCCCTAGCAGCCTGAGCGCCAGCGTGGGCGACAGAGTGA CCATCACCTGCCGGTCCAGCCAGAACATCGTGCACAGCAACGGCAACAC
RPL27	NM_000 988.3	23-123	GGGCCGGGTGGTGTGTGCCGAAATGGGCAAGTTTCATGAAACCTGGGAAGGTG GTGCTTGTCTGGCTGGACGCTACTCCGGACGCAAAGCTGTCATCGTG
OAZ1	NM_004 152.2	313-413	GGTGGGCGAGGGAATAGTCAGAGGGATCACAATCTTTCAGCTAACTTATTCT ACTCCGATGATCGGCTGAATGTAACAGAGGAACTAACGTCCAACGACA
GABPa	NM_002 040.3	1160-1260	GACCAAGTCCTGCATTGGGTGGTTTGGGTAATGAAGGAATTCAGCATGACCG ATATAGACCTCACCACACTCAACATTTGGGGGAGAGAATTATGTAGTC
XBP-1	NM_005 080.2	440-540	GGAGTTAAGACAGCGCTTGGGGATGGATGCCCTGGTTGCTGAAGAGGAGGCG GAAGCCAAGGGGAATGAAGTGAGGCCAGTGGCCGGGTCTGCTGAGTCC
MBD2	NM_003 927.3	2015-2115	ATTTACATTCAACTCTGATCCCTGGGCCTTAGGTTTGACATGGAGGTGGAGGA AGATAGCGCATATATTTGCAGTATGAACTATTGCCTCTGGACGTTGT
Bcl6b	NM_181 844.3	2135-2235	CTTTATTTGTTCTAGGGCAGCTCTGGGAACATGCGGGATTGTGGAATTGGGTC AGGAACCCTCTCTGGTATTCTGGATGTTGTAGGTTCTCTAGCAGTCT
TSLP-R	NM_022 148.2	1420-1520	CAAGGCAGCACGTCCAAAATGCTGTAAAACCATCTTCCACTCTGTGAGTCCC CAGTTCGCTCCATGTACCTGTTCCATAGCATTGGATTCTCGGAGGAT
BTLA	NM_001 085357.1	890-990	GCACCAACAGAATATGCATCCATATGTGTGAGGAGTTAAGTCTGTTTCTGACT CCAACAGGGACCATTGAATGATCAGCATGTTGACATCATTGTCTGGG
HVEM	NM_003 820.2	916-1016	CTCAGGGAGCCTCGTCATCGTCATTGTTTGCTCCACAGTTGGCCTAATCATAT GTGTGAAAAGAAGAAAGCCAAGGGGTGATGTAGTCAAGGTGATCGTC
LTbR	NM_002 342.1	1435-1535	CTAACAGGGGCCCCAAGGAACCAATTTATCACCCATGACTGACGGAGTCTGAG AAAAGGCAGAGAAGGGGGGCACAAGGGCACTTTCTCCCTTGAGGCTG
CD43	NM_001 030288.1	2798-2898	AAGCCAGGCTTCATGGAAGATCGTATGTGTGACCCAAATATGAGTTCCTTCA GCTCAGCCATGGTAATCCCTTCCTTGAAGTCTCCATTTCTGCAGTACA
mTOR	NM_004 958.2	5095-5195	TTAGTGTTGCTCCTGGGAGTTGATCCGTCTCGGCAACTTGACCATCCTCTGCC AACAGTTCACCCTCAGGTGACCTATGCCTACATGAAAAACATGTGGA
AMPK	NM_006 252.2	975-1075	ATAGTGGTGACCCTCAAGACCAGCTTGCAGTGGCTTATCATCTTATCATTGAC AATCGGAGAATAATGAACCAAGCCAGTGAGTTCTACCTCGCCTCTAG
SIP1	NM_001 009182.1	537-637	ACAAGCAACAGTAACTAGTGTCTTGGAAATATCTGAGTAATTGGTTTGAGAA AGAGACTTTACTCCAGAATTGGGAAGATGGCTTTATGCTTTATTGGCT
EphA2	NM_004 431.2	1525-1625	GAGCCGAGTGTGGAAGTACGAGGTCACTTACCGCAAGAAGGGAGACTCCAAC AGCTACAATGTGCGCCGACCGAGGGTTTCTCCGTGACCCTGGACGAC
CD254	NM_003 701.2	490-590	TACCTGATTATGTAGGAGAATTAAACAGGCCTTTCAAGGAGCTGTGCAAAA GGAATTACAACATATCGTTGGATCACAGCACATCAGAGCAGAGAAAAGC
BCLxL	NM_001 191.2	260-360	ATCTTGGCTTTGGATCTTAGAAGAGAATCACTAACCAGAGACGAGACTCAGT GAGTGAGCAGGTGTTTTGGACAATGGACTGGTTGAGCCCATCCCTATT
Xbp1	NM_001 079539.1	935-1035	ATTCATTGTCTCAGTGAAGGAAGAACCTGTAGAAGATGACCTCGTTCCGGAG CTGGGTATCTCAAATCTGCTTTCATCCAGCCACTGCCCAAAGCCATCT
IL27	NM_145 659.3	143-243	CAGGAGCTGCGGAGGGAGTTCACAGTCAGCCTGCATCTCGCCAGGAAGCTGC TCTCCGAGGTTCGGGGCCAGGCCACCGCTTTGCGGAATCTCACCTGC



IKZF2	NM_001 079526.1	945- 1045	CCATGTACCTCCTATGGAAGATTGTAAGGAACAAGAGCCTATTATGGACAAC AATATTTCTCTGGTGCCTTTTGAGAGACCTGCTGTCATAGAGAAGCTC
GNLY	NM_006 433.2	305-405	CAGGAGCTGGGCCGTGACTACAGGACCTGTCTGACGATAGTCCAAAACTGA AGAAGATGGTGGATAAGCCCACCCAGAGAAGTGTTTCCAATGCTGCGA
NFkB	NM_001 165412.1	2305- 2405	CTTGGGTAACTCTGTTTTGCACCTAGCTGCCAAAGAAGGACATGATAAAGTTC TCAGTATCTTACTCAAGCACAAAAAGGCAGCACTACTTCTTGACCAC
GADD45alp ha	NM_001 924.2	865-965	GTTACTCCCTACACTGATGCAAGGATTACAGAACTGATGCCAAGGGGCTGA GTGAGTTCAACTACATGTTCTGGGGGCCCGGAGATAGATGACTTTGCA
GADD45bet a	NM_015 675.2	365-465	TGTGGACCCAGACAGCGTGGTCCTCTGCCTCTTGGCCATTGACGAGGAGGAG GAGGATGACATCGCCCTGCAAATCCACTTCACGCTCATCCAGTCCTTC
ATF3	NM_001 030287.2	600-700	GGCTCAGAAATGGGAGGACTCCAGAAGATGAGAGAAACCTCTTTATCCAACAG ATAAAAGAAGGAACATTGCAGAGCTAAGCAGTCGTGGTATGGGGGCGA
MAD	NM_002 357.2	880-980	GAGAATAAAGCTGCAGGACAGTCACAAGGCGTGTCTTGGTCTCTAAGAGAGT GGGCACTGCGGCTGTCTCCTTGAAGGTTCTCCCTGTTGGTTCTGATTA
Crem	NM_001 881.2	260-360	CTCCACCTCCTCGCGTCCGTAATCAGTGACGAGGTCCGCTACGTAAATCCCTT TGCGGGCGACAAATGACCATGGAACAGTTGAATCCCAGCATGATGG
SOCS1	NM_003 745.1	1025- 1125	TTAACTGTATCTGGAGCCAGGACCTGAACTCGCACCTCCTACCTCTTCATGTT TACATATACCCAGTATCTTTGCACAAACCAGGGGTGGGGGAGGGTC
SOCS3	NM_003 955.3	1870- 1970	GGAGGATGGAGGAGACGGGACATCTTTCACCTCAGGCTCCTGGTAGAGAAGA CAGGGGATTCTACTCTGTGCCTCCTGACTATGTCTGGCTAAGAGATTC
DUSP16	NM_030 640.2	615-715	ATGGGTTTAACTCTCCTTTTGCCAGTCACCACCAGCCTGACCTCATACACTTTT AGTACAATGGAGTGCGTGAGCCTTTGAGCACACCACCATTACATCA
Rps13	NM_001 017.2	331-431	GCATCTTGAGAGGAACAGAAAGGATAAGGATGCTAAATTCCGTCTGATTCTA ATAGAGAGCCGGATTACCGGTTTGGCTCGATATTATAAGACCAAGCGA
TBP	NM_003 194.3	25-125	CGCCGGCTGTTTAACTTCGCTTCCGCTGGCCCATAGTGATCTTTGCAGTGACC CAGCAGCATCACTGTTTCTTGGCGTGTGAAGATAACCCAAGGAATTG
G6PD	NM_000 402.2	1155- 1255	ACAACATCGCCTGCGTTATCCTCACCTTCAAGGAGCCCTTTGGCACTGAGGGT CGCGGGGGCTATTTTCGATGAATTTGGGATCATCCGGGACGTGATGCA
Rbpms	NM_001 008710.1	842-942	AAACAGCCTGTAGGTTTTGTGAGTTTTGACAGTCGCTCAGAAGCAGAGGGCTGC AAAGAATGCTTTGAATGGCATCCGCTTCGATCCTGAAATTCCGCAA
KLF7	NM_001 270943.1	1546- 1646	GTAATTTGAGATCTTTCGCGTCGATCCCAACGGCCTTAGCGGGCGGAGACTG GAATAACACCTTACACCTTTCTGGCCTGCATTTCTGTAGACTTCACT
Vax2	NM_012 476.2	871-971	CAGCGCCAGCAGCTGCAAGAAAGCTAACTTAAGACTCCCACCCTGTGACA CTGAGTCCCGAGCACAGCACCTTCCAGTCTCCTGTGCCCCAGCGGAC
RUNX3	NM_004 350.1	2085- 2185	GTGGTCTCATAATTCCATTTGTGGAGAGAACAGGAGGGCCAGATAGATAGGT CCTAGCAGAAGGCATTGAGGTGAGGGATCATTTTGGGTCAGACATCAA
ERK	NM_017 449.2	785-885	CAAAGCAGGCTTCGAGGCCGTTGAGAATGGCACCGTCTGCCGAGGTTGTCCA TCTGGGACTTTCAAGGCCAACCAAGGGGATGAGGCTGTACCCACTGT
ITCH	NM_031 483.4	155-255	ACTGTGAGAACTTCAGGTTTTCCAACCTATTGGTGGTATGTCTGACAGTGGAT CACAACCTGGTTCAATGGGTAGCCTCACCATGAAATCACAGCTTCAG
CBLB	NM_170 662.3	3195- 3295	TAATGTGCAAGTTGCCCGGAGCATCCTCCGAGAATTTGCCTTCCCTCCTCCAG TATCCCCACGTCTAAATCTATAGCAGCCAGAACTGTAGACACCAAAA
DGKA	NM_001 345.4	1375- 1475	TTCTTAACACCCACCCACTTCTCGTCTTTGTCAATCCTAAGAGTGGCGGGAAG CAGGGGCAAAGGGTGCTCTGGAAGTTCCAGTATATATTAACCCCTCG
LTA	NM_000 595.2	885-985	CTGATCAAGTCACCGGAGCTTTCAAAGAAGGAATTCTAGGCATCCCAGGGGA CCACACCTCCCTGAACCATCCCTGATGTCTGTCTGGCTGAGGATTTCA

FoxP1	NM_032 682.5	6758- 6858	CCTGAAAATCAGATTTACAATGCTGAAGGCATTCTTGGGCCCAAGTGTAGCTC ACGCAATCTCTGCTACCCATAAGCCTTGATGAAGATGATACAGTCCG
CD223 (LAG3)	NM_002 286.5	1735- 1835	CTTTTGGTGACTGGAGCCTTTGGCTTTCACCTTTGGAGAAGACAGTGGCGACC AAGACGATTTTCTGCCTTAGAGCAAGGGATTACCCCTCCGCAGGCTC
CD118	NM_002 310.3	2995- 3095	CCTATTGTCCACCCATCATTGAGGAAGAAATACCAAACCCAGCCGCAGATGA AGCTGGAGGGACTGCACAGGTTATTACATTGATGTTTCAGTCGATGTA
Txk	NM_003 328.1	800-900	ATGACTCGTCTCCGATATCCAGTTGGGCTGATGGGCAGTTGTTTACCAGCCAC AGCTGGGTTTAGCTACGAAAAGTGGGAGATAGATCCATCTGAGTTGG
Prkcq	NM_006 257.2	1325- 1425	GATGGACGATGATGTTGAGTGCACGATGGTAGAGAAGAGAGTTCTTTCTCTTG GCCTGGGAGCATCCGTTTCTGACGCACATGTTTGTACATTCCAGACC
STS2 (Ubash3a)	NM_001 001895.1	1970- 2070	GAGATGCTGCTGTTTCCAGAGGCGTCTTAGTCTCACCCAAATGTGATTGTGAGA AGCACGAGACGCACTTTTATATCCCGGAATATTTCCCTCCGGCTTTC
RNF125	NM_017 831.3	790-890	GCAAGGTGTGTATGTCCCTTTTGTGTCAGAGGGAAGTGTATGAAGACAGCTTGCT GGATCATTGTATTACTCATCACAGATCGGAACGGAGGCTGTGTTCT
Lat	NM_001 014987.1	1290- 1390	TGTGTAATAGAATAAAGGCCTGCGTGTGTCTGTGTTGAGCGTGCGTCTGTGTG TGCCTGTGTGCGAGTCTGAGTCAGAGATTGGAGATGTCTCTGTGTG
Skap1	NM_003 726.3	1360- 1460	AAGTGGGAAGAGGCACGTTTCATCAAACCTGTTACTAAACCAGCCTAGTCATA GCTCATCCCCATCTCTAAATGTGTCCACACAACCACATCTGCCTTTTC
Dok2	NM_003 974.2	650-750	GCCAGGGACCCAGCTGTACGACTGGCCCTACAGGTTTCTGCGGCGCTTTGGGC GGGACAAGGTAACCTTTTCTTTGAGGCAGGCCGTCGCTGCGTCTCT
Axin2	NM_004 655.3	1035- 1135	CTTGTCAGCAAACTCTGAGGGCCACGGCGAGTGTGAGGTCCACGAAACT GTTGACAGTGGATACAGGTCCTTCAAGAGGAGCGATCCTGTTAATCCT
Sh2d2a	NM_001 161443.1	341-441	TGCTGGAGCCCAAGCCTCAGGGGTGCTACTTGGTGCGGTTACAGCGAGAGCGC GGTGACCTTCGTGCTGACTTACAGGAGCCGGACTTGCTGCCGCCACTT
Klra5 (Ly49E)	NR_028 045.1	414-514	CCTTCAGAGTCACAGAATAGATTAAGGCCTGATGATACTCAAAGGCCTGGGA AAACTGATGACAAAGAATTTTCAGTGCCCTGGCACCTCATTGCAGTGA
CD7	NM_006 137.6	440-540	CCTACACCTGCCAGGCCATCACGGAGGTCAATGTCTACGGCTCCGGCACCT GGTCCTGGTGACAGAGGAACAGTCCCAAGGATGGCACAGATGCTCGGA
CD11c	NM_000 887.3	700-800	CCCCTCAGCCTGTTGGCTTCTGTTACCCAGCTGCAAGGGTTTACATACACGGC CACCGCCATCCAAAATGTCGTGCACCGATTGTTCCATGCCTCATATG
Syk	NM_003 177.3	1685- 1785	CGGACTCTCCAAAGCACTGCGTGCTGATGAAAATACTACAAGGCCAGACC CATGGAAAGTGGCCTGTCAAGTGGTACGCTCCGGAATGCATCAACTAC
Lyn	NM_002 350.1	1285- 1385	TCCTGAAGAGCGATGAAGGTGGCAAAGTGCTGCTTCCAAAGCTCATTGACTTT TCTGCTCAGATTGCAGAGGGAATGGCATAACATCGAGCGGAAGAACTA
Lat2	NM_014 146.3	1863- 1963	TGCAGAGCTGATTAACAGTGTGTGACTGTCTCATGGGAAGAGCTGGGGCC CAGAGGGACCTTGAGTCAGAAATGTTGCCAGAAAAAGTATCTCCTCCA
Clnk	NM_052 964.2	1108- 1208	GAAGGAGAAACAAGGATGGTAGTTTCTTGGTCCGAGATTGTTCCACAAAATCC AAGGAAGAGCCCTATGTTTTGGCTGTGTTTTATGAGAACAAAGTCTAC
Car2	NM_000 067.2	575-675	AGCTGTGCAGCAACCTGATGGACTGGCCGTTCTAGGTATTTTTTTGAAGGTTG GCAGCGCTAAACCGGCCCTTCAGAAAGTTGTTGATGTGCTGGATTCC
Fgl2	NM_006 682.2	250-350	CAATTCAGCAGGATCGAGGAGGTGTTCAAAGAAGTCCAAAACCTCAAGGAAA TCGTAAATAGTCTAAAGAAATCTTGCCAAGACTGCAAGCTGCAGGCTG
cathepsinC	NM_001 114173.1	260-360	TGCTCGGTTATGGGACCACAAGAAAAAAGTAGTGGTGTACCTTCAGAAGC TGGATACAGCATATGATGACCTTGGAATTCTGGCCATTTACCATCA
CathepsinD	NM_001 909.3	1495- 1595	GAAGCCGGCGGCCCAAGCCCGACTTGCTGTTTTGTCTGTGGTTTTCCCTCC CTGGGTTTCAGAAATGCTGCCTGCCTGTCTGTCTCTCCATCTGTTTGG

Rab31	NM_006 868.3	3800- 3900	TTTTGTAAAGAGCTTCCATCTGGGCTGGACCCAGTTCTTGCACATACAAGACA CCGCTGCAGTCAGCTAGGACCTTTCCGCCATGTATTCTATTCTGTAG
Spry2	NM_005 842.2	85-185	AAAGAGGAAATACTCCGCGTGCCTTGTAGAAGGGGAGTCGTCTCCAGCTCC GAACCCCGGAGTGTTCATCAGCGGGGAATCTGGCTCCGAATTCTCTTT
S100A6	NM_014 624.3	539-639	TTCTTGGGGGCCTTGGCTTTGATCTACAATGAAGCCCTCAAGGGCTGAAAATA AATAGGGAAGATGGAGACACCTCTGGGGGTCTCTCTGAGTCAAAT
Lgals1	NM_002 305.3	60-160	GGTGCCTGCCCCGGGAACATCCTCTGGACTCAATCATGGCTTGTGGTCTGG 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	TGCGAGATGACAAATGGGAACATCCTAGACCCTGACGAAACCAGCACCATTGC CCTCTTCAAGGCCCATGAGGTGGCCTCCAAAAGGATTGAGGAAAAGAT
Ptpn4	NM_002 830.2	705-805	TCGAGGCTTTTTTCTCCAGCCGAGAGACGCGGCTGTGATATACGAAGACTT TGTGTGGACAGTAATGACCTCACGTTCCGATTGCCTGCTGGCAGAA
PLZF	NM_006 006.4	1585- 1685	TCCTGGATAGTTTTCGGGTGAGAATGCACTTACTGGCTCATTCAGCGGGTGCC AAAGCCTTTGTCTGTGATCAGTGCCTGTCACAGTTTTCGAAGGAGGA
Foxo1	NM_002 015.3	1526- 1626	TCTCATCACCAACATCATTAAGTGTTCGACCCAGTCTCACCTGGCACCATTG ATGCAGCAGACGCCGTGCTACTCGTTTGCGCCACCAACACCAGTTT
Foxo3	NM_001 455.2	1860- 1960	CCGGAACGTGATGCTTCGCAATGATCCGATGATGTCCTTTGCTGCCAGCCTA ACCAGGGAAGTTTGGTCAATCAGAACTTGCTCCACCACCAGCACCAA
ID3	NM_002 167.3	195-295	AGGAAGCCTGTTTGCAATTTAAGCGGGCTGTGAACGCCAGGGCCGGCGGGG GCAGGGCCGAGGCGGGCCATTTTGAATAAAGAGGCGTGCCTTCCAGGC
ZEB2	NM_014 795.2	20-120	TCCCAGAGAGAAAAGTGGCGATCACGTTTTTACATGATGCTCACGCTCAGGGC GCTTCAATTATCCCTCCCCACAAAGATAGGTGGCGCGTGTTCAGGG
SMAD4	NM_005 359.3	1370- 1470	AGGTTGCACATAGGCAAAGGTGTGCAGTTGGAATGTAAAGGTGAAGGTGATG TTTGGGTGAGGTGCCTTAGTGACCACGCGGTCTTTGTACAGAGTTACT
YAP	NM_139 118.2	755-855	ATGGGAGCTATGCAGCTGATTGAAGACTTCAGCACACATGTCAGCATTGACT GCAGCCCTCATAAACTGTCAAGAAGACTGCCAATGAATTTCCCTGTT
E2A	NM_003 200.2	4325- 4425	ATACGTGTCAACACAGCTGGCTGGATGATTGGGACTTTAAAACGACCCTCTTT CAGGTGGATTTCAGAGACCTGTCTGTATATAACAGCACTGTAGCAAT
Nanog	NM_024 865.2	1100- 1200	CTACTCCATGAACATGCAACCTGAAGACGTGTGAAGATGAGTGAACTGATA TTACTCAATTTAGTCTGGACACTGGCTGAATCCTTCTCTCCCCCTCC
OCT4	NM_002 701.4	1225- 1325	AAGTTCTTCATTCATAAGGAAGGAATTGGGAACACAAAGGGTGGGGGCAGG GGAGTTTGGGGCAACTGGTTGGAGGGAAGGTGAAGTTCAATGATGCTC
Sox2	NM_003 106.2	151-251	CTTAAGCCTTTCCAAAAAATAATAATAACAATCATCGGCGGCGGCAGGATCG GCCAGAGGAGGAGGGAAGCGCTTTTTTTGATCCTGATTCCAGTTTGCC
TAL1	NM_003 189.2	4635- 4735	ACAGCATCTGTAGTCAGCCGACAACCTATTTCCGGCTTTTGGGGGTGGGTCTGG CCGTACTTGTGATTTTCGATGGTACGTGACCCTCTGCTGAAGACTTGC
ELF1	NM_032 377.3	125-225	AGACCCAGTTCACCTGCCCTTCTGCAACCACGAGAAATCCTGTGATGTGAAA ATGGACCGTGCCCGCAACACCGGAGTCATCTTGTACCGTGTGCCT
SOX13	NM_005 686.2	3039- 3139	ATTTATTGAGTGCCCACTACGTGCCAGGCACTGTTGCTGAGTTTCTGTGGGTG TGTCTCTCGATGCCACTCTGCTTCTCTGGGGGCCTTTTCTGTGCT
Nrp1	NM_003 873.5	370-470	GCCTCGCTGCTTTCTTTTCTCCAAGACGGGCTGAGGATTGTACAGCTCTAGGC GGAGTTGGGGCTCTTCGGATCGCTTAGATTCTCTCTTTGCTGCATT

Blk	NM_001 715.2	990- 1090	AGCTTCTTGCTCCAATCAACAAGGCCGGCTCCTTTCTTATCAGAGAGAGTGAA ACCAACAAAGGTGCCTTCTCCCTGTCTGTGAAGGATGTACCACCCA
CCR10	NM_001 296.3	1345- 1445	GAACAGATGGGAACCAGCTCAATTGGGTGTCCACTCAAAGTGCTCTCTCCAG GGGCCTCAGTGA CTGTGTTGCTAAACCCAGTGGTCAGTTCTCAGTTCT
ITGB7	NM_000 889.1	1278- 1378	CAACGTGGTACAGCTCATCATGGATGCTTATAATAGCCTGTCTTCCACCGTGA CCCTTGAACACTCTTCACTCCCTCCTGGGGTCCACATTTCTTACGAA
Sox5	NM_152 989.2	1885- 1985	TAGCCATGCAATGATGGATTCAATCTGAGTGGAGATTCTGATGGAAGTGCTG GAGTCTCAGAGTCAAGAATTTATAGGGAATCCCGAGGGCGTGGTAGC
Bcl11b	NM_022 898.1	3420- 3520	GAGATGTAGCACTCATGTGCTCCCGAGTCAAGCGGCCTTTTCTGTGTTGATTT CGGCTTTCATATTACATAAGGGAAACCTTGAGTGGTGGTGTCTGGGGG
SOX4	NM_003 107.2	3040- 3140	GTTACACGGTCAAACTGAAATGGATTGTCACGTTGGGGAGCTGGCGGCGGCGG CTGCTGGGCCTCCGCCTTCTTTCTACGTGAAATCAGTGAGGTGAGAC
Tcf12	NM_207 037.1	1105- 1205	CACATGACCGCTTGAGTTATCCTCCACACTCAGTTTCACCAACAGACATAAAC ACGAGTCTTCCACCAATGTCCAGCTTTCATCGCGGCAGTACCAGCAG
Dapl 1	NM_001 017920.2	190-290	CGAGAAAACAAGTGCCATTGCAAATGTTGCCAAAATACAGACACTGGATGCC CTGAATGACGCACTGGAGAAGCTCAACTATAAATTTCCAGCAACAGTG
Trf	NM_003 218.3	1037- 1137	CTGAAAGCAGAATACCTGTTTCAAAGAGTCAGCCGGTAACTCCTGAAAAACA TCGAGCTAGAAAAAGACAGGCATGGCTTTGGGAAGAAGACAAGAATTT
Cpt1	NM_020 244.2	1303- 1403	GATATGGTGATATACTTTAGTGCTTTGTGCCTGCAAATTTCAAGACACCTTCA TCTAAATATATTCAAGACTGCATGTCATCAAGCACCTGAACAGGTTT
Bim	NM_138 621.4	257-357	CGGACTGAGAAACGCAAGAAAAAAGACCAAATGGCAAAGCAACCTTCTGA TGTAAGTTCTGAGTGTGACCGAGAAGGTAGACAATTGCAGCCTGCGGAG
C-flip	NM_001 127183.1	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	H1B19	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
TCR $\alpha\beta$	WT31	BD Biosciences
TCR $\gamma\delta$	B1	BD Biosciences
TCR $\gamma\delta$	IMMU510	Thermo Fisher
TCR $\delta 1$	TS-1	Thermo/Pierce

TCR $\delta$ 2	B6	BD Biosciences
TCR $\gamma$ 9	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
IFN $\gamma$	4S.B3	BD Biosciences
TNF $\alpha$	MAb11	BD Biosciences

\* To detect CAR expression

\*\* To detect CD19-specific CAR expression

\*\*\* Used at 1:67 dilution

## BIBLIOGRAPHY

1. <http://www.cdc.gov/nchs/fastats/lcod.htm>. 2010. Leading Causes of Death. In FastStats Homepage. National Center for Health Statistics, Centers for Disease Control and Prevention.
2. Siegel, R., C. DeSantis, K. Virgo, K. Stein, A. Mariotto, T. Smith, D. Cooper, T. Gansler, C. Lerro, S. Fedewa, C. Lin, C. Leach, R. S. Cannady, H. Cho, S. Scoppa, M. Hachey, R. Kirch, A. Jemal, and E. Ward. 2012. Cancer treatment and survivorship statistics, 2012. *CA Cancer J Clin* 62:220-241.
3. Matasar, M. J., E. K. Ritchie, N. Consedine, C. Magai, and A. I. Neugut. 2006. Incidence rates of the major leukemia subtypes among US Hispanics, Blacks, and non-Hispanic Whites. *Leukemia & Lymphoma* 47:2365-2370.
4. Grupp, S. A., M. Kalos, D. Barrett, R. Aplenc, D. L. Porter, S. R. Rheingold, D. T. Teachey, A. Chew, B. Hauck, J. F. Wright, M. C. Milone, B. L. Levine, and C. H. June. 2013. Chimeric antigen receptor-modified T cells for acute lymphoid leukemia. *The New England Journal of Medicine* 368:1509-1518.
5. Kochenderfer, J. N., M. E. Dudley, S. A. Feldman, W. H. Wilson, D. E. Spaner, I. Maric, M. Stetler-Stevenson, G. Q. Phan, M. S. Hughes, R. M. Sherry, J. C. Yang, U. S. Kammula, L. Devillier, R. Carpenter, D. A. Nathan, R. A. Morgan, C. Laurencot, and S. A. Rosenberg. 2012. B-cell depletion and remissions of malignancy along with cytokine-associated toxicity in a clinical trial of anti-CD19 chimeric-antigen-receptor-transduced T cells. *Blood* 119:2709-2720.
6. Brentjens, R. J., I. Riviere, J. H. Park, M. L. Davila, X. Wang, J. Stefanski, C. Taylor, R. Yeh, S. Bartido, O. Borquez-Ojeda, M. Olszewska, Y. Bernal, H.



- Pegram, M. Przybylowski, D. Hollyman, Y. Usachenko, D. Pirraglia, J. Hosey, E. Santos, E. Halton, P. Maslak, D. Scheinberg, J. Jurcic, M. Heaney, G. Heller, M. Frattini, and M. Sadelain. 2011. Safety and persistence of adoptively transferred autologous CD19-targeted T cells in patients with relapsed or chemotherapy refractory B-cell leukemias. *Blood* 118:4817-4828.
7. Porter, D. L., B. L. Levine, M. Kalos, A. Bagg, and C. H. June. 2011. Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia. *The New England Journal of Medicine* 365:725-733.
  8. McNeer, J. L., and E. A. Raetz. 2012. Acute lymphoblastic leukemia in young adults: which treatment? *Current Opinion in Oncology* 24:487-494.
  9. Lo Nigro, L. 2013. Biology of childhood acute lymphoblastic leukemia. *Journal of Pediatric Hematology/Oncology* 35:245-252.
  10. Belson, M., B. Kingsley, and A. Holmes. 2007. Risk factors for acute leukemia in children: a review. *Environmental Health Perspectives* 115:138-145.
  11. Cobaleda, C., and I. Sanchez-Garcia. 2009. B-cell acute lymphoblastic leukaemia: towards understanding its cellular origin. *BioEssays : news and reviews in molecular, cellular and developmental biology* 31:600-609.
  12. Aspland, S. E., H. H. Bendall, and C. Murre. 2001. The role of E2A-PBX1 in leukemogenesis. *Oncogene* 20:5708-5717.
  13. Bicocca, V. T., B. H. Chang, B. K. Masouleh, M. Muschen, M. M. Loriaux, B. J. Druker, and J. W. Tyner. 2012. Crosstalk between ROR1 and the Pre-B cell receptor promotes survival of t(1;19) acute lymphoblastic leukemia. *Cancer Cell* 22:656-667.

14. Onciu, M. 2009. Acute lymphoblastic leukemia. *Hematology/Oncology Clinics of North America* 23:655-674.
15. Faderl, S., S. O'Brien, C. H. Pui, W. Stock, M. Wetzler, D. Hoelzer, and H. M. Kantarjian. 2010. Adult acute lymphoblastic leukemia: concepts and strategies. *Cancer* 116:1165-1176.
16. Oliansky, D. M., B. Camitta, P. Gaynon, M. L. Nieder, S. K. Parsons, M. A. Pulsipher, H. Dillon, T. A. Ratko, D. Wall, P. L. McCarthy, Jr., T. Hahn, B. American Society for, and T. Marrow. 2012. Role of cytotoxic therapy with hematopoietic stem cell transplantation in the treatment of pediatric acute lymphoblastic leukemia: update of the 2005 evidence-based review. *Biology of Blood and Marrow Transplantation : Journal of the American Society for Blood and Marrow Transplantation* 18:505-522.
17. Pulsipher, M. A., C. Peters, and C. H. Pui. 2011. High-risk pediatric acute lymphoblastic leukemia: to transplant or not to transplant? *Biology of Blood and Marrow Transplantation : Journal of the American Society for Blood and Marrow Transplantation* 17:S137-148.
18. Forman, S. J., and J. M. Rowe. 2013. The myth of the second remission of acute leukemia in the adult. *Blood* 121:1077-1082.
19. Borowitz, M. J., M. Devidas, S. P. Hunger, W. P. Bowman, A. J. Carroll, W. L. Carroll, S. Linda, P. L. Martin, D. J. Pullen, D. Viswanatha, C. L. Willman, N. Winick, B. M. Camitta, and G. Children's Oncology. 2008. Clinical significance of minimal residual disease in childhood acute lymphoblastic leukemia and its

- relationship to other prognostic factors: a Children's Oncology Group study. *Blood* 111:5477-5485.
20. Pullen, J., J. J. Shuster, M. Link, M. Borowitz, M. Amylon, A. J. Carroll, V. Land, A. T. Look, B. McIntyre, and B. Camitta. 1999. Significance of commonly used prognostic factors differs for children with T cell acute lymphocytic leukemia (ALL), as compared to those with B-precursor ALL. A Pediatric Oncology Group (POG) study. *Leukemia* 13:1696-1707.
  21. Shimizu, H., H. Handa, N. Hatsumi, S. Takada, T. Saitoh, T. Sakura, S. Miyawaki, and Y. Nojima. 2013. Distinctive disease subgroups according to differentiation stages in adult patients with T-cell acute lymphoblastic leukemia. *European Journal of Haematology* 90:301-307.
  22. Fielding, A. K., L. Banerjee, and D. I. Marks. 2012. Recent developments in the management of T-cell precursor acute lymphoblastic leukemia/lymphoma. *Current Hematologic Malignancy Reports* 7:160-169.
  23. Szczepanski, T., V. H. van der Velden, E. Waanders, R. P. Kuiper, P. Van Vlierberghe, B. Gruhn, C. Eckert, R. Panzer-Grumayer, G. Basso, H. Cave, U. Z. Stadt, D. Campana, A. Schrauder, R. Sutton, E. van Wering, J. P. Meijerink, and J. J. van Dongen. 2011. Late recurrence of childhood T-cell acute lymphoblastic leukemia frequently represents a second leukemia rather than a relapse: first evidence for genetic predisposition. *Journal of Clinical Oncology : Official Journal of the American Society of Clinical Oncology* 29:1643-1649.
  24. Van Vlierberghe, P., and A. Ferrando. 2012. The molecular basis of T cell acute lymphoblastic leukemia. *The Journal of clinical investigation* 122:3398-3406.

25. McGregor, S., J. McNeer, and S. Gurbuxani. 2012. Beyond the 2008 World Health Organization classification: the role of the hematopathology laboratory in the diagnosis and management of acute lymphoblastic leukemia. *Seminars in Diagnostic Pathology* 29:2-11.
26. Tam, C. S., and M. J. Keating. 2010. Chemoimmunotherapy of chronic lymphocytic leukemia. *Nature reviews. Clinical Oncology* 7:521-532.
27. Chiorazzi, N., K. R. Rai, and M. Ferrarini. 2005. Chronic lymphocytic leukemia. *The New England Journal of Medicine* 352:804-815.
28. Binet, J. L., F. Caligaris-Cappio, D. Catovsky, B. Cheson, T. Davis, G. Dighiero, H. Dohner, M. Hallek, P. Hillmen, M. Keating, E. Montserrat, T. J. Kipps, K. Rai, and L. International Workshop on Chronic Lymphocytic. 2006. Perspectives on the use of new diagnostic tools in the treatment of chronic lymphocytic leukemia. *Blood* 107:859-861.
29. Yee, K. W., and S. M. O'Brien. 2006. Chronic lymphocytic leukemia: diagnosis and treatment. *Mayo Clinic proceedings. Mayo Clinic* 81:1105-1129.
30. Wierda, W. G., T. J. Kipps, and M. J. Keating. 2005. Novel immune-based treatment strategies for chronic lymphocytic leukemia. *Journal of Clinical Oncology : Official Journal of the American Society of Clinical Oncology* 23:6325-6332.
31. Cooper, L. J., Z. Al-Kadhimi, D. DiGiusto, M. Kalos, D. Colcher, A. Raubitschek, S. J. Forman, and M. C. Jensen. 2004. Development and application of CD19-specific T cells for adoptive immunotherapy of B cell malignancies. *Blood Cells Mol Dis* 33:83-89.

32. Kalos, M., B. L. Levine, D. L. Porter, S. Katz, S. A. Grupp, A. Bagg, and C. H. June. 2011. T cells with chimeric antigen receptors have potent antitumor effects and can establish memory in patients with advanced leukemia. *Science Translational Medicine* 3:95ra73.
33. Rauh-Hain, J. A., T. C. Krivak, M. G. Del Carmen, and A. B. Olawaiye. 2011. Ovarian cancer screening and early detection in the general population. *Rev Obstet Gynecol* 4:15-21.
34. Lowe, K. A., V. M. Chia, A. Taylor, C. O'Malley, M. Kelsh, M. Mohamed, F. S. Mowat, and B. Goff. 2013. An international assessment of ovarian cancer incidence and mortality. *Gynecologic Oncology*.
35. Baldwin, L. A., B. Huang, R. W. Miller, T. Tucker, S. T. Goodrich, I. Podzielinski, C. P. DeSimone, F. R. Ueland, J. R. van Nagell, and L. G. Seamon. 2012. Ten-year relative survival for epithelial ovarian cancer. *Obstetrics and Gynecology* 120:612-618.
36. Pignata, S., L. Cannella, D. Leopardo, C. Pisano, G. S. Bruni, and G. Facchini. 2011. Chemotherapy in epithelial ovarian cancer. *Cancer Letters* 303:73-83.
37. Lengyel, E. 2010. Ovarian cancer development and metastasis. *The American journal of pathology* 177:1053-1064.
38. Moore, R. G., S. MacLaughlan, and R. C. Bast, Jr. 2010. Current state of biomarker development for clinical application in epithelial ovarian cancer. *Gynecologic Oncology* 116:240-245.

39. Mould, T. 2012. An overview of current diagnosis and treatment in ovarian cancer. *International Journal of Gynecological Cancer : Official Journal of the International Gynecological Cancer Society* 22 Suppl 1:S2-4.
40. Le, X. F., and R. C. Bast, Jr. 2011. Src family kinases and paclitaxel sensitivity. *Cancer Biology & Therapy* 12:260-269.
41. Brahmer, J. R., S. S. Tykodi, L. Q. Chow, W. J. Hwu, S. L. Topalian, P. Hwu, C. G. Drake, L. H. Camacho, J. Kauh, K. Odunsi, H. C. Pitot, O. Hamid, S. Bhatia, R. Martins, K. Eaton, S. Chen, T. M. Salay, S. Alaparthi, J. F. Grosso, A. J. Korman, S. M. Parker, S. Agrawal, S. M. Goldberg, D. M. Pardoll, A. Gupta, and J. M. Wigginton. 2012. Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. *The New England Journal of Medicine* 366:2455-2465.
42. Kershaw, M. H., J. A. Westwood, L. L. Parker, G. Wang, Z. Eshhar, S. A. Mavroukakis, D. E. White, J. R. Wunderlich, S. Canevari, L. Rogers-Freezer, C. C. Chen, J. C. Yang, S. A. Rosenberg, and P. Hwu. 2006. A phase I study on adoptive immunotherapy using gene-modified T cells for ovarian cancer. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research* 12:6106-6115.
43. Fujita, K., H. Ikarashi, K. Takakuwa, S. Kodama, A. Tokunaga, T. Takahashi, and K. Tanaka. 1995. Prolonged disease-free period in patients with advanced epithelial ovarian cancer after adoptive transfer of tumor-infiltrating lymphocytes. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research* 1:501-507.

44. Hung, C. F., T. C. Wu, A. Monie, and R. Roden. 2008. Antigen-specific immunotherapy of cervical and ovarian cancer. *Immunological Reviews* 222:43-69.
45. Saif, M. W. 2013. Advancements in the management of pancreatic cancer: 2013. *JOP : Journal of the Pancreas* 14:112-118.
46. Sharma, C., K. M. Eltawil, P. D. Renfrew, M. J. Walsh, and M. Molinari. 2011. Advances in diagnosis, treatment and palliation of pancreatic carcinoma: 1990-2010. *World Journal of Gastroenterology : WJG* 17:867-897.
47. DiMagno, E. P. 1999. Pancreatic cancer: clinical presentation, pitfalls and early clues. *Annals of Oncology : Official Journal of the European Society for Medical Oncology / ESMO* 10 Suppl 4:140-142.
48. Paulson, A. S., H. S. Tran Cao, M. A. Tempero, and A. M. Lowy. 2013. Therapeutic advances in pancreatic cancer. *Gastroenterology* 144:1316-1326.
49. Di Marco, M., R. Di Cicilia, M. Macchini, E. Nobili, S. Vecchiarelli, G. Brandi, and G. Biasco. 2010. Metastatic pancreatic cancer: is gemcitabine still the best standard treatment? (Review). *Oncology Reports* 23:1183-1192.
50. el-Kamar, F. G., M. L. Grossbard, and P. S. Kozuch. 2003. Metastatic pancreatic cancer: emerging strategies in chemotherapy and palliative care. *Oncologist* 8:18-34.
51. Koido, S., S. Homma, A. Takahara, Y. Namiki, S. Tsukinaga, J. Mitobe, S. Odahara, T. Yukawa, H. Matsudaira, K. Nagatsuma, K. Uchiyama, K. Satoh, M. Ito, H. Komita, H. Arakawa, T. Ohkusa, J. Gong, and H. Tajiri. 2011. Current

- immunotherapeutic approaches in pancreatic cancer. *Clinical & Developmental Immunology* 2011:267539.
52. Plate, J. M. 2012. Advances in therapeutic vaccines for pancreatic cancer. *Discovery Medicine* 14:89-94.
  53. Gordan, J. D., and R. H. Vonderheide. 2002. Universal tumor antigens as targets for immunotherapy. *Cytotherapy* 4:317-327.
  54. Offringa, R. 2009. Antigen choice in adoptive T-cell therapy of cancer. *Current Opinion in Immunology* 21:190-199.
  55. Zhou, G., and H. Levitsky. 2012. Towards curative cancer immunotherapy: overcoming posttherapy tumor escape. *Clinical & Developmental Immunology* 2012:124187.
  56. Kochenderfer, J. N., W. H. Wilson, J. E. Janik, M. E. Dudley, M. Stetler-Stevenson, S. A. Feldman, I. Maric, M. Raffeld, D. A. Nathan, B. J. Lanier, R. A. Morgan, and S. A. Rosenberg. 2010. Eradication of B-lineage cells and regression of lymphoma in a patient treated with autologous T cells genetically engineered to recognize CD19. *Blood* 116:4099-4102.
  57. Singh, H., M. J. Figliola, M. J. Dawson, H. Huls, S. Olivares, K. Switzer, T. Mi, S. Maiti, P. Kebriaei, D. A. Lee, R. E. Champlin, and L. J. Cooper. 2011. Reprogramming CD19-Specific T Cells with IL-21 Signaling Can Improve Adoptive Immunotherapy of B-Lineage Malignancies. *Cancer Research* 71:3516-3527.
  58. Chaplin, D. D. 2010. Overview of the immune response. *J Allergy Clin Immunol* 125:S3-23.



59. Boackle, S. A., M. A. Morris, V. M. Holers, and D. R. Karp. 1998. Complement opsonization is required for presentation of immune complexes by resting peripheral blood B cells. *Journal of Immunology* 161:6537-6543.
60. Rickert, R. C. 2005. Regulation of B lymphocyte activation by complement C3 and the B cell coreceptor complex. *Current Opinion in Immunology* 17:237-243.
61. Cherukuri, A., T. Shoham, H. W. Sohn, S. Levy, S. Brooks, R. Carter, and S. K. Pierce. 2004. The tetraspanin CD81 is necessary for partitioning of coligated CD19/CD21-B cell antigen receptor complexes into signaling-active lipid rafts. *Journal of Immunology* 172:370-380.
62. Carter, R. H., Y. Wang, and S. Brooks. 2002. Role of CD19 signal transduction in B cell biology. *Immunologic Research* 26:45-54.
63. Jolles, S., W. A. Sewell, and S. A. Misbah. 2005. Clinical uses of intravenous immunoglobulin. *Clin Exp Immunol* 142:1-11.
64. Rebagay, G., S. Yan, C. Liu, and N. K. Cheung. 2012. ROR1 and ROR2 in Human Malignancies: Potentials for Targeted Therapy. *Frontiers in Oncology* 2:34.
65. Baskar, S., K. Y. Kwong, T. Hofer, J. M. Levy, M. G. Kennedy, E. Lee, L. M. Staudt, W. H. Wilson, A. Wiestner, and C. Rader. 2008. Unique cell surface expression of receptor tyrosine kinase ROR1 in human B-cell chronic lymphocytic leukemia. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research* 14:396-404.
66. Broome, H. E., L. Z. Rassenti, H. Y. Wang, L. M. Meyer, and T. J. Kipps. 2011. ROR1 is expressed on hematogones (non-neoplastic human B-lymphocyte

- precursors) and a minority of precursor-B acute lymphoblastic leukemia. *Leukemia Research* 35:1390-1394.
67. Zhang, S., L. Chen, J. Wang-Rodriguez, L. Zhang, B. Cui, W. Frankel, R. Wu, and T. J. Kipps. 2012. The onco-embryonic antigen ROR1 is expressed by a variety of human cancers. *The American Journal of Pathology* 181:1903-1910.
68. Masiakowski, P., and R. D. Carroll. 1992. A novel family of cell surface receptors with tyrosine kinase-like domain. *The Journal of Biological Chemistry* 267:26181-26190.
69. Nomi, M., I. Oishi, S. Kani, H. Suzuki, T. Matsuda, A. Yoda, M. Kitamura, K. Itoh, S. Takeuchi, K. Takeda, S. Akira, M. Ikeya, S. Takada, and Y. Minami. 2001. Loss of mRor1 enhances the heart and skeletal abnormalities in mRor2-deficient mice: redundant and pleiotropic functions of mRor1 and mRor2 receptor tyrosine kinases. *Molecular and Cellular Biology* 21:8329-8335.
70. Takeuchi, S., K. Takeda, I. Oishi, M. Nomi, M. Ikeya, K. Itoh, S. Tamura, T. Ueda, T. Hatta, H. Otani, T. Terashima, S. Takada, H. Yamamura, S. Akira, and Y. Minami. 2000. Mouse Ror2 receptor tyrosine kinase is required for the heart development and limb formation. *Genes to Cells : Devoted to Molecular & Cellular Mechanisms* 5:71-78.
71. Reddy, U. R., S. Phatak, C. Allen, L. M. Nycum, E. P. Sulman, P. S. White, and J. A. Biegel. 1997. Localization of the human Ror1 gene (NTRKR1) to chromosome 1p31-p32 by fluorescence in situ hybridization and somatic cell hybrid analysis. *Genomics* 41:283-285.

72. Schwabe, G. C., B. Trepczik, K. Suring, N. Brieske, A. S. Tucker, P. T. Sharpe, Y. Minami, and S. Mundlos. 2004. Ror2 knockout mouse as a model for the developmental pathology of autosomal recessive Robinow syndrome. *Developmental Dynamics : An Official Publication of the American Association of Anatomists* 229:400-410.
73. van Bokhoven, H., J. Celli, H. Kayserili, E. van Beusekom, S. Balci, W. Brussel, F. Skovby, B. Kerr, E. F. Percin, N. Akarsu, and H. G. Brunner. 2000. Mutation of the gene encoding the ROR2 tyrosine kinase causes autosomal recessive Robinow syndrome. *Nature Genetics* 25:423-426.
74. Oldridge, M., A. M. Fortuna, M. Maringa, P. Propping, S. Mansour, C. Pollitt, T. M. DeChiara, R. B. Kimble, D. M. Valenzuela, G. D. Yancopoulos, and A. O. Wilkie. 2000. Dominant mutations in ROR2, encoding an orphan receptor tyrosine kinase, cause brachydactyly type B. *Nature Genetics* 24:275-278.
75. Fukuda, T., L. Chen, T. Endo, L. Tang, D. Lu, J. E. Castro, G. F. Widhopf, 2nd, L. Z. Rassenti, M. J. Cantwell, C. E. Prussak, D. A. Carson, and T. J. Kipps. 2008. Antisera induced by infusions of autologous Ad-CD154-leukemia B cells identify ROR1 as an oncofetal antigen and receptor for Wnt5a. *Proceedings of the National Academy of Sciences of the United States of America* 105:3047-3052.
76. Daneshmanesh, A. H., E. Mikaelsson, M. Jeddi-Tehrani, A. A. Bayat, R. Ghods, M. Ostadkarampour, M. Akhondi, S. Lagercrantz, C. Larsson, A. Osterborg, F. Shokri, H. Mellstedt, and H. Rabbani. 2008. Ror1, a cell surface receptor

- tyrosine kinase is expressed in chronic lymphocytic leukemia and may serve as a putative target for therapy. *International Journal of Cancer* 123:1190-1195.
77. Hudecek, M., T. M. Schmitt, S. Baskar, M. T. Lupo-Stanghellini, T. Nishida, T. N. Yamamoto, M. Bleakley, C. J. Turtle, W. C. Chang, H. A. Greisman, B. Wood, D. G. Maloney, M. C. Jensen, C. Rader, and S. R. Riddell. 2010. The B-cell tumor-associated antigen ROR1 can be targeted with T cells modified to express a ROR1-specific chimeric antigen receptor. *Blood* 116:4532-4541.
  78. Gentile, A., L. Lazzari, S. Benvenuti, L. Trusolino, and P. M. Comoglio. 2011. Ror1 is a pseudokinase that is crucial for Met-driven tumorigenesis. *Cancer Research* 71:3132-3141.
  79. Zhang, S., L. Chen, B. Cui, H. Y. Chuang, J. Yu, J. Wang-Rodriguez, L. Tang, G. Chen, G. W. Basak, and T. J. Kipps. 2012. ROR1 is expressed in human breast cancer and associated with enhanced tumor-cell growth. *PloS One* 7:e31127.
  80. Hojjat-Farsangi, M., F. Ghaemimanesh, A. H. Daneshmanesh, A. A. Bayat, J. Mahmoudian, M. Jeddi-Tehrani, H. Rabbani, and H. Mellstedt. 2013. Inhibition of the Receptor Tyrosine Kinase ROR1 by Anti-ROR1 Monoclonal Antibodies and siRNA Induced Apoptosis of Melanoma Cells. *PloS One* 8:e61167.
  81. Dave, H., M. R. Anver, D. O. Butcher, P. Brown, J. Khan, A. S. Wayne, S. Baskar, and C. Rader. 2012. Restricted cell surface expression of receptor tyrosine kinase ROR1 in pediatric B-lineage acute lymphoblastic leukemia suggests targetability with therapeutic monoclonal antibodies. *PloS One* 7:e52655.

82. Li, P., D. Harris, Z. Liu, J. Liu, M. Keating, and Z. Estrov. 2010. Stat3 activates the receptor tyrosine kinase like orphan receptor-1 gene in chronic lymphocytic leukemia cells. *PloS One* 5:e11859.
83. Medzhitov, R., and C. Janeway, Jr. 2000. Innate immunity. *The New England Journal of Medicine* 343:338-344.
84. Hoebe, K., E. Janssen, and B. Beutler. 2004. The interface between innate and adaptive immunity. *Nature Immunology* 5:971-974.
85. Schenten, D., and R. Medzhitov. 2011. The control of adaptive immune responses by the innate immune system. *Advances in Immunology* 109:87-124.
86. Vesely, M. D., M. H. Kershaw, R. D. Schreiber, and M. J. Smyth. 2011. Natural innate and adaptive immunity to cancer. *Annual Review of Immunology* 29:235-271.
87. Janeway, C. A., Jr., and R. Medzhitov. 2002. Innate immune recognition. *Annual review of Immunology* 20:197-216.
88. Clem, A. S. 2011. Fundamentals of vaccine immunology. *Journal of Global Infectious Diseases* 3:73-78.
89. Elgueta, R., V. C. de Vries, and R. J. Noelle. 2010. The immortality of humoral immunity. *Immunological Reviews* 236:139-150.
90. Koretzky, G. A. 2010. Multiple roles of CD4 and CD8 in T cell activation. *Journal of Immunology* 185:2643-2644.
91. Nishana, M., and S. C. Raghavan. 2012. Role of recombination activating genes in the generation of antigen receptor diversity and beyond. *Immunology* 137:271-281.

92. Kreslavsky, T., M. Gleimer, A. I. Garbe, and H. von Boehmer. 2010. alphabeta versus gammadelta fate choice: counting the T-cell lineages at the branch point. *Immunological Reviews* 238:169-181.
93. Mackelprang, R., C. S. Carlson, L. Subrahmanyam, R. J. Livingston, M. A. Eberle, and D. A. Nickerson. 2002. Sequence variation in the human T-cell receptor loci. *Immunological Reviews* 190:26-39.
94. Marrack, P., J. P. Scott-Browne, S. Dai, L. Gapin, and J. W. Kappler. 2008. Evolutionarily conserved amino acids that control TCR-MHC interaction. *Annual review of Immunology* 26:171-203.
95. Bednarski, J. J., and B. P. Sleckman. 2012. Lymphocyte development: integration of DNA damage response signaling. *Advances in Immunology* 116:175-204.
96. Ramsden, D. A., B. D. Weed, and Y. V. Reddy. 2010. V(D)J recombination: Born to be wild. *Seminars in Cancer Biology* 20:254-260.
97. Spits, H. 2002. Development of alphabeta T cells in the human thymus. *Nature Reviews. Immunology* 2:760-772.
98. Robins, H. S., P. V. Campregher, S. K. Srivastava, A. Wacher, C. J. Turtle, O. Kahsai, S. R. Riddell, E. H. Warren, and C. S. Carlson. 2009. Comprehensive assessment of T-cell receptor beta-chain diversity in alphabeta T cells. *Blood* 114:4099-4107.
99. Stritesky, G. L., S. C. Jameson, and K. A. Hogquist. 2012. Selection of self-reactive T cells in the thymus. *Annual Review of Immunology* 30:95-114.

100. Halkias, J., H. J. Melichar, K. T. Taylor, J. O. Ross, B. Yen, S. B. Cooper, A. Winoto, and E. A. Robey. 2013. Opposing chemokine gradients control human thymocyte migration in situ. *The Journal of Clinical Investigation*.
101. Stefanski, H. E., D. Mayerova, S. C. Jameson, and K. A. Hogquist. 2001. A low affinity TCR ligand restores positive selection of CD8<sup>+</sup> T cells in vivo. *Journal of Immunology* 166:6602-6607.
102. Yin, L., J. Scott-Browne, J. W. Kappler, L. Gapin, and P. Marrack. 2012. T cells and their eons-old obsession with MHC. *Immunological Reviews* 250:49-60.
103. Capone, M., P. Romagnoli, F. Beermann, H. R. MacDonald, and J. P. van Meerwijk. 2001. Dissociation of thymic positive and negative selection in transgenic mice expressing major histocompatibility complex class I molecules exclusively on thymic cortical epithelial cells. *Blood* 97:1336-1342.
104. Akirav, E. M., N. H. Ruddle, and K. C. Herold. 2011. The role of AIRE in human autoimmune disease. *Nature Reviews. Endocrinology* 7:25-33.
105. Germain, R. N. 2002. T-cell development and the CD4-CD8 lineage decision. *Nature reviews. Immunology* 2:309-322.
106. Van Laethem, F., A. N. Tikhonova, and A. Singer. 2012. MHC restriction is imposed on a diverse T cell receptor repertoire by CD4 and CD8 co-receptors during thymic selection. *Trends in Immunology* 33:437-441.
107. Bretscher, P. A. 1999. A two-step, two-signal model for the primary activation of precursor helper T cells. *Proceedings of the National Academy of Sciences of the United States of America* 96:185-190.

108. Groux, H., M. Bigler, J. E. de Vries, and M. G. Roncarolo. 1996. Interleukin-10 induces a long-term antigen-specific anergic state in human CD4<sup>+</sup> T cells. *The Journal of Experimental Medicine* 184:19-29.
109. Appleman, L. J., and V. A. Boussiotis. 2003. T cell anergy and costimulation. *Immunological Reviews* 192:161-180.
110. Gascoigne, N. R., T. Zal, P. P. Yachi, and J. A. Hoerter. 2010. Co-receptors and recognition of self at the immunological synapse. *Current Topics in Microbiology and Immunology* 340:171-189.
111. Kuhns, M. S., and H. B. Badgandi. 2012. Piecing together the family portrait of TCR-CD3 complexes. *Immunological Reviews* 250:120-143.
112. Love, P. E., and S. M. Hayes. 2010. ITAM-mediated signaling by the T-cell antigen receptor. *Cold Spring Harbor Perspectives in Biology* 2:a002485.
113. Smith-Garvin, J. E., G. A. Koretzky, and M. S. Jordan. 2009. T cell activation. *Annual Review of Immunology* 27:591-619.
114. Vigano, S., M. Perreau, G. Pantaleo, and A. Harari. 2012. Positive and negative regulation of cellular immune responses in physiologic conditions and diseases. *Clinical & Developmental Immunology* 2012:485781.
115. Kinnear, G., N. D. Jones, and K. J. Wood. 2013. Costimulation blockade: current perspectives and implications for therapy. *Transplantation* 95:527-535.
116. Bertram, E. M., W. Dawicki, and T. H. Watts. 2004. Role of T cell costimulation in anti-viral immunity. *Seminars in Immunology* 16:185-196.
117. Lenschow, D. J., T. L. Walunas, and J. A. Bluestone. 1996. CD28/B7 system of T cell costimulation. *Annual Review of Immunology* 14:233-258.



118. Croft, M. 2003. Costimulation of T cells by OX40, 4-1BB, and CD27. *Cytokine & Growth Factor Reviews* 14:265-273.
119. Quezada, S. A., and K. S. Peggs. 2013. Exploiting CTLA-4, PD-1 and PD-L1 to reactivate the host immune response against cancer. *British Journal of Cancer* 108:1560-1565.
120. Janeway, C. A., Jr. 2001. How the immune system protects the host from infection. *Microbes and Infection / Institut Pasteur* 3:1167-1171.
121. Martin-Fontecha, A., A. Lanzavecchia, and F. Sallusto. 2009. Dendritic cell migration to peripheral lymph nodes. *Handbook of Experimental Pharmacology*:31-49.
122. Alvarez, D., E. H. Vollmann, and U. H. von Andrian. 2008. Mechanisms and consequences of dendritic cell migration. *Immunity* 29:325-342.
123. Curtsinger, J. M., and M. F. Mescher. 2010. Inflammatory cytokines as a third signal for T cell activation. *Current Opinion in Immunology* 22:333-340.
124. Zou, W., and N. P. Restifo. 2010. T(H)17 cells in tumour immunity and immunotherapy. *Nature Reviews. Immunology* 10:248-256.
125. Zhu, J., and W. E. Paul. 2010. Peripheral CD4<sup>+</sup> T-cell differentiation regulated by networks of cytokines and transcription factors. *Immunological Reviews* 238:247-262.
126. Zhu, J., and W. E. Paul. 2008. CD4 T cells: fates, functions, and faults. *Blood* 112:1557-1569.
127. Muranski, P., and N. P. Restifo. 2013. Essentials of Th17 cell commitment and plasticity. *Blood* 121:2402-2414.

128. Taylor, A., J. Verhagen, K. Blaser, M. Akdis, and C. A. Akdis. 2006. Mechanisms of immune suppression by interleukin-10 and transforming growth factor-beta: the role of T regulatory cells. *Immunology* 117:433-442.
129. Bendelac, A., P. B. Savage, and L. Teyton. 2007. The biology of NKT cells. *Annual Review of Immunology* 25:297-336.
130. Brennan, P. J., M. Brigl, and M. B. Brenner. 2013. Invariant natural killer T cells: an innate activation scheme linked to diverse effector functions. *Nature reviews. Immunology* 13:101-117.
131. Klebanoff, C. A., L. Gattinoni, and N. P. Restifo. 2006. CD8+ T-cell memory in tumor immunology and immunotherapy. *Immunological Reviews* 211:214-224.
132. Wherry, E. J., and R. Ahmed. 2004. Memory CD8 T-cell differentiation during viral infection. *Journal of Virology* 78:5535-5545.
133. Harty, J. T., and V. P. Badovinac. 2008. Shaping and reshaping CD8+ T-cell memory. *Nature reviews. Immunology* 8:107-119.
134. Gattinoni, L., E. Lugli, Y. Ji, Z. Pos, C. M. Paulos, M. F. Quigley, J. R. Almeida, E. Gostick, Z. Yu, C. Carpenito, E. Wang, D. C. Douek, D. A. Price, C. H. June, F. M. Marincola, M. Roederer, and N. P. Restifo. 2011. A human memory T cell subset with stem cell-like properties. *Nature Medicine* 17:1290-1297.
135. Geginat, J., A. Lanzavecchia, and F. Sallusto. 2003. Proliferation and differentiation potential of human CD8+ memory T-cell subsets in response to antigen or homeostatic cytokines. *Blood* 101:4260-4266.

136. Butcher, E. C., and L. J. Picker. 1996. Lymphocyte homing and homeostasis. *Science* 272:60-66.
137. Harrington, L. E., K. M. Janowski, J. R. Oliver, A. J. Zajac, and C. T. Weaver. 2008. Memory CD4 T cells emerge from effector T-cell progenitors. *Nature* 452:356-360.
138. MacLeod, M. K., J. W. Kappler, and P. Marrack. 2010. Memory CD4 T cells: generation, reactivation and re-assignment. *Immunology* 130:10-15.
139. Shedlock, D. J., and H. Shen. 2003. Requirement for CD4 T cell help in generating functional CD8 T cell memory. *Science* 300:337-339.
140. Vantourout, P., and A. Hayday. 2013. Six-of-the-best: unique contributions of gammadelta T cells to immunology. *Nature reviews. Immunology* 13:88-100.
141. Kabelitz, D., D. Wesch, and W. He. 2007. Perspectives of gammadelta T cells in tumor immunology. *Cancer Research* 67:5-8.
142. Roden, A. C., W. G. Morice, and C. A. Hanson. 2008. Immunophenotypic attributes of benign peripheral blood gammadelta T cells and conditions associated with their increase. *Archives of Pathology & Laboratory Medicine* 132:1774-1780.
143. Lopez, R. D. 2002. Human gammadelta-T cells in adoptive immunotherapy of malignant and infectious diseases. *Immunologic Research* 26:207-221.
144. Carding, S. R., and P. J. Egan. 2002. Gammadelta T cells: functional plasticity and heterogeneity. *Nature Reviews. Immunology* 2:336-345.
145. Poggi, A., S. Catellani, A. Musso, and M. R. Zocchi. 2009. Gammadelta T lymphocytes producing IFNgamma and IL-17 in response to *Candida albicans*

- or mycobacterial antigens: possible implications for acute and chronic inflammation. *Current Medicinal Chemistry* 16:4743-4749.
146. Bonneville, M., R. L. O'Brien, and W. K. Born. 2010. Gammadelta T cell effector functions: a blend of innate programming and acquired plasticity. *Nature Reviews. Immunology* 10:467-478.
  147. Xiong, N., and D. H. Raulet. 2007. Development and selection of gammadelta T cells. *Immunological Reviews* 215:15-31.
  148. Hayday, A. C. 2009. Gammadelta T cells and the lymphoid stress-surveillance response. *Immunity* 31:184-196.
  149. Hao, J., X. Wu, S. Xia, Z. Li, T. Wen, N. Zhao, Z. Wu, P. Wang, L. Zhao, and Z. Yin. 2010. Current progress in gammadelta T-cell biology. *Cellular & Molecular Immunology* 7:409-413.
  150. Ishikawa, H., T. Naito, T. Iwanaga, H. Takahashi-Iwanaga, M. Suematsu, T. Hibi, and M. Nanno. 2007. Curriculum vitae of intestinal intraepithelial T cells: their developmental and behavioral characteristics. *Immunological Reviews* 215:154-165.
  151. Pang, D. J., J. F. Neves, N. Sumaria, and D. J. Pennington. 2012. Understanding the complexity of gammadelta T-cell subsets in mouse and human. *Immunology* 136:283-290.
  152. Xu, B., J. C. Pizarro, M. A. Holmes, C. McBeth, V. Groh, T. Spies, and R. K. Strong. 2011. Crystal structure of a gammadelta T-cell receptor specific for the human MHC class I homolog MICA. *Proceedings of the National Academy of Sciences of the United States of America* 108:2414-2419.

153. Steinle, A., V. Groh, and T. Spies. 1998. Diversification, expression, and gamma delta T cell recognition of evolutionarily distant members of the MIC family of major histocompatibility complex class I-related molecules. *Proceedings of the National Academy of Sciences of the United States of America* 95:12510-12515.
154. Groh, V., R. Rhinehart, H. Secrist, S. Bauer, K. H. Grabstein, and T. Spies. 1999. Broad tumor-associated expression and recognition by tumor-derived gamma delta T cells of MICA and MICB. *Proceedings of the National Academy of Sciences of the United States of America* 96:6879-6884.
155. Li, J., L. Cui, and W. He. 2005. Distinct pattern of human Vdelta1 gammadelta T cells recognizing MICA. *Cellular & Molecular Immunology* 2:253-258.
156. Spada, F. M., E. P. Grant, P. J. Peters, M. Sugita, A. Melian, D. S. Leslie, H. K. Lee, E. van Donselaar, D. A. Hanson, A. M. Krensky, O. Majdic, S. A. Porcelli, C. T. Morita, and M. B. Brenner. 2000. Self-recognition of CD1 by gamma/delta T cells: implications for innate immunity. *The Journal of Experimental Medicine* 191:937-948.
157. Ciocca, D. R., and S. K. Calderwood. 2005. Heat shock proteins in cancer: diagnostic, prognostic, predictive, and treatment implications. *Cell Stress & Chaperones* 10:86-103.
158. Borg, N. A., K. S. Wun, L. Kjer-Nielsen, M. C. Wilce, D. G. Pellicci, R. Koh, G. S. Besra, M. Bharadwaj, D. I. Godfrey, J. McCluskey, and J. Rossjohn. 2007. CD1d-lipid-antigen recognition by the semi-invariant NKT T-cell receptor. *Nature* 448:44-49.

159. Paget, C., M. T. Chow, H. Duret, S. R. Mattarollo, and M. J. Smyth. 2012. Role of gammadelta T cells in alpha-galactosylceramide-mediated immunity. *Journal of Immunology* 188:3928-3939.
160. Dieude, M., H. Striegl, A. J. Tyznik, J. Wang, S. M. Behar, C. A. Piccirillo, J. S. Levine, D. M. Zajonc, and J. Rauch. 2011. Cardiolipin binds to CD1d and stimulates CD1d-restricted gammadelta T cells in the normal murine repertoire. *Journal of Immunology* 186:4771-4781.
161. Nanno, M., T. Shiohara, H. Yamamoto, K. Kawakami, and H. Ishikawa. 2007. gammadelta T cells: firefighters or fire boosters in the front lines of inflammatory responses. *Immunological Reviews* 215:103-113.
162. Chodaczek, G., V. Papanna, M. A. Zal, and T. Zal. 2012. Body-barrier surveillance by epidermal gammadelta TCRs. *Nature Immunology* 13:272-282.
163. Xiong, N., C. Kang, and D. H. Raulet. 2004. Positive selection of dendritic epidermal gammadelta T cell precursors in the fetal thymus determines expression of skin-homing receptors. *Immunity* 21:121-131.
164. Wesch, D., T. Hinz, and D. Kabelitz. 1998. Analysis of the TCR Vgamma repertoire in healthy donors and HIV-1-infected individuals. *International Immunology* 10:1067-1075.
165. Knight, A., A. J. Madrigal, S. Grace, J. Sivakumaran, P. Kottaridis, S. Mackinnon, P. J. Travers, and M. W. Lowdell. 2010. The role of Vdelta2-negative gammadelta T cells during cytomegalovirus reactivation in recipients of allogeneic stem cell transplantation. *Blood* 116:2164-2172.

166. Brandes, M., K. Willmann, and B. Moser. 2005. Professional antigen-presentation function by human gammadelta T Cells. *Science* 309:264-268.
167. Bukowski, J. F., C. T. Morita, and M. B. Brenner. 1999. Human gamma delta T cells recognize alkylamines derived from microbes, edible plants, and tea: implications for innate immunity. *Immunity* 11:57-65.
168. Wang, L., A. Kamath, H. Das, L. Li, and J. F. Bukowski. 2001. Antibacterial effect of human V gamma 2V delta 2 T cells in vivo. *The Journal of Clinical Investigation* 108:1349-1357.
169. Munk, M. E., C. Elser, and S. H. Kaufmann. 1996. Human gamma/delta T-cell response to *Listeria monocytogenes* protein components in vitro. *Immunology* 87:230-235.
170. Green, A. E., A. Lissina, S. L. Hutchinson, R. E. Hewitt, B. Temple, D. James, J. M. Boulter, D. A. Price, and A. K. Sewell. 2004. Recognition of nonpeptide antigens by human V gamma 9V delta 2 T cells requires contact with cells of human origin. *Clin Exp Immunol* 136:472-482.
171. Mookerjee-Basu, J., P. Vantourout, L. O. Martinez, B. Perret, X. Collet, C. Perigaud, S. Peyrottes, and E. Champagne. 2010. F1-adenosine triphosphatase displays properties characteristic of an antigen presentation molecule for Vgamma9Vdelta2 T cells. *Journal of Immunology* 184:6920-6928.
172. Vantourout, P., J. Mookerjee-Basu, C. Rolland, F. Pont, H. Martin, C. Davrinche, L. O. Martinez, B. Perret, X. Collet, C. Perigaud, S. Peyrottes, and E. Champagne. 2009. Specific requirements for Vgamma9Vdelta2 T cell

- stimulation by a natural adenylylated phosphoantigen. *Journal of Immunology* 183:3848-3857.
173. D'Asaro, M., C. La Mendola, D. Di Liberto, V. Orlando, M. Todaro, M. Spina, G. Guggino, S. Meraviglia, N. Caccamo, A. Messina, A. Salerno, F. Di Raimondo, P. Vigneri, G. Stassi, J. J. Fournie, and F. Dieli. 2010. V gamma 9V delta 2 T lymphocytes efficiently recognize and kill zoledronate-sensitized, imatinib-sensitive, and imatinib-resistant chronic myelogenous leukemia cells. *Journal of Immunology* 184:3260-3268.
174. Thedrez, A., C. Harly, A. Morice, S. Salot, M. Bonneville, and E. Scotet. 2009. IL-21-mediated potentiation of antitumor cytolytic and proinflammatory responses of human V gamma 9V delta 2 T cells for adoptive immunotherapy. *Journal of Immunology* 182:3423-3431.
175. Chiplunkar, S., S. Dhar, D. Wesch, and D. Kabelitz. 2009. gammadelta T cells in cancer immunotherapy: current status and future prospects. *Immunotherapy* 1:663-678.
176. Thompson, K., A. J. Roelofs, M. Jauhainen, H. Monkkonen, J. Monkkonen, and M. J. Rogers. 2010. Activation of gammadelta T cells by bisphosphonates. *Advances in Experimental Medicine and Biology* 658:11-20.
177. Gomes, A. Q., D. S. Martins, and B. Silva-Santos. 2010. Targeting gammadelta T lymphocytes for cancer immunotherapy: from novel mechanistic insight to clinical application. *Cancer Research* 70:10024-10027.



178. Nagamine, I., Y. Yamaguchi, M. Ohara, T. Ikeda, and M. Okada. 2009. Induction of gamma delta T cells using zoledronate plus interleukin-2 in patients with metastatic cancer. *Hiroshima J Med Sci* 58:37-44.
179. Wilhelm, M., V. Kunzmann, S. Eckstein, P. Reimer, F. Weissinger, T. Ruediger, and H. P. Tony. 2003. Gammadelta T cells for immune therapy of patients with lymphoid malignancies. *Blood* 102:200-206.
180. Nicol, A. J., H. Tokuyama, S. R. Mattarollo, T. Hagi, K. Suzuki, K. Yokokawa, and M. Nieda. 2011. Clinical evaluation of autologous gamma delta T cell-based immunotherapy for metastatic solid tumours. *British Journal of Cancer* 105:778-786.
181. Lang, J. M., M. R. Kaikobad, M. Wallace, M. J. Staab, D. L. Horvath, G. Wilding, G. Liu, J. C. Eickhoff, D. G. McNeel, and M. Malkovsky. 2011. Pilot trial of interleukin-2 and zoledronic acid to augment gammadelta T cells as treatment for patients with refractory renal cell carcinoma. *Cancer Immunology, Immunotherapy : CII* 60:1447-1460.
182. Kobayashi, H., Y. Tanaka, J. Yagi, N. Minato, and K. Tanabe. 2011. Phase I/II study of adoptive transfer of gammadelta T cells in combination with zoledronic acid and IL-2 to patients with advanced renal cell carcinoma. *Cancer Immunology, Immunotherapy : CII* 60:1075-1084.
183. Kabelitz, D., T. Hinz, T. Dobmeyer, U. Mentzel, S. Marx, A. Bohme, B. Arden, R. Rossol, and D. Hoelzer. 1997. Clonal expansion of Vgamma3/Vdelta3-expressing gammadelta T cells in an HIV-1/2-negative patient with CD4 T-cell deficiency. *British Journal of Haematology* 96:266-271.

184. Cooper, L. J. 2008. Test-driving CARs. *Blood* 112:2172-2173.
185. June, C. H. 2007. Principles of adoptive T cell cancer therapy. *The Journal of Clinical Investigation* 117:1204-1212.
186. Jena, B., G. Dotti, and L. J. Cooper. 2010. Redirecting T-cell specificity by introducing a tumor-specific chimeric antigen receptor. *Blood* 116:1035-1044.
187. Sadelain, M., R. Brentjens, and I. Riviere. 2009. The promise and potential pitfalls of chimeric antigen receptors. *Current Opinion in Immunology* 21:215-223.
188. Gross, G., T. Waks, and Z. Eshhar. 1989. Expression of immunoglobulin-T-cell receptor chimeric molecules as functional receptors with antibody-type specificity. *Proceedings of the National Academy of Sciences of the United States of America* 86:10024-10028.
189. Kowolik, C. M., M. S. Topp, S. Gonzalez, T. Pfeiffer, S. Olivares, N. Gonzalez, D. D. Smith, S. J. Forman, M. C. Jensen, and L. J. Cooper. 2006. CD28 costimulation provided through a CD19-specific chimeric antigen receptor enhances in vivo persistence and antitumor efficacy of adoptively transferred T cells. *Cancer Research* 66:10995-11004.
190. Milone, M. C., J. D. Fish, C. Carpenito, R. G. Carroll, G. K. Binder, D. Teachey, M. Samanta, M. Lakhali, B. Gloss, G. Danet-Desnoyers, D. Campana, J. L. Riley, S. A. Grupp, and C. H. June. 2009. Chimeric receptors containing CD137 signal transduction domains mediate enhanced survival of T cells and increased antileukemic efficacy in vivo. *Molecular Therapy : The Journal of the American Society of Gene Therapy* 17:1453-1464.

191. Cartellieri, M., M. Bachmann, A. Feldmann, C. Bippes, S. Stamova, R. Wehner, A. Temme, and M. Schmitz. 2010. Chimeric antigen receptor-engineered T cells for immunotherapy of cancer. *Journal of Biomedicine & Biotechnology* 2010:956304.
192. Brentjens, R. J., E. Santos, Y. Nikhamin, R. Yeh, M. Matsushita, K. La Perle, A. Quintas-Cardama, S. M. Larson, and M. Sadelain. 2007. Genetically targeted T cells eradicate systemic acute lymphoblastic leukemia xenografts. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research* 13:5426-5435.
193. Imai, C., K. Mihara, M. Andreansky, I. C. Nicholson, C. H. Pui, T. L. Geiger, and D. Campana. 2004. Chimeric receptors with 4-1BB signaling capacity provoke potent cytotoxicity against acute lymphoblastic leukemia. *Leukemia* 18:676-684.
194. Zhao, Y., Q. J. Wang, S. Yang, J. N. Kochenderfer, Z. Zheng, X. Zhong, M. Sadelain, Z. Eshhar, S. A. Rosenberg, and R. A. Morgan. 2009. A herceptin-based chimeric antigen receptor with modified signaling domains leads to enhanced survival of transduced T lymphocytes and antitumor activity. *Journal of Immunology* 183:5563-5574.
195. Wang, J., M. Jensen, Y. Lin, X. Sui, E. Chen, C. G. Lindgren, B. Till, A. Raubitschek, S. J. Forman, X. Qian, S. James, P. Greenberg, S. Riddell, and O. W. Press. 2007. Optimizing adoptive polyclonal T cell immunotherapy of lymphomas, using a chimeric T cell receptor possessing CD28 and CD137 costimulatory domains. *Human Gene Therapy* 18:712-725.

196. Carpenito, C., M. C. Milone, R. Hassan, J. C. Simonet, M. Lakhali, M. M. Suhoski, A. Varela-Rohena, K. M. Haines, D. F. Heitjan, S. M. Albelda, R. G. Carroll, J. L. Riley, I. Pastan, and C. H. June. 2009. Control of large, established tumor xenografts with genetically retargeted human T cells containing CD28 and CD137 domains. *Proceedings of the National Academy of Sciences of the United States of America* 106:3360-3365.
197. Finney, H. M., A. D. Lawson, C. R. Bebbington, and A. N. Weir. 1998. Chimeric receptors providing both primary and costimulatory signaling in T cells from a single gene product. *Journal of Immunology* 161:2791-2797.
198. James, S. E., P. D. Greenberg, M. C. Jensen, Y. Lin, J. Wang, B. G. Till, A. A. Raubitschek, S. J. Forman, and O. W. Press. 2008. Antigen sensitivity of CD22-specific chimeric TCR is modulated by target epitope distance from the cell membrane. *Journal of Immunology* 180:7028-7038.
199. Hudecek, M., M. T. Lupo Stanghellini, P. L. Kosasih, D. Sommermeyer, M. Jensen, C. Rader, and S. Riddell. 2013. Receptor affinity and extracellular domain modifications affect tumor recognition by ROR1-specific chimeric antigen receptor T-cells. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research*.
200. Till, B. G., M. C. Jensen, J. Wang, X. Qian, A. K. Gopal, D. G. Maloney, C. G. Lindgren, Y. Lin, J. M. Pagel, L. E. Budde, A. Raubitschek, S. J. Forman, P. D. Greenberg, S. R. Riddell, and O. W. Press. 2012. CD20-specific adoptive immunotherapy for lymphoma using a chimeric antigen receptor with both CD28 and 4-1BB domains: pilot clinical trial results. *Blood* 119:3940-3950.

201. Giordano Attianese, G. M., V. Marin, V. Hoyos, B. Savoldo, I. Pizzitola, S. Tettamanti, V. Agostoni, M. Parma, M. Ponzoni, M. T. Bertilaccio, P. Ghia, A. Biondi, G. Dotti, and E. Biagi. 2011. In vitro and in vivo model of a novel immunotherapy approach for chronic lymphocytic leukemia by anti-CD23 chimeric antigen receptor. *Blood* 117:4736-4745.
202. Park, J. H., and R. J. Brentjens. 2010. Adoptive immunotherapy for B-cell malignancies with autologous chimeric antigen receptor modified tumor targeted T cells. *Discovery Medicine* 9:277-288.
203. Jensen, M. C., L. J. Cooper, A. M. Wu, S. J. Forman, and A. Raubitschek. 2003. Engineered CD20-specific primary human cytotoxic T lymphocytes for targeting B-cell malignancy. *Cytherapy* 5:131-138.
204. Vera, J., B. Savoldo, S. Vigouroux, E. Biagi, M. Pule, C. Rossig, J. Wu, H. E. Heslop, C. M. Rooney, M. K. Brenner, and G. Dotti. 2006. T lymphocytes redirected against the kappa light chain of human immunoglobulin efficiently kill mature B lymphocyte-derived malignant cells. *Blood* 108:3890-3897.
205. Di Stasi, A., B. De Angelis, C. M. Rooney, L. Zhang, A. Mahendravada, A. E. Foster, H. E. Heslop, M. K. Brenner, G. Dotti, and B. Savoldo. 2009. T lymphocytes coexpressing CCR4 and a chimeric antigen receptor targeting CD30 have improved homing and antitumor activity in a Hodgkin tumor model. *Blood* 113:6392-6402.
206. Savoldo, B., C. M. Rooney, A. Di Stasi, H. Abken, A. Hombach, A. E. Foster, L. Zhang, H. E. Heslop, M. K. Brenner, and G. Dotti. 2007. Epstein Barr virus specific cytotoxic T lymphocytes expressing the anti-CD30zeta artificial

- chimeric T-cell receptor for immunotherapy of Hodgkin disease. *Blood* 110:2620-2630.
207. Hombach, A., C. Heuser, R. Sircar, T. Tillmann, V. Diehl, C. Pohl, and H. Abken. 1998. An anti-CD30 chimeric receptor that mediates CD3-zeta-independent T-cell activation against Hodgkin's lymphoma cells in the presence of soluble CD30. *Cancer Research* 58:1116-1119.
208. Haso, W., D. W. Lee, N. N. Shah, M. Stetler-Stevenson, C. M. Yuan, I. H. Pastan, D. S. Dimitrov, R. A. Morgan, D. J. FitzGerald, D. M. Barrett, A. S. Wayne, C. L. Mackall, and R. J. Orentas. 2013. Anti-CD22-chimeric antigen receptors targeting B-cell precursor acute lymphoblastic leukemia. *Blood* 121:1165-1174.
209. Wang, D., L. Zhang, Y. Li, H. Wang, Q. Xiao, W. Cao, and W. Feng. 2012. Construction and expression of humanized chimeric T cell receptor specific for chronic myeloid leukemia cells. *Biotechnology Letters* 34:1193-1201.
210. Tettamanti, S., V. Marin, I. Pizzitola, C. F. Magnani, G. M. Giordano Attianese, E. Cribioli, F. Maltese, S. Galimberti, A. F. Lopez, A. Biondi, D. Bonnet, and E. Biagi. 2013. Targeting of acute myeloid leukaemia by cytokine-induced killer cells redirected with a novel CD123-specific chimeric antigen receptor. *British Journal of Haematology* 161:389-401.
211. Dutour, A., V. Marin, I. Pizzitola, S. Valsesia-Wittmann, D. Lee, E. Yvon, H. Finney, A. Lawson, M. Brenner, A. Biondi, E. Biagi, and R. Rousseau. 2012. In Vitro and In Vivo Antitumor Effect of Anti-CD33 Chimeric Receptor-

- Expressing EBV-CTL against CD33 Acute Myeloid Leukemia. *Advances in Hematology* 2012:683065.
212. Pizzitola, I., V. Agostoni, E. Cribioli, M. Pule, R. Rousseau, H. Finney, A. Lawson, A. Biondi, E. Biagi, and V. Marin. 2011. In vitro comparison of three different chimeric receptor-modified effector T-cell populations for leukemia cell therapy. *Journal of Immunotherapy* 34:469-479.
213. Schmitt, N., M. C. Cumont, M. T. Nugeyre, B. Hurtrel, F. Barre-Sinoussi, D. Scott-Algara, and N. Israel. 2007. Ex vivo characterization of human thymic dendritic cell subsets. *Immunobiology* 212:167-177.
214. Garnache-Ottou, F., L. Chaperot, S. Biichle, C. Ferrand, J. P. Remy-Martin, E. Deconinck, P. D. de Tailly, B. Bulabois, J. Poulet, E. Kuhlein, M. C. Jacob, V. Salaun, M. Arock, B. Drenou, F. Schillinger, E. Seilles, P. Tiberghien, J. C. Bensa, J. Plumas, and P. Saas. 2005. Expression of the myeloid-associated marker CD33 is not an exclusive factor for leukemic plasmacytoid dendritic cells. *Blood* 105:1256-1264.
215. Song, D. G., Q. Ye, C. Carpenito, M. Poussin, L. P. Wang, C. Ji, M. Figini, C. H. June, G. Coukos, and D. J. Powell, Jr. 2011. In vivo persistence, tumor localization, and antitumor activity of CAR-engineered T cells is enhanced by costimulatory signaling through CD137 (4-1BB). *Cancer Research* 71:4617-4627.
216. Kandalaft, L. E., D. J. Powell, Jr., and G. Coukos. 2012. A phase I clinical trial of adoptive transfer of folate receptor-alpha redirected autologous T cells for recurrent ovarian cancer. *Journal of Translational Medicine* 10:157.

217. Parker, L. L., M. T. Do, J. A. Westwood, J. R. Wunderlich, M. E. Dudley, S. A. Rosenberg, and P. Hwu. 2000. Expansion and characterization of T cells transduced with a chimeric receptor against ovarian cancer. *Human Gene Therapy* 11:2377-2387.
218. Tchou, J., L. C. Wang, B. Selven, H. Zhang, J. Conejo-Garcia, H. Borghaei, M. Kalos, R. H. Vondeheide, S. M. Albelda, C. H. June, and P. J. Zhang. 2012. Mesothelin, a novel immunotherapy target for triple negative breast cancer. *Breast Cancer Research and Treatment* 133:799-804.
219. Lanitis, E., M. Poussin, I. S. Hagemann, G. Coukos, R. Sandaltzopoulos, N. Scholler, and D. J. Powell, Jr. 2012. Redirected antitumor activity of primary human lymphocytes transduced with a fully human anti-mesothelin chimeric receptor. *Molecular Therapy : The Journal of the American Society of Gene Therapy* 20:633-643.
220. Lamers, C. H., S. Sleijfer, S. van Steenbergen, P. van Elzaker, B. van Krimpen, C. Groot, A. Vulto, M. den Bakker, E. Oosterwijk, R. Debets, and J. W. Gratama. 2013. Treatment of metastatic renal cell carcinoma with CAIX CAR-engineered T cells: clinical evaluation and management of on-target toxicity. *Molecular Therapy : The Journal of the American Society of Gene Therapy* 21:904-912.
221. Lamers, C. H., R. Willemsen, P. van Elzaker, S. van Steenbergen-Langeveld, M. Broertjes, J. Oosterwijk-Wakka, E. Oosterwijk, S. Sleijfer, R. Debets, and J. W. Gratama. 2011. Immune responses to transgene and retroviral vector in patients treated with ex vivo-engineered T cells. *Blood* 117:72-82.



222. Genega, E. M., M. Ghebremichael, R. Najarian, Y. Fu, Y. Wang, P. Argani, C. Grisanzio, and S. Signoretti. 2010. Carbonic anhydrase IX expression in renal neoplasms: correlation with tumor type and grade. *American Journal of Clinical Pathology* 134:873-879.
223. Chmielewski, M., O. Hahn, G. Rappl, M. Nowak, I. H. Schmidt-Wolf, A. A. Hombach, and H. Abken. 2012. T cells that target carcinoembryonic antigen eradicate orthotopic pancreatic carcinomas without inducing autoimmune colitis in mice. *Gastroenterology* 143:1095-1107 e1092.
224. Schlimper, C., A. A. Hombach, H. Abken, and I. G. Schmidt-Wolf. 2012. Improved activation toward primary colorectal cancer cells by antigen-specific targeting autologous cytokine-induced killer cells. *Clinical & Developmental Immunology* 2012:238924.
225. Nakazawa, Y., L. E. Huye, V. S. Salsman, A. M. Leen, N. Ahmed, L. Rollins, G. Dotti, S. M. Gottschalk, M. H. Wilson, and C. M. Rooney. 2011. PiggyBac-mediated cancer immunotherapy using EBV-specific cytotoxic T-cells expressing HER2-specific chimeric antigen receptor. *Molecular Therapy : The Journal of the American Society of Gene Therapy* 19:2133-2143.
226. Li, S., J. Yang, F. A. Urban, J. N. MacGregor, D. P. Hughes, A. E. Chang, K. T. McDonagh, and Q. Li. 2008. Genetically engineered T cells expressing a HER2-specific chimeric receptor mediate antigen-specific tumor regression. *Cancer Gene Therapy* 15:382-392.
227. Ahmed, N., M. Ratnayake, B. Savoldo, L. Perlaky, G. Dotti, W. S. Wels, M. B. Bhattacharjee, R. J. Gilbertson, H. D. Shine, H. L. Weiss, C. M. Rooney, H. E.

- Heslop, and S. Gottschalk. 2007. Regression of experimental medulloblastoma following transfer of HER2-specific T cells. *Cancer Research* 67:5957-5964.
228. Stancovski, I., D. G. Schindler, T. Waks, Y. Yarden, M. Sela, and Z. Eshhar. 1993. Targeting of T lymphocytes to Neu/HER2-expressing cells using chimeric single chain Fv receptors. *Journal of Immunology* 151:6577-6582.
229. Morgan, R. A., J. C. Yang, M. Kitano, M. E. Dudley, C. M. Laurencot, and S. A. Rosenberg. 2010. Case report of a serious adverse event following the administration of T cells transduced with a chimeric antigen receptor recognizing ERBB2. *Molecular Therapy : The Journal of the American Society of Gene Therapy* 18:843-851.
230. Zhou, X., J. Li, Z. Wang, Z. Chen, J. Qiu, Y. Zhang, W. Wang, Y. Ma, N. Huang, K. Cui, J. Li, and Y. Q. Wei. 2013. Cellular Immunotherapy for Carcinoma Using Genetically Modified EGFR-Specific T Lymphocytes. *Neoplasia* 15:544-553.
231. Ohno, M., A. Natsume, K. Ichiro Iwami, H. Iwamizu, K. Noritake, D. Ito, Y. Toi, M. Ito, K. Motomura, J. Yoshida, K. Yoshikawa, and T. Wakabayashi. 2010. Retrovirally engineered T-cell-based immunotherapy targeting type III variant epidermal growth factor receptor, a glioma-associated antigen. *Cancer Science* 101:2518-2524.
232. Morgan, R. A., L. A. Johnson, J. L. Davis, Z. Zheng, K. D. Woolard, E. A. Reap, S. A. Feldman, N. Chinnasamy, C. T. Kuan, H. Song, W. Zhang, H. A. Fine, and S. A. Rosenberg. 2012. Recognition of glioma stem cells by

- genetically modified T cells targeting EGFRvIII and development of adoptive cell therapy for glioma. *Human Gene Therapy* 23:1043-1053.
233. Westwood, J. A., M. J. Smyth, M. W. Teng, M. Moeller, J. A. Trapani, A. M. Scott, F. E. Smyth, G. A. Cartwright, B. E. Power, D. Honemann, H. M. Prince, P. K. Darcy, and M. H. Kershaw. 2005. Adoptive transfer of T cells modified with a humanized chimeric receptor gene inhibits growth of Lewis-Y-expressing tumors in mice. *Proceedings of the National Academy of Sciences of the United States of America* 102:19051-19056.
  234. Orentas, R. J., D. W. Lee, and C. Mackall. 2012. Immunotherapy targets in pediatric cancer. *Frontiers in Oncology* 2:3.
  235. Park, J. R., D. L. Digiusto, M. Slovak, C. Wright, A. Naranjo, J. Wagner, H. B. Meechoovet, C. Bautista, W. C. Chang, J. R. Ostberg, and M. C. Jensen. 2007. Adoptive transfer of chimeric antigen receptor re-directed cytolytic T lymphocyte clones in patients with neuroblastoma. *Molecular Therapy : The Journal of the American Society of Gene Therapy* 15:825-833.
  236. Pule, M. A., B. Savoldo, G. D. Myers, C. Rossig, H. V. Russell, G. Dotti, M. H. Huls, E. Liu, A. P. Gee, Z. Mei, E. Yvon, H. L. Weiss, H. Liu, C. M. Rooney, H. E. Heslop, and M. K. Brenner. 2008. Virus-specific T cells engineered to coexpress tumor-specific receptors: persistence and antitumor activity in individuals with neuroblastoma. *Nature Medicine* 14:1264-1270.
  237. Louis, C. U., B. Savoldo, G. Dotti, M. Pule, E. Yvon, G. D. Myers, C. Rossig, H. V. Russell, O. Diouf, E. Liu, H. Liu, M. F. Wu, A. P. Gee, Z. Mei, C. M. Rooney, H. E. Heslop, and M. K. Brenner. 2011. Antitumor activity and long-

- term fate of chimeric antigen receptor-positive T cells in patients with neuroblastoma. *Blood* 118:6050-6056.
238. Kailayangiri, S., B. Altvater, J. Meltzer, S. Pscherer, A. Luecke, C. Dierkes, U. Titze, K. Leuchte, S. Landmeier, M. Hotfilder, U. Dirksen, J. Harges, G. Gosheger, H. Juergens, and C. Rossig. 2012. The ganglioside antigen G(D2) is surface-expressed in Ewing sarcoma and allows for MHC-independent immune targeting. *British Journal of Cancer* 106:1123-1133.
239. Yvon, E., M. Del Vecchio, B. Savoldo, V. Hoyos, A. Dutour, A. Anichini, G. Dotti, and M. K. Brenner. 2009. Immunotherapy of metastatic melanoma using genetically engineered GD2-specific T cells. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research* 15:5852-5860.
240. Burns, W. R., Y. Zhao, T. L. Frankel, C. S. Hinrichs, Z. Zheng, H. Xu, S. A. Feldman, S. Ferrone, S. A. Rosenberg, and R. A. Morgan. 2010. A high molecular weight melanoma-associated antigen-specific chimeric antigen receptor redirects lymphocytes to target human melanomas. *Cancer Research* 70:3027-3033.
241. Morgan, R. A., M. E. Dudley, and S. A. Rosenberg. 2010. Adoptive cell therapy: genetic modification to redirect effector cell specificity. *Cancer Journal* 16:336-341.
242. Rosenberg, S. A. 2012. Raising the bar: the curative potential of human cancer immunotherapy. *Science Translational Medicine* 4:127ps128.

243. Zhong, X. S., M. Matsushita, J. Plotkin, I. Riviere, and M. Sadelain. 2010. Chimeric antigen receptors combining 4-1BB and CD28 signaling domains augment PI3kinase/AKT/Bcl-XL activation and CD8<sup>+</sup> T cell-mediated tumor eradication. *Molecular Therapy : The Journal of the American Society of Gene Therapy* 18:413-420.
244. Morgenroth, A., M. Cartellieri, M. Schmitz, S. Gunes, B. Weigle, M. Bachmann, H. Abken, E. P. Rieber, and A. Temme. 2007. Targeting of tumor cells expressing the prostate stem cell antigen (PSCA) using genetically engineered T-cells. *The Prostate* 67:1121-1131.
245. Gade, T. P., W. Hassen, E. Santos, G. Gunset, A. Saudemont, M. C. Gong, R. Brentjens, X. S. Zhong, M. Stephan, J. Stefanski, C. Lyddane, J. R. Osborne, I. M. Buchanan, S. J. Hall, W. D. Heston, I. Riviere, S. M. Larson, J. A. Koutcher, and M. Sadelain. 2005. Targeted elimination of prostate cancer by genetically directed human T lymphocytes. *Cancer Research* 65:9080-9088.
246. Sanchez, C., R. Chan, P. Bajgain, S. Rambally, G. Palapattu, M. Mims, C. M. Rooney, A. M. Leen, M. K. Brenner, and J. F. Vera. 2013. Combining T-cell immunotherapy and anti-androgen therapy for prostate cancer. *Prostate Cancer and Prostatic Diseases*.
247. Wilkie, S., G. Picco, J. Foster, D. M. Davies, S. Julien, L. Cooper, S. Arif, S. J. Mather, J. Taylor-Papadimitriou, J. M. Burchell, and J. Maher. 2008. Retargeting of human T cells to tumor-associated MUC1: the evolution of a chimeric antigen receptor. *Journal of Immunology* 180:4901-4909.

248. Sharifzadeh, Z., F. Rahbarizadeh, M. A. Shokrgozar, D. Ahmadvand, F. Mahboudi, F. R. Jamnani, and S. M. Moghimi. 2012. Genetically engineered T cells bearing chimeric nanoconstructed receptors harboring TAG-72-specific camelid single domain antibodies as targeting agents. *Cancer Letters*.
249. Ren-Heidenreich, L., G. T. Hayman, and K. T. Trevor. 2000. Specific targeting of EGP-2+ tumor cells by primary lymphocytes modified with chimeric T cell receptors. *Human Gene Therapy* 11:9-19.
250. Chinnasamy, D., Z. Yu, S. P. Kerkar, L. Zhang, R. A. Morgan, N. P. Restifo, and S. A. Rosenberg. 2012. Local delivery of interleukin-12 using T cells targeting VEGF receptor-2 eradicates multiple vascularized tumors in mice. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research* 18:1672-1683.
251. Chinnasamy, D., Z. Yu, M. R. Theoret, Y. Zhao, R. K. Shrimali, R. A. Morgan, S. A. Feldman, N. P. Restifo, and S. A. Rosenberg. 2010. Gene therapy using genetically modified lymphocytes targeting VEGFR-2 inhibits the growth of vascularized syngenic tumors in mice. *The Journal of Clinical Investigation* 120:3953-3968.
252. Kahlon, K. S., C. Brown, L. J. Cooper, A. Raubitschek, S. J. Forman, and M. C. Jensen. 2004. Specific recognition and killing of glioblastoma multiforme by interleukin 13-zetakine redirected cytolytic T cells. *Cancer Research* 64:9160-9166.

253. Stastny, M. J., C. E. Brown, C. Ruel, and M. C. Jensen. 2007. Medulloblastomas expressing IL13Ralpha2 are targets for IL13-zetakine+ cytolytic T cells. *Journal of Pediatric Hematology/Oncology* 29:669-677.
254. Brown, C. E., R. Starr, B. Aguilar, A. F. Shami, C. Martinez, M. D'Apuzzo, M. E. Barish, S. J. Forman, and M. C. Jensen. 2012. Stem-like tumor-initiating cells isolated from IL13Ralpha2 expressing gliomas are targeted and killed by IL13-zetakine-redirected T Cells. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research* 18:2199-2209.
255. Kong, S., S. Sengupta, B. Tyler, A. J. Bais, Q. Ma, S. Doucette, J. Zhou, A. Sahin, B. S. Carter, H. Brem, R. P. Junghans, and P. Sampath. 2012. Suppression of human glioma xenografts with second-generation IL13R-specific chimeric antigen receptor-modified T cells. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research* 18:5949-5960.
256. June, C. H. 2007. Adoptive T cell therapy for cancer in the clinic. *The Journal of Clinical Investigation* 117:1466-1476.
257. Lipowska-Bhalla, G., D. E. Gilham, R. E. Hawkins, and D. G. Rothwell. 2012. Targeted immunotherapy of cancer with CAR T cells: achievements and challenges. *Cancer Immunology, Immunotherapy : CII* 61:953-962.
258. Hosing, C., P. Kebriaei, W. Wierda, B. Jena, L. J. Cooper, and E. Shpall. 2013. CARs in chronic lymphocytic leukemia -- ready to drive. *Current Hematologic Malignancy Reports* 8:60-70.

259. Jena, B., S. Maiti, H. Huls, H. Singh, D. A. Lee, R. E. Champlin, and L. J. Cooper. 2013. Chimeric antigen receptor (CAR)-specific monoclonal antibody to detect CD19-specific T cells in clinical trials. *PloS One* 8:e57838.
260. Kochenderfer, J. N., and S. A. Rosenberg. 2013. Treating B-cell cancer with T cells expressing anti-CD19 chimeric antigen receptors. *Nature reviews. Clinical Oncology* 10:267-276.
261. Brentjens, R., R. Yeh, Y. Bernal, I. Riviere, and M. Sadelain. 2010. Treatment of chronic lymphocytic leukemia with genetically targeted autologous T cells: case report of an unforeseen adverse event in a phase I clinical trial. *Molecular Therapy : The Journal of the American Society of Gene Therapy* 18:666-668.
262. Ertl, H. C., J. Zaia, S. A. Rosenberg, C. H. June, G. Dotti, J. Kahn, L. J. Cooper, J. Corrigan-Curay, and S. E. Strome. 2011. Considerations for the clinical application of chimeric antigen receptor T cells: observations from a recombinant DNA Advisory Committee Symposium held June 15, 2010. *Cancer Research* 71:3175-3181.
263. Maiti, S. N., H. Huls, H. Singh, M. Dawson, M. Figliola, S. Olivares, P. Rao, Y. J. Zhao, A. Multani, G. Yang, L. Zhang, D. Crossland, S. Ang, H. Torikai, B. Rabinovich, D. A. Lee, P. Kebriaei, P. Hackett, R. E. Champlin, and L. J. Cooper. 2013. Sleeping beauty system to redirect T-cell specificity for human applications. *Journal of Immunotherapy* 36:112-123.
264. Hackett, P. B., D. A. Largaespada, and L. J. Cooper. 2010. A transposon and transposase system for human application. *Molecular Therapy : The Journal of the American Society of Gene Therapy* 18:674-683.



265. Wadman, S. A., K. J. Clark, and P. B. Hackett. 2005. Fishing for answers with transposons. *Marine Biotechnology* 7:135-141.
266. Liu, G., E. L. Aronovich, Z. Cui, C. B. Whitley, and P. B. Hackett. 2004. Excision of Sleeping Beauty transposons: parameters and applications to gene therapy. *The Journal of Gene Medicine* 6:574-583.
267. Hackett, P. B., D. A. Largaespada, K. C. Switzer, and L. J. Cooper. 2013. Evaluating risks of insertional mutagenesis by DNA transposons in gene therapy. *Translational Research : The Journal of Laboratory and Clinical Medicine* 161:265-283.
268. Hackett, P. B., Jr., E. L. Aronovich, D. Hunter, M. Urness, J. B. Bell, S. J. Kass, L. J. Cooper, and S. McIvor. 2011. Efficacy and safety of Sleeping Beauty transposon-mediated gene transfer in preclinical animal studies. *Current Gene Therapy* 11:341-349.
269. Liu, G., A. M. Geurts, K. Yae, A. R. Srinivasan, S. C. Fahrenkrug, D. A. Largaespada, J. Takeda, K. Horie, W. K. Olson, and P. B. Hackett. 2005. Target-site preferences of Sleeping Beauty transposons. *Journal of Molecular Biology* 346:161-173.
270. Howe, S. J., M. R. Mansour, K. Schwarzwaelder, C. Bartholomae, M. Hubank, H. Kempster, M. H. Brugman, K. Pike-Overzet, S. J. Chatters, D. de Ridder, K. C. Gilmour, S. Adams, S. I. Thornhill, K. L. Parsley, F. J. Staal, R. E. Gale, D. C. Linch, J. Bayford, L. Brown, M. Quaye, C. Kinnon, P. Ancliff, D. K. Webb, M. Schmidt, C. von Kalle, H. B. Gaspar, and A. J. Thrasher. 2008. Insertional mutagenesis combined with acquired somatic mutations causes leukemogenesis

- following gene therapy of SCID-X1 patients. *The Journal of Clinical Investigation* 118:3143-3150.
271. Hacein-Bey-Abina, S., A. Garrigue, G. P. Wang, J. Soulier, A. Lim, E. Morillon, E. Clappier, L. Caccavelli, E. Delabesse, K. Beldjord, V. Asnafi, E. MacIntyre, L. Dal Cortivo, I. Radford, N. Brousse, F. Sigaux, D. Moshous, J. Hauer, A. Borkhardt, B. H. Belohradsky, U. Wintergerst, M. C. Velez, L. Leiva, R. Sorensen, N. Wulffraat, S. Blanche, F. D. Bushman, A. Fischer, and M. Cavazzana-Calvo. 2008. Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1. *The Journal of Clinical Investigation* 118:3132-3142.
  272. Singh, H., P. R. Manuri, S. Olivares, N. Dara, M. J. Dawson, H. Huls, P. B. Hackett, D. B. Kohn, E. J. Shpall, R. E. Champlin, and L. J. Cooper. 2008. Redirecting specificity of T-cell populations for CD19 using the Sleeping Beauty system. *Cancer Research* 68:2961-2971.
  273. Huls, M. H., M. J. Figliola, M. J. Dawson, S. Olivares, P. Kebriaei, E. J. Shpall, R. E. Champlin, H. Singh, and L. J. Cooper. 2013. Clinical application of Sleeping Beauty and artificial antigen presenting cells to genetically modify T cells from peripheral and umbilical cord blood. *Journal of Visualized Experiments : JoVE*:e50070.
  274. Ye, Q., M. Loisiou, B. L. Levine, M. M. Suhoski, J. L. Riley, C. H. June, G. Coukos, and D. J. Powell, Jr. 2011. Engineered artificial antigen presenting cells facilitate direct and efficient expansion of tumor infiltrating lymphocytes. *Journal of Translational Medicine* 9:131.

275. Denman, C. J., V. V. Senyukov, S. S. Somanchi, P. V. Phatarpekar, L. M. Kopp, J. L. Johnson, H. Singh, L. Hurton, S. N. Maiti, M. H. Huls, R. E. Champlin, L. J. Cooper, and D. A. Lee. 2012. Membrane-bound IL-21 promotes sustained ex vivo proliferation of human natural killer cells. *PloS One* 7:e30264.
276. Butler, M. O., S. Ansen, M. Tanaka, O. Imataki, A. Berezovskaya, M. M. Mooney, G. Metzler, M. I. Milstein, L. M. Nadler, and N. Hirano. 2010. A panel of human cell-based artificial APC enables the expansion of long-lived antigen-specific CD4+ T cells restricted by prevalent HLA-DR alleles. *International Immunology* 22:863-873.
277. Suhoski, M. M., T. N. Golovina, N. A. Aquil, V. C. Tai, A. Varela-Rohena, M. C. Milone, R. G. Carroll, J. L. Riley, and C. H. June. 2007. Engineering artificial antigen-presenting cells to express a diverse array of co-stimulatory molecules. *Molecular Therapy : The Journal of the American Society of Gene Therapy* 15:981-988.
278. Maus, M. V., A. K. Thomas, D. G. Leonard, D. Allman, K. Addya, K. Schlienger, J. L. Riley, and C. H. June. 2002. Ex vivo expansion of polyclonal and antigen-specific cytotoxic T lymphocytes by artificial APCs expressing ligands for the T-cell receptor, CD28 and 4-1BB. *Nature Biotechnology* 20:143-148.
279. Manuri, P. V., M. H. Wilson, S. N. Maiti, T. Mi, H. Singh, S. Olivares, M. J. Dawson, H. Huls, D. A. Lee, P. H. Rao, J. M. Kaminski, Y. Nakazawa, S. Gottschalk, P. Kebriaei, E. J. Shpall, R. E. Champlin, and L. J. Cooper. 2010.

- piggyBac transposon/transposase system to generate CD19-specific T cells for the treatment of B-lineage malignancies. *Human Gene Therapy* 21:427-437.
280. Numbenjapon, T., L. M. Serrano, H. Singh, C. M. Kowolik, S. Olivares, N. Gonzalez, W. C. Chang, S. J. Forman, M. C. Jensen, and L. J. Cooper. 2006. Characterization of an artificial antigen-presenting cell to propagate cytolytic CD19-specific T cells. *Leukemia* 20:1889-1892.
281. Singh, H., L. M. Serrano, T. Pfeiffer, S. Olivares, G. McNamara, D. D. Smith, Z. Al-Kadhimi, S. J. Forman, S. D. Gillies, M. C. Jensen, D. Colcher, A. Raubitschek, and L. J. Cooper. 2007. Combining adoptive cellular and immunocytokine therapies to improve treatment of B-lineage malignancy. *Cancer Research* 67:2872-2880.
282. Choudhury, A., K. Derkow, A. H. Daneshmanesh, E. Mikaelsson, S. Kiaii, P. Kokhaei, A. Osterborg, and H. Mellstedt. 2010. Silencing of ROR1 and FMOD with siRNA results in apoptosis of CLL cells. *British Journal of Haematology* 151:327-335.
283. Baskar, S., A. Wiestner, W. H. Wilson, I. Pastan, and C. Rader. 2012. Targeting malignant B cells with an immunotoxin against ROR1. *mAbs* 4:349-361.
284. Stonier, S. W., and K. S. Schluns. 2010. Trans-presentation: a novel mechanism regulating IL-15 delivery and responses. *Immunology Letters* 127:85-92.
285. Stonier, S. W., L. J. Ma, E. F. Castillo, and K. S. Schluns. 2008. Dendritic cells drive memory CD8 T-cell homeostasis via IL-15 transpresentation. *Blood* 112:4546-4554.

286. Smetak, M., B. Kimmel, J. Birkmann, K. Schaefer-Eckart, H. Einsele, M. Wilhelm, and V. Kunzmann. 2008. Clinical-scale single-step CD4(+) and CD8(+) cell depletion for donor innate lymphocyte infusion (DILI). *Bone Marrow Transplant* 41:643-650.
287. Kelly-Rogers, J., L. Madrigal-Estebas, T. O'Connor, and D. G. Doherty. 2006. Activation-induced expression of CD56 by T cells is associated with a reprogramming of cytolytic activity and cytokine secretion profile in vitro. *Human Immunology* 67:863-873.
288. Gattinoni, L., X. S. Zhong, D. C. Palmer, Y. Ji, C. S. Hinrichs, Z. Yu, C. Wrzesinski, A. Boni, L. Cassard, L. M. Garvin, C. M. Paulos, P. Muranski, and N. P. Restifo. 2009. Wnt signaling arrests effector T cell differentiation and generates CD8+ memory stem cells. *Nature Medicine* 15:808-813.
289. Di Mitri, D., R. I. Azevedo, S. M. Henson, V. Libri, N. E. Riddell, R. Macaulay, D. Kipling, M. V. Soares, L. Battistini, and A. N. Akbar. 2011. Reversible senescence in human CD4+CD45RA+CD27- memory T cells. *Journal of Immunology* 187:2093-2100.
290. Zhang, M., S. Maiti, C. Bernatchez, H. Huls, B. Rabinovich, R. E. Champlin, L. M. Vence, P. Hwu, L. Radvanyi, and L. J. Cooper. 2012. A New Approach to Simultaneously Quantify Both TCR alpha- and beta-Chain Diversity after Adoptive Immunotherapy. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research*.

291. Chatila, T., L. Silverman, R. Miller, and R. Geha. 1989. Mechanisms of T cell activation by the calcium ionophore ionomycin. *Journal of Immunology* 143:1283-1289.
292. Iwata, M., Y. Ohoka, T. Kuwata, and A. Asada. 1996. Regulation of T cell apoptosis via T cell receptors and steroid receptors. *Stem Cells* 14:632-641.
293. Yang, J., S. Baskar, K. Y. Kwong, M. G. Kennedy, A. Wiestner, and C. Rader. 2011. Therapeutic potential and challenges of targeting receptor tyrosine kinase ROR1 with monoclonal antibodies in B-cell malignancies. *PloS One* 6:e21018.
294. Straathof, K. C., M. A. Pule, P. Yotnda, G. Dotti, E. F. Vanin, M. K. Brenner, H. E. Heslop, D. M. Spencer, and C. M. Rooney. 2005. An inducible caspase 9 safety switch for T-cell therapy. *Blood* 105:4247-4254.
295. Gattinoni, L., C. A. Klebanoff, D. C. Palmer, C. Wrzesinski, K. Kerstann, Z. Yu, S. E. Finkelstein, M. R. Theoret, S. A. Rosenberg, and N. P. Restifo. 2005. Acquisition of full effector function in vitro paradoxically impairs the in vivo antitumor efficacy of adoptively transferred CD8<sup>+</sup> T cells. *The Journal of Clinical Investigation* 115:1616-1626.
296. Cooper, L. J., M. S. Topp, L. M. Serrano, S. Gonzalez, W. C. Chang, A. Naranjo, C. Wright, L. Popplewell, A. Raubitschek, S. J. Forman, and M. C. Jensen. 2003. T-cell clones can be rendered specific for CD19: toward the selective augmentation of the graft-versus-B-lineage leukemia effect. *Blood* 101:1637-1644.

297. Castella, B., C. Vitale, M. Coscia, and M. Massaia. 2011. Vgamma9Vdelta2 T cell-based immunotherapy in hematological malignancies: from bench to bedside. *Cell Mol Life Sci* 68:2419-2432.
298. Brandes, M., K. Willimann, G. Bioley, N. Levy, M. Eberl, M. Luo, R. Tampe, F. Levy, P. Romero, and B. Moser. 2009. Cross-presenting human gammadelta T cells induce robust CD8+ alphabeta T cell responses. *Proceedings of the National Academy of Sciences of the United States of America* 106:2307-2312.
299. Scotet, E., L. O. Martinez, E. Grant, R. Barbaras, P. Jenot, M. Guiraud, B. Monsarrat, X. Saulquin, S. Maillet, J. P. Esteve, F. Lopez, B. Perret, X. Collet, M. Bonneville, and E. Champagne. 2005. Tumor recognition following Vgamma9Vdelta2 T cell receptor interactions with a surface F1-ATPase-related structure and apolipoprotein A-I. *Immunity* 22:71-80.
300. Hayday, A. C. 2000. [gamma][delta] cells: a right time and a right place for a conserved third way of protection. *Annual Review of Immunology* 18:975-1026.
301. Kondo, M., K. Sakuta, A. Noguchi, N. Ariyoshi, K. Sato, S. Sato, A. Hosoi, J. Nakajima, Y. Yoshida, K. Shiraishi, K. Nakagawa, and K. Kakimi. 2008. Zoledronate facilitates large-scale ex vivo expansion of functional gammadelta T cells from cancer patients for use in adoptive immunotherapy. *Cytherapy* 10:842-856.
302. Lamb, L. S., Jr., P. J. Henslee-Downey, R. S. Parrish, K. Godder, J. Thompson, C. Lee, and A. P. Gee. 1996. Increased frequency of TCR gamma delta + T cells in disease-free survivors following T cell-depleted, partially mismatched, related donor bone marrow transplantation for leukemia. *J Hematother* 5:503-509.

303. Lamb, L. S., Jr., A. P. Gee, L. J. Hazlett, P. Musk, R. S. Parrish, T. P. O'Hanlon, S. S. Geier, R. S. Folk, W. G. Harris, K. McPherson, C. Lee, and P. J. Henslee-Downey. 1999. Influence of T cell depletion method on circulating gammadelta T cell reconstitution and potential role in the graft-versus-leukemia effect. *Cytotherapy* 1:7-19.
304. Lamb, L. S., Jr., P. Musk, Z. Ye, F. van Rhee, S. S. Geier, J. J. Tong, K. M. King, and P. J. Henslee-Downey. 2001. Human gammadelta(+) T lymphocytes have in vitro graft vs leukemia activity in the absence of an allogeneic response. *Bone Marrow Transplant* 27:601-606.
305. Godder, K. T., P. J. Henslee-Downey, J. Mehta, B. S. Park, K. Y. Chiang, S. Abhyankar, and L. S. Lamb. 2007. Long term disease-free survival in acute leukemia patients recovering with increased gammadelta T cells after partially mismatched related donor bone marrow transplantation. *Bone Marrow Transplant* 39:751-757.
306. Rischer, M., S. Pscherer, S. Duwe, J. Vormoor, H. Jurgens, and C. Rossig. 2004. Human gammadelta T cells as mediators of chimaeric-receptor redirected anti-tumour immunity. *British Journal of Haematology* 126:583-592.
307. Hanrahan, C. F., W. G. Kimpton, C. J. Howard, K. R. Parsons, M. R. Brandon, A. E. Andrews, and A. D. Nash. 1997. Cellular requirements for the activation and proliferation of ruminant gammadelta T cells. *Journal of Immunology* 159:4287-4294.
308. Garcia, V. E., D. Jullien, M. Song, K. Uyemura, K. Shuai, C. T. Morita, and R. L. Modlin. 1998. IL-15 enhances the response of human gamma delta T cells to



- nonpeptide [correction of nonpetide] microbial antigens. *Journal of Immunology* 160:4322-4329.
309. Do, J. S., and B. Min. 2009. IL-15 produced and trans-presented by DCs underlies homeostatic competition between CD8 and  $\gamma\delta$  T cells in vivo. *Blood* 113:6361-6371.
310. O'Connor, C. M., S. Sheppard, C. A. Hartline, H. Huls, M. Johnson, S. L. Palla, S. Maiti, W. Ma, R. E. Davis, S. Craig, D. A. Lee, R. Champlin, H. Wilson, and L. J. Cooper. 2012. Adoptive T-cell therapy improves treatment of canine non-Hodgkin lymphoma post chemotherapy. *Sci Rep* 2:249.
311. 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. *Molecular Therapy : The Journal of the American Society of Gene Therapy* 21:638-647.
312. Kulkarni, M. M. 2011. Digital multiplexed gene expression analysis using the NanoString nCounter system. *Curr Protoc Mol Biol* Chapter 25:Unit25B 10.
313. Kang, N., J. Zhou, T. Zhang, L. Wang, F. Lu, Y. Cui, L. Cui, and W. He. 2009. Adoptive immunotherapy of lung cancer with immobilized anti-TCRgammadelta antibody-expanded human gammadelta T-cells in peripheral blood. *Cancer Biology & Therapy* 8:1540-1549.
314. Dokouhaki, P., M. Han, B. Joe, M. Li, M. R. Johnston, M. S. Tsao, and L. Zhang. 2010. Adoptive immunotherapy of cancer using ex vivo expanded human gammadelta T cells: A new approach. *Cancer Letters* 297:126-136.

315. Lopez, R. D., S. Xu, B. Guo, R. S. Negrin, and E. K. Waller. 2000. CD2-mediated IL-12-dependent signals render human gamma delta-T cells resistant to mitogen-induced apoptosis, permitting the large-scale ex vivo expansion of functionally distinct lymphocytes: implications for the development of adoptive immunotherapy strategies. *Blood* 96:3827-3837.
316. Newton, D. J., E. M. Andrew, J. E. Dalton, R. Mears, and S. R. Carding. 2006. Identification of novel gammadelta T-cell subsets following bacterial infection in the absence of Vgamma1+ T cells: homeostatic control of gammadelta T-cell responses to pathogen infection by Vgamma1+ T cells. *Infect Immun* 74:1097-1105.
317. Olofsson, K., S. Hellstrom, and M. L. Hammarstrom. 1998. The surface epithelium of recurrent infected palatine tonsils is rich in gammadelta T cells. *Clin Exp Immunol* 111:36-47.
318. Stresing, V., F. Daubine, I. Benzaid, H. Monkkonen, and P. Clezardin. 2007. Bisphosphonates in cancer therapy. *Cancer Letters* 257:16-35.
319. Angelini, D. F., G. Borsellino, M. Poupot, A. Diamantini, R. Poupot, G. Bernardi, F. Poccia, J. J. Fournie, and L. Battistini. 2004. FcgammaRIII discriminates between 2 subsets of Vgamma9Vdelta2 effector cells with different responses and activation pathways. *Blood* 104:1801-1807.
320. Haas, J. D., K. Nistala, F. Petermann, N. Saran, V. Chennupati, S. Schmitz, T. Korn, L. R. Wedderburn, R. Forster, A. Krueger, and I. Prinz. 2011. Expression of miRNAs miR-133b and miR-206 in the Il17a/f locus is co-regulated with IL-17 production in alphabeta and gammadelta T cells. *PloS One* 6:e20171.

321. Kryczek, I., S. Wei, L. Zou, S. Altuwaijri, W. Szeliga, J. Kolls, A. Chang, and W. Zou. 2007. Cutting edge: Th17 and regulatory T cell dynamics and the regulation by IL-2 in the tumor microenvironment. *Journal of Immunology* 178:6730-6733.
322. Lai, D., F. Wang, Y. Chen, C. Wang, S. Liu, B. Lu, X. Ge, and L. Guo. 2011. Human ovarian cancer stem-like cells can be efficiently killed by gammadelta T lymphocytes. *Cancer Immunology, Immunotherapy : CII*.
323. Middleton, G. W., N. E. Annels, and H. S. Pandha. 2011. Are we ready to start studies of Th17 cell manipulation as a therapy for cancer? *Cancer Immunology, Immunotherapy : CII*.
324. Cua, D. J., and C. M. Tato. 2010. Innate IL-17-producing cells: the sentinels of the immune system. *Nature Reviews. Immunology* 10:479-489.
325. Haas, J. D., F. H. Gonzalez, S. Schmitz, V. Chennupati, L. Fohse, E. Kremmer, R. Forster, and I. Prinz. 2009. CCR6 and NK1.1 distinguish between IL-17A and IFN-gamma-producing gammadelta effector T cells. *European Journal of Immunology* 39:3488-3497.
326. Paulos, C. M., C. Carpenito, G. Plesa, M. M. Suhoski, A. Varela-Rohena, T. N. Golovina, R. G. Carroll, J. L. Riley, and C. H. June. 2010. The inducible costimulator (ICOS) is critical for the development of human T(H)17 cells. *Science translational medicine* 2:55ra78.
327. Turchinovich, G., and D. J. Pennington. 2011. T cell receptor signalling in gammadelta cell development: strength isn't everything. *Trends in Immunology* 32:567-573.

328. Janssen, O., S. Wesselborg, B. Heckl-Ostreicher, K. Pechhold, A. Bender, S. Schondelmaier, G. Moldenhauer, and D. Kabelitz. 1991. T cell receptor/CD3-signaling induces death by apoptosis in human T cell receptor gamma delta + T cells. *Journal of Immunology* 146:35-39.
329. Numbenjapon, T., L. M. Serrano, W. C. Chang, S. J. Forman, M. C. Jensen, and L. J. Cooper. 2007. Antigen-independent and antigen-dependent methods to numerically expand CD19-specific CD8+ T cells. *Exp Hematol* 35:1083-1090.
330. Sallusto, F., D. Lenig, R. Forster, M. Lipp, and A. Lanzavecchia. 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 401:708-712.
331. Unsoeld, H., and H. Pircher. 2005. Complex memory T-cell phenotypes revealed by coexpression of CD62L and CCR7. *Journal of Virology* 79:4510-4513.
332. Tomiyama, H., T. Matsuda, and M. Takiguchi. 2002. Differentiation of human CD8(+) T cells from a memory to memory/effector phenotype. *Journal of Immunology* 168:5538-5550.
333. Caccamo, N., C. La Mendola, V. Orlando, S. Meraviglia, M. Todaro, G. Stassi, G. Sireci, J. J. Fournie, and F. Dieli. 2011. Differentiation, phenotype, and function of interleukin-17-producing human Vgamma9Vdelta2 T cells. *Blood* 118:129-138.
334. Rostene, W., P. Kitabgi, and S. M. Parsadaniantz. 2007. Chemokines: a new class of neuromodulator? *Nat Rev Neurosci* 8:895-903.

335. Gilfillan, S., C. J. Chan, M. Cella, N. M. Haynes, A. S. Rapaport, K. S. Boles, D. M. Andrews, M. J. Smyth, and M. Colonna. 2008. DNAM-1 promotes activation of cytotoxic lymphocytes by nonprofessional antigen-presenting cells and tumors. *The Journal of Experimental Medicine* 205:2965-2973.
336. Bauer, S., V. Groh, J. Wu, A. Steinle, J. H. Phillips, L. L. Lanier, and T. Spies. 1999. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. *Science* 285:727-729.
337. Bottino, C., R. Castriconi, D. Pende, P. Rivera, M. Nanni, B. Carnemolla, C. Cantoni, J. Grassi, S. Marcenaro, N. Reymond, M. Vitale, L. Moretta, M. Lopez, and A. Moretta. 2003. Identification of PVR (CD155) and Nectin-2 (CD112) as cell surface ligands for the human DNAM-1 (CD226) activating molecule. *The Journal of Experimental Medicine* 198:557-567.
338. Jensen, H., M. Hagemann-Jensen, F. Lauridsen, and S. Skov. 2013. Regulation of NKG2D-ligand cell surface expression by intracellular calcium after HDAC-inhibitor treatment. *Molecular Immunology* 53:255-264.
339. Pende, D., P. Rivera, S. Marcenaro, C. C. Chang, R. Biassoni, R. Conte, M. Kubin, D. Cosman, S. Ferrone, L. Moretta, and A. Moretta. 2002. Major histocompatibility complex class I-related chain A and UL16-binding protein expression on tumor cell lines of different histotypes: analysis of tumor susceptibility to NKG2D-dependent natural killer cell cytotoxicity. *Cancer Research* 62:6178-6186.
340. Pende, D., C. Cantoni, P. Rivera, M. Vitale, R. Castriconi, S. Marcenaro, M. Nanni, R. Biassoni, C. Bottino, A. Moretta, and L. Moretta. 2001. Role of

- NKG2D in tumor cell lysis mediated by human NK cells: cooperation with natural cytotoxicity receptors and capability of recognizing tumors of nonepithelial origin. *European Journal of Immunology* 31:1076-1086.
341. Turchinovich, G., and A. C. Hayday. 2011. Skint-1 identifies a common molecular mechanism for the development of interferon-gamma-secreting versus interleukin-17-secreting gammadelta T cells. *Immunity* 35:59-68.
  342. Lam, V., R. DeMars, B. P. Chen, J. A. Hank, S. Kovats, P. Fisch, and P. M. Sondel. 1990. Human T cell receptor-gamma delta-expressing T-cell lines recognize MHC-controlled elements on autologous EBV-LCL that are not HLA-A, -B, -C, -DR, -DQ, or -DP. *Journal of Immunology* 145:36-45.
  343. Bhaduri-McIntosh, S., M. J. Rotenberg, B. Gardner, M. Robert, and G. Miller. 2008. Repertoire and frequency of immune cells reactive to Epstein-Barr virus-derived autologous lymphoblastoid cell lines. *Blood* 111:1334-1343.
  344. Micklethwaite, K. P., B. Savoldo, P. J. Hanley, A. M. Leen, G. J. Demmler-Harrison, L. J. Cooper, H. Liu, A. P. Gee, E. J. Shpall, C. M. Rooney, H. E. Heslop, M. K. Brenner, C. M. Bollard, and G. Dotti. 2010. Derivation of human T lymphocytes from cord blood and peripheral blood with antiviral and antileukemic specificity from a single culture as protection against infection and relapse after stem cell transplantation. *Blood* 115:2695-2703.
  345. Torikai, H., A. Reik, P. Q. Liu, Y. Zhou, L. Zhang, S. Maiti, H. Huls, J. C. Miller, P. Kebriaei, B. Rabinovitch, D. A. Lee, R. E. Champlin, C. Bonini, L. Naldini, E. J. Rebar, P. D. Gregory, M. C. Holmes, and L. J. Cooper. 2012. A foundation for universal T-cell based immunotherapy: T cells engineered to

- express a CD19-specific chimeric-antigen-receptor and eliminate expression of endogenous TCR. *Blood* 119:5697-5705.
346. Kebriaei, P., H. Huls, B. Jena, M. Munsell, R. Jackson, D. A. Lee, P. B. Hackett, G. Rondon, E. Shpall, R. E. Champlin, and L. J. Cooper. 2012. Infusing CD19-directed T cells to augment disease control in patients undergoing autologous hematopoietic stem-cell transplantation for advanced B-lymphoid malignancies. *Human Gene Therapy* 23:444-450.
347. Anderson, J., K. Gustafsson, and N. Himoudi. 2012. Licensing of killer dendritic cells in mouse and humans: functional similarities between IKDC and human blood gammadelta T-lymphocytes. *Journal of Immunotoxicology* 9:259-266.
348. Himoudi, N., D. A. Morgenstern, M. Yan, B. Vernay, L. Saraiva, Y. Wu, C. J. Cohen, K. Gustafsson, and J. Anderson. 2012. Human gammadelta T lymphocytes are licensed for professional antigen presentation by interaction with opsonized target cells. *Journal of Immunology* 188:1708-1716.
349. Anderson, J., K. Gustafsson, N. Himoudi, M. Yan, and J. Heuijerjans. 2012. Licensing of gammadeltaT cells for professional antigen presentation: A new role for antibodies in regulation of antitumor immune responses. *Oncoimmunology* 1:1652-1654.
350. Kryczek, I., M. Banerjee, P. Cheng, L. Vatan, W. Szeliga, S. Wei, E. Huang, E. Finlayson, D. Simeone, T. H. Welling, A. Chang, G. Coukos, R. Liu, and W. Zou. 2009. Phenotype, distribution, generation, and functional and clinical relevance of Th17 cells in the human tumor environments. *Blood* 114:1141-1149.

351. DeBarros, A., M. Chaves-Ferreira, F. d'Orey, J. C. Ribot, and B. Silva-Santos. 2011. CD70-CD27 interactions provide survival and proliferative signals that regulate T cell receptor-driven activation of human gammadelta peripheral blood lymphocytes. *European Journal of Immunology* 41:195-201.
352. Ribot, J. C., A. deBarros, D. J. Pang, J. F. Neves, V. Peperzak, S. J. Roberts, M. Girardi, J. Borst, A. C. Hayday, D. J. Pennington, and B. Silva-Santos. 2009. CD27 is a thymic determinant of the balance between interferon-gamma- and interleukin 17-producing gammadelta T cell subsets. *Nature Immunology* 10:427-436.
353. Di Ianni, M., L. Moretti, A. Terenzi, F. Bazzucchi, B. Del Papa, M. Bazzucchi, R. Ciurnelli, A. Lucchesi, P. Sportoletti, E. Rosati, P. F. Marconi, F. Falzetti, and A. Tabilio. 2009. Activated autologous T cells exert an anti-B-cell chronic lymphatic leukemia effect in vitro and in vivo. *Cytotherapy* 11:86-96.
354. Kang, N., L. Tang, X. Li, D. Wu, W. Li, X. Chen, L. Cui, D. Ba, and W. He. 2009. Identification and characterization of Foxp3(+) gammadelta T cells in mouse and human. *Immunology Letters* 125:105-113.
355. Lu, X., and Y. Kang. 2010. Hypoxia and hypoxia-inducible factors: master regulators of metastasis. *Clinical cancer research : an official journal of the American Association for Cancer Research* 16:5928-5935.
356. Zhu, P., Y. Ning, L. Yao, M. Chen, and C. Xu. 2010. The proliferation, apoptosis, invasion of endothelial-like epithelial ovarian cancer cells induced by hypoxia. *J Exp Clin Cancer Res* 29:124.



357. Turkman, N., A. Shavrin, R. A. Ivanov, B. Rabinovich, A. Volgin, J. G. Gelovani, and M. M. Alauddin. 2011. Fluorinated cannabinoid CB2 receptor ligands: synthesis and in vitro binding characteristics of 2-oxoquinoline derivatives. *Bioorg Med Chem* 19:5698-5707.

## VITA

Drew Caldwell Deniger was born on January 2, in Dallas, TX to William Southgate Deniger, Jr. and Jeanne Caldwell Deniger. He attended Dallas' Hillcrest High School where he served as the art and design editor for the school's newspaper, *The Hurricane*, and played varsity soccer and football. After graduating from Hillcrest in 1998, he enrolled at The University of Texas at Austin where he earned B.S. degrees in Chemistry and Biochemistry. He then worked as a research associate for three years in the lab of Robert A. Davey, Ph.D. at The University of Texas Medical Branch in Galveston, TX. In 2006, he enrolled at The University of Texas Graduate School of Biomedical Sciences in the M.S. program under the mentorship of Madeleine Duvic, M.D. and graduated two years later with a thesis focused on metastatic melanoma. He then enrolled in the Ph.D. program at The University of Texas Graduate School of Biomedical Sciences and joined the Immunology Program and the laboratory of Laurence J.N. Cooper, M.D., Ph.D. in the Division of Pediatrics at The University of Texas M.D. Anderson Cancer Center.