Mechanisms of FOXP3-mediated resistance to restimulation-induced cell death in regulatory and conventional T cells

by

Kelsey Voss

Dissertation submitted to the Faculty of the
Emerging Infectious Diseases Graduate Program
Uniformed Services University of the Health Sciences
In partial fulfillment of the requirements for the degree of
Doctor of Philosophy 2019

Distribution Statement

Distribution A: Public Release.

The views presented here are those of the author and are not to be construed as official or reflecting the views of the Uniformed Services University of the Health Sciences, the Department of Defense or the U.S. Government.

UNIFORMED SERVICES UNIVERSITY, SCHOOL OF MEDICINE GRADUATE PROGRAMS
Graduate Education Office (A 1045), 4301 Jones Bridge Road, Bethesda, MD 20814

APPROVAL OF THE DOCTORAL DISSERTATION IN THE EMERGING INFECTIOUS DISEASES GRADUATE PROGRAM

Title of Dissertation: "Mechanisms of FOXP3-mediated Resistance to Restimulation-induced Cell Death in Regulatory and Conventional T Cells"

Name of Candidate: Kelsey Voss
Doctor of Philosophy
March 20, 2019

DISSERTATION AND ABSTRACT APPROVED:

DATE:

[redacted] 4/4/19

Dr. Brian Schaefer
DEPARTMENT OF MICROBIOLOGY & IMMUNOLOGY
Committee Chairperson

[redacted] 3/20/19

Dr. Andrew Snow
DEPARTMENT OF PHARMACOLOGY & MOLECULAR THERAPEUTICS
Dissertation Advisor

[redacted] 3/20/19

Dr. Chou-Zen Giam
DEPARTMENT OF MICROBIOLOGY & IMMUNOLOGY
Committee Member

[redacted] 3/20/19

Dr. Robert Kortum
DEPARTMENT OF PHARMACOLOGY & MOLECULAR THERAPEUTICS
Committee Member

UNIFORMED SERVICES UNIVERSITY, SCHOOL OF MEDICINE GRADUATE PROGRAMS
Graduate Education Office (A 1045), 4301 Jones Bridge Road, Bethesda, MD 20814

FINAL EXAMINATION/PRIVATE DEFENSE FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
IN THE EMERGING INFECTIOUS DISEASES GRADUATE PROGRAM

Name of Student: Kelsey Voss

Date of Examination: Wednesday, March 20, 2019
Time: 2:30 PM
Place: Room C2015

DECISION OF EXAMINATION COMMITTEE MEMBERS:

	PASS	FAIL
[redacted]	X	___
Dr. Brian Schaefer DEPARTMENT OF MICROBIOLOGY & IMMUNOLOGY Committee Chairperson		
[redacted]	X	___
Dr. Andrew Snow DEPARTMENT OF PHARMACOLOGY & MOLECULAR THERAPEUTICS Dissertation Advisor		
[redacted]	X	___
Dr. Chou-Zen Giam DEPARTMENT OF MICROBIOLOGY & IMMUNOLOGY Committee Member		
[redacted]	X	___
Dr. Robert Kortum DEPARTMENT OF PHARMACOLOGY & MOLECULAR THERAPEUTICS Committee Member		

ACKNOWLEDGMENTS

Moushimi Amaya. Moushimi was my first true scientific mentor. She not only taught me technical skills in the lab, but was also instrumental to building my confidence enough to pursue my Ph.D.. She has always supported me, and I credit her with teaching me the amazing potential of teamwork in the lab.

Sasha Larsen-Akins. When I rotated in Dr. Snow's lab, I was overwhelmed and intimidated by the field of immunology. Only with Sasha's guidance was I able to continue building confidence while trying something that was new and challenging. Jeff Stinson has also been an amazing source of support during this process and continues to provide help and guidance even after moving on to a new position.

Elisa Ruffo. An additional lab member who came into my life when I least expected it, but when I needed her the most. She has supported me and was an amazing team member in our lab that helped me get through difficult times in my Ph.D. Her friendship is absolutely special.

Nathaniel Lott. Nate was tasked with working with me on this project in the final stages of my degree. Thank you for working so hard with me, dedicating so much time and care into my project, and keeping me excited about my work in the final stage of my degree.

My thesis committee: Brian Schaefer, Chou-Zen Giam, and Robert Kortum. Every member of my committee was reliable, supportive, and kind; their guidance and support was absolutely critical for my success. Dr. Schaefer challenged me with difficult questions that ultimately raised the quality of my dissertation work. Dr. Giam generously

offered lab supplies, cell lines, and antibodies without hesitation. Dr. Kortum was willing to get in the trenches with me to complete some technically challenging experiments, generously offering his lab supplies, expertise, and time.

My lab members: Batsukh Dorjbal, Swadhinya Arjunaraja, Camille Lake, Brad Bauman, and Chris Luthers. You made coming into work every day something to look forward to. I would not be here without you and I love you all dearly.

My friends who made graduate school special. The volleyball crew, BRK, Britney, Kariana, and Patrick who made getting through the rigorous EID program a much better experience.

My family who continue to be my biggest fans. My dad who showed me how to be a leader, and my mom for setting an example for me of how to be a good person. My sister, Ashley, who has been an amazing and supportive sister. I know she has my back no matter what. Adam Webster who gave me love and support at all times.

Andrew Snow. Honestly, it's difficult to put into words how much his support and mentorship has meant to me. I wasn't sure what career path I wanted to pursue after my Ph.D., but Andy gave me the confidence I needed to pursue a career in academia and have my own lab one day. Not only did he set an amazing example for me, but he was there for me on the good days and the bad days. I credit him (although I'm sure he will deny it) with giving me the best lab experience I could have dreamed of for my graduate education. I am not sure what he saw in me years ago, but I'm so grateful that he did.

DEDICATION

I dedicate this dissertation to my wonderful niece, Reese Aitken.

May you always believe in yourself and go after your dreams.

COPYRIGHT STATEMENT

The author hereby certifies that the use of any copyrighted material in the dissertation manuscript entitled: “Mechanisms of FOXP3-mediated resistance to restimulation-induced cell death in regulatory and conventional T cells” is appropriately acknowledged and, beyond brief excerpts, is with the permission of the copyright owner.



Kelsey Voss

May 17, 2019

DISCLAIMER

The views presented here are those of the author and are not to be construed as official or reflecting the views of the Uniformed Services University of the Health Sciences, the Department of Defense or the U.S. Government.

ABSTRACT

Mechanisms of FOXP3-mediated resistance to restimulation-induced cell death in regulatory and conventional T cells:

Kelsey Voss, Doctor of Philosophy, Emerging Infectious Diseases 2019

Thesis directed by: Andrew L. Snow, Associate Professor, Department of Pharmacology & Molecular Therapeutics

The adaptive immune response relies on a multitude of factors to maintain homeostasis. T cell homeostasis is interconnected with the number of T cells responding to an antigen and is guided by pre-programmed death pathways critical for self-regulation. Upon antigen stimulation, conventional effector T cell (Tcon) proliferation is constrained by both forkhead box P3 (FOXP3)+ regulatory T cells (Tregs) and restimulation-induced cell death (RICD). Restimulation-induced cell death (RICD) is an apoptotic program triggered by repeated stimulation through the T cell receptor (TCR) in the presence of interleukin-2 (IL-2). Tregs are considered key mediators of immune homeostasis, mainly by their ability to suppress the activation and function of other T cells. Although regulatory T cells (Tregs) consume IL-2 and experience frequent TCR stimulation, they are highly resistant to RICD. Resistance in Tregs is dependent on the forkhead box P3 (FOXP3) transcription factor, although the mechanism remains unclear. T cells from patients with X-linked lymphoproliferative disease (XLP-1) lack the adaptor

molecule SLAM-associated protein (SAP), and are resistant to RICD due to reduced TCR signal strength upon restimulation. Here we demonstrate that FOXP3 reduces SAP expression by directly binding to and repressing the *SH2D1A* (SAP) promoter, which ensures low SAP levels in order to reduce TCR signal strength upon restimulation. Our findings illuminate the SAP-dependent mechanism behind FOXP3-mediated RICD resistance in Tregs, providing new insight into their long-term persistence.

Furthermore, Tcons are resistant to RICD during activation-induced expansion when FOXP3 is expressed, but the function of transient FOXP3 induction in human Tcons remains unresolved. Here we describe a novel role for FOXP3 in protecting both CD4 and CD8 human Tcons from premature RICD during expansion. Unlike late-stage effector T cells which require SAP expression for optimal RICD, low levels of RICD in expanding Tcons was not affected by SAP. Subsequent transcriptome profiling and functional analyses revealed that FOXP3 enhances expression of the SLAM family receptor CD48, which in turn sustains basal autophagy. Higher basal autophagy levels corresponded to increased RICD resistance in expanding Tcons. Therefore, FOXP3 governs a distinct transcriptional program that maintains RICD resistance in early-stage effector Tcons via CD48-dependent protective autophagy.

TABLE OF CONTENTS

LIST OF FIGURES xii

CHAPTER 1: Introduction 13

Immune homeostasis 13
T cell subsets 16
Regulatory T cells (Tregs) 18
Tregs in clinical therapy 20
Forkhead box P3 (FOXP3) 21
FOXP3 expression in other cell types 24
Programmed cell death 25
Apoptosis 26
Restimulation-induced cell death (RICD) 29
SLAM-associated protein (SAP) 31
Cellular metabolism and autophagy influence apoptosis sensitivity 32
Summary 33
Hypothesis 34
Importance 35

CHAPTER 2: FOXP3 renders activated human regulatory T cells resistant to restimulation-induced cell death by suppressing SAP expression 36

Abstract 37
Introduction 37
Materials and Methods 39
Cell isolations and culture conditions 39
Flow cytometry and apoptosis assays 41
Western blotting 41
Quantitative RT-PCR 42
SAP promoter cloning and luciferase assays 42
Chromatin immunoprecipitation 43
Lentiviral infections and siRNA transfections 44
Statistics 45
Results 45
Activated human Tregs display minimal SAP expression and are resistant to RICD 45
Expression of FOXP3 suppresses *SH2D1A* promoter activity 47
FOXP3 directly binds to the *SH2D1A* promoter 49
FOXP3-mediated RICD resistance in Tregs is dependent on repression of SAP expression 51
Discussion 53

CHAPTER 3: FOXP3 protects conventional human T cells from premature restimulation-induced cell death 56

Abstract 56
Introduction 57
Materials and Methods 60
T cell isolation and culture conditions 60
siRNA-mediated knockdowns in primary T cells 61
Restimulation-induced cell death (RICD) assays 61
Flow cytometry 62
Western blotting 63
Transcriptome profiling of primary T lymphocytes 64
Statistics 64
Results 65
Activation-induced FOXP3 expression in conventional T cells varies across multiple human donors 65
FOXP3 expression protects newly activated T cells from restimulation-induced cell death 66
FOXP3 knockdown status does not impact T cell proliferation or SAP expression 68
RICD in expanding T cells is dependent on de novo transcription 71
FOXP3 promotes high CD48 expression in activated T cells 71
CD48 protects activated CD4 and CD8 T cells from RICD 74
CD48 ligand interactions demonstrate variable effects on RICD sensitivity in human donors 74
FOXP3 and CD48 expression protect cells from RICD via promotion of autophagy 77
Expanded View (EV) Figures 81
Discussion 85
Acknowledgements 90
Author Contributions 90

CHAPTER 4: Metabolic reprogramming and apoptosis sensitivity: defining the contours of a T cell response 91

Abstract 91
Introduction 92
Changes in T cell metabolism impact apoptosis sensitivity 94
The size of the responding effector T cell pool is shaped by the interplay between metabolic reprogramming and specific apoptosis pathways 98
T cell metabolism and death sensitivity can influence immunological memory 100
The tumor microenvironment alters T lymphocyte metabolism and the anti-tumor response by tuning apoptosis sensitivity 101
Conclusion 106

CHAPTER 5: Discussion 107

Regulatory T cells 108
Unanswered questions and future directions 110
Conventional T cells 112
Unanswered questions and future directions 115
FOXP3 in Tcons: the million-dollar question 118

RICD: who lives and who dies? 120
Appendix A: Supplementary data 122
Introduction 122
Figures 122
Single-cell (sc) RNA-Seq 126
REFERENCES 128

LIST OF FIGURES

Figure 1. The balance that maintains a healthy T cell response. 15
Figure 2. FOXP3 expression in Tcons inversely correlates with RICD sensitivity..... 34
Figure 3. Gating strategy to sort human Tregs and Tcons. 40
Figure 4. Human Tregs are resistant to RICD, but not FAS-induced death. 46
Figure 5. Inverse correlation between FOXP3 and SAP expression in human Tregs. 47
Figure 6. FOXP3 represses *SH2D1A* promoter activity. 48
Figure 7. FOXP3 binds directly to the *SH2D1A* promoter. 50
Figure 8. FOXP3 confers RICD resistance in Tregs by a SAP-dependent mechanism. .. 52
Figure 9. FOXP3 protects expanding T cells from RICD. 67
Figure 10. RICD in expanding CD4 and CD8 T cells is SAP-independent, but requires *de novo* transcription..... 70
Figure 11. CD48 expression is impaired in FOXP3 KD T cells..... 73
Figure 12. CD48 expression protects expanding CD4 and CD8 T cells from RICD. 76
Figure 13. FOXP3 and CD48 expression promote autophagy to protect T cells from RICD. 79
Figure EV 1. FOXP3 expression in multiple human donors. 81
Figure EV 2. FOXP3 knockdown does not impact T cell proliferation, cell cycle status, or Fas ligand and BIM expression..... 82
Figure EV 3. FOXP3 and ATG3 silencing on T cell activation markers. 84
Figure 14. Sensitivity to critical apoptosis pathways correlates with metabolic reprogramming over the course of the T cell response. 93
Figure 15. Exhausted TIL (Tex) escape apoptosis via altered metabolism. 104
Figure 16. Model of FOXP3-mediated resistance to RICD in Tregs. 108
Figure 17. Model of FOXP3-mediated resistance to RICD in expanding Tcons. 112
Figure 18. Day 7 FOXP3 knockdown T cells show an intermediate phenotype of RICD protection. 122
Figure 19. TCR restimulation decreases autophagy. 123
Figure 20. FOXP3 knockdown T cells exhibit slightly decreased mitochondrial membrane potential..... 123
Figure 21. FOXP3 knockdown and glycolysis. 124
Figure 22. FOXP3 and CD48 knockdown T cells have reduced BATF expression..... 125
Figure 23. t-distributed stochastic neighborhood embedding (t-SNE) cluster analysis.. 126
Figure 24. FOXP3 detection in scRNA-Seq dataset. 127

CHAPTER 1: Introduction

IMMUNE HOMEOSTASIS

The immune system is responsible for protecting us effectively against disease. To accomplish this, immune cells must first recognize the presence of an infection and then carry out the appropriate effector functions to contain the infection. At the same time, the immune response has to be kept under control to ensure minimal harm to the host. Therefore, the immune system has been programmed with the ability to regulate itself. Auto-regulation is absolutely essential for maintaining homeostasis of the immune system, both innate and adaptive. This dissertation will discuss multiple features of immune homeostasis, focusing specifically on factors that regulate T cell responses of the adaptive immune system (Figure 1).

The immune system is comprised of innate and adaptive arms. The innate immune system serves as the first line of defense from pathogens that have penetrated epithelial surfaces of the body. Microorganisms are met by neutrophils and macrophages, which engulf them and release biologically active molecules such as cytokines and chemokines that initiate inflammation and recruit additional immune cells to the area. If the innate immune system is unable to eliminate the pathogen, the lymphocytes of the adaptive immune system can assist. Adaptive immune responses require pathogens to be ingested by an immature dendritic cell. Once a dendritic cell engulfs a pathogen, it becomes activated and travels to a lymph node to serve as an antigen-presenting cell (APC) for activating pathogen-specific T cells.

Adaptive immunity is instrumental for forming a robust effector response to eliminate pathogens, but also for creating immunological memory that confers long-term protection. Upon specific antigen recognition via antigen receptors, T and B lymphocytes react to mount an adaptive immune defense. Naïve T cells recognize antigen epitopes through the T cell receptor (TCR) in the context of major histocompatibility complexes (MHCs) on antigen presenting cells (APCs). In addition to TCR engagement, APCs also provide co-stimulatory signals via CD28 and a third signal consisting of cytokines such as interleukin-2 (IL-2) to reach full activation in the naïve T cell. Upon activation, the naïve T cell then undergoes massive clonal proliferation and expansion to respond to the infection. The activated T cells are also called effector T cells, because they are capable of effector functions such as killing infected cells in the body or secreting cytokines to enhance the immune response. As the infection is cleared and the antigen dissipates, effector T cell numbers contract as the massive army of clonally expanded T cells cannot (and should not) be reasonably sustained long-term. Only a small number of T cells remain behind to form a population memory T cells. If the pathogen returns, memory T cells will become quickly activated, and respond rapidly to their specific antigen by creating a new army of effector T cells.

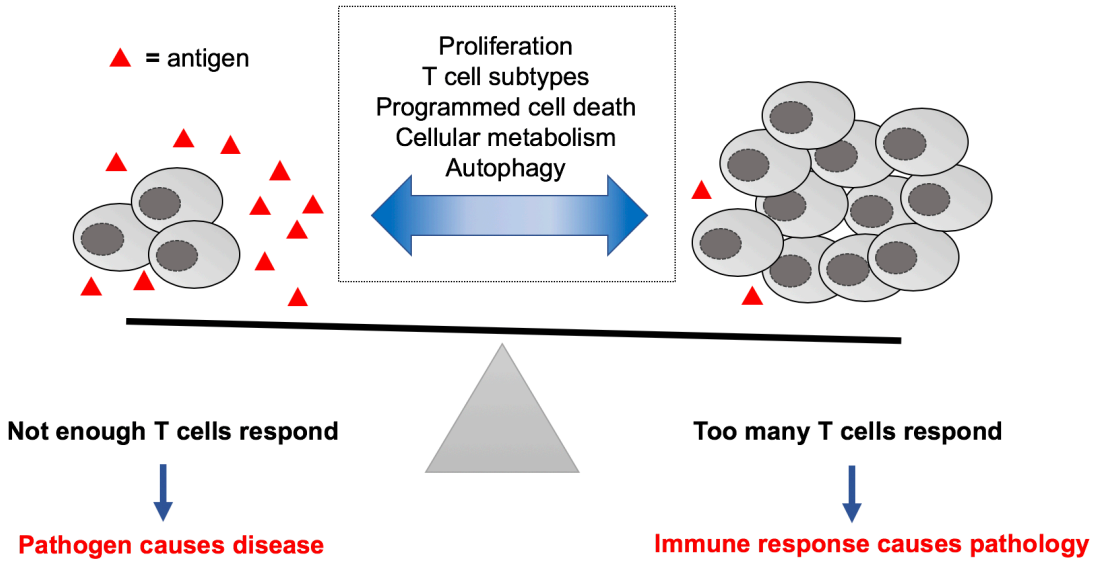


Figure 1. The balance that maintains a healthy T cell response.
The number of T cells responding to a pathogen-derived antigen can determine the outcome of an infection. This balance is regulated by many factors including the rate of T cell proliferation, the presence and actions of specific T cell subtypes, programmed cell death, cellular metabolism, and autophagy.

T cell homeostasis is closely related to the number of T cells responding to a pathogen-derived antigen during the course of the adaptive immune response (Figure 1). If T cell numbers are insufficient, the pathogen will not be cleared, leading to disease. On the other hand, if T cell numbers are excessive, the immune response itself may damage the host. *In vivo* studies of *Listeria monocytogenes* and lymphocytic choriomeningitis virus (LCMV) infections in mice elegantly demonstrated that T cells are pre-programmed for self-regulation (13). In these models of infection, the extent of T cell expansion depends on the initial infection dose or antigen as well as the rate of pathogen clearance. However, the kinetics of T cell contraction after infection was unrelated to the magnitude of expansion or antigen dose, suggesting that the contraction phase of a T cell response is programmed early after infection to ensure homeostasis. A failure of self-regulation in

the T cell response can lead to immunopathology, but mechanisms of self-regulation in different types of T cells can differ considerably. Molecular therapeutic strategies to restore balance therefore require a mechanistic understanding of self-regulation among different T cell subsets.

T CELL SUBSETS

Our immune systems are challenged daily with invading microbes, and yet also need to be properly educated to recognize friend from foe. Antigens that are components of pathogens, opportunistic pathogens, commensal microbes, and "self" must be distinguished and acted upon accordingly. To accomplish this, T cells require the flexibility to react to unique challenges appropriately, and therefore have evolved multiple cell lineages with distinct abilities to regulate immune responses.

Human T cells consist of many subtypes or subsets, the majority of which are TCRαβ-bearing conventional T cells (Tcons). Tcons include CD4 "helper" T cells and CD8 "cytotoxic" T cells. Tcons exit the thymus as naïve T cells. Upon activation by APCs in secondary lymphoid organs, they undergo clonal expansion and gain unique effector functions. Differentiation into disparate T cell lineages is multi-factorial, but involves cytokine signals during naïve T cell activation. The cytokine milieu during activation drives distinct transcriptional programs and epigenetic regulation in the T cells that drive their lineage commitments (174; 236).

Naïve CD8 T cells are typically activated in response to intracellular pathogens including viruses and some bacteria. Upon rapid clonal proliferation, the resulting effector pool of cytotoxic T cells (CTLs) kills infected cells with granzymes and perforin and secrete cytokines such as interferon-γ (IFN-γ) and tumor necrosis factor (TNF) to

amplify host defenses. In addition to infected host cells, CTLs are able to recognize tumor antigens to eliminate cancerous cells. CTLs engage the cognate peptide-MHC I complex on the surface of the infected cells for killing. CTL effector cells are further classified into two subsets: short-lived effector cells (SLECs) and memory precursor effector cells (MPECs). Although SLECs and MPECs have similar cytotoxicity profiles (227), the majority of SLECs die after pathogen clearance, whereas MPECs survive to form a pool of long-lived memory cells (41).

CD4 T cells are also referred to as T helper (Th) cells because of their ability to coordinate immune responses, including antibody production by B cells mediated primarily by T follicular helper (Tfh) cells. Some CD4 populations exit the thymus as a pre-determined lineage, such as natural regulatory T (nTreg) cells (235). Others such as conventional naïve CD4 T cells begin as a Th0 precursor. Upon activation, Th0 cells further differentiate into Th1, Th2, Th17, or induced regulatory T (iTreg) cells among others. Th1 cells are best suited for fighting intracellular pathogens and Th2 cells are better suited for extracellular pathogens (and helminths in particular) (136). Th17 cells are efficient for defense against extracellular bacteria and fungi, although if dysregulated can contribute to inflammatory diseases (191). The importance of activating the correct subsets of CD4 T cells to control infections is best exemplified by results showing that pathogens such as *Yersinia pseudotuberculosis* and *Schistosoma mansoni* have evolved to directly influence Th differentiation processes (135). Additionally, perturbations in the balance of different Th subsets is associated with disorders of the immune system. For example, dysregulated Th2 cytokines underlie the pathogenesis of asthma (59), and Th17

cells that produce both IL-17 and IFN-γ drive autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, and inflammatory bowel disease (190).

Regulatory T cells (Tregs)

A special subset of CD4 T cell is the regulatory T cell (Treg). Tregs were first described as "suppressor T cells", characterized for their unique ability to suppress the proliferation of neighboring effector T cells. It is now appreciated that Tregs have many important functions including regulation of lymphocyte homeostasis and prevention of autoimmunity (111; 157). Tregs are divided into two major types: natural Tregs (nTregs) and induced Tregs (iTregs), depending on whether they exited thymic development as a Treg or were induced to become a Treg in the periphery (79). As such, iTregs are also referred to as peripheral Tregs (pTregs). Typically, pTreg induction requires naïve CD4 T cells to be exposed to IL-2 and transforming growth factor β (TGF-β) (22; 158). All Treg types express the transcription factor forkhead box P3 (FOXP3), which will be discussed in detail later in this section.

In the thymus, both affinity-based and avidity-based models have been proposed for determining whether T cells are deleted or diverted into the Treg repertoire (92). In the affinity-based model, T cells with the highest affinity (strength of the interaction between the TCR and its peptide-MHC ligand) are removed by clonal deletion, and T cells with intermediate affinity are destined for the Treg lineage. T cells with lower affinity join the repertoire as Tcons. In the avidity-based model of development, affinity interactions are implicated, but the density of the peptide-MHC ligand is also important (92). An increased presence of antigen presented on thymic APCs favors clonal deletion, whereas a decreased density favors Treg differentiation. In addition to these models, Yen

et al demonstrated that developing Tregs display asymmetric cell division with unequal Foxp3 distribution in daughter T cells, which could be considered a stochastic model of Treg development (225).

Tregs are considered key mediators of immune homeostasis, mainly performed by their ability to suppress the activation and function of other T cells. Suppression can occur by multiple mechanisms such as release of suppressive cytokines, consumption of IL-2, and cytolysis (181). Suppressive molecules are plentiful and can either be cell-associated such as co-inhibitory receptors or secreted factors such as inhibitory cytokines. Inhibitory cytokines released by Tregs include IL-10, IL-35 and TGF-β (207). These cytokines typically exert their suppressive effects on nearby effector Tcons. Secreted factors other than cytokines also inhibit nearby effector T cells: galectin-1 induces cell cycle arrest and apoptosis, and adenosine mono phosphate (AMP) suppresses proliferation of target cells. Co-inhibitory receptors include lymphocyte activation gene-3 (LAG-3) and cytotoxic T lymphocyte antigen-4 (CTLA-4), which disrupt dendritic cell (DC) function upon cell to cell interactions (207); ultimately, the antigen presenting cell is maintained in an immature state leading to incomplete T cell activation. Consumption of IL-2 and other cytokines is another major suppressive feature of Tregs. Unlike Tcons, Tregs do not synthesize their own IL-2 to promote survival and expansion upon activation, although they depend on IL-2 survival (146). Instead, Tregs compete for IL-2 produced by activated effector T cells. Given their high expression level of the high-affinity IL-2 receptor α (CD25), Tregs are optimally suited for competing for IL-2 in the microenvironment (108). The competitive nature of IL-2 consumption limits the nearby effector cell clonal expansion and also sensitizes this cell type to cytokine withdrawal-

induced death (CWID), which will be discussed in more detail in the 'programmed cell death' section. Finally, Tregs have cytotoxic functions similar to CD8 CTLs, consisting of direct target cell lysis with granzyme B and perforin (36).

Tregs in clinical therapy

The Treg population is critical for maintaining peripheral tolerance and has therefore been studied extensively for possible use as immune therapies against tumors, autoimmune diseases, and transplant rejection (3; 69; 211). Efforts are underway not only to boost Treg function in cases of autoimmune diseases, but also to deplete Tregs to boost antitumor responses (52; 57; 143). In the case of autoimmune diseases, typically a loss of Treg numbers and/or functionality correlates with severity of disease. Therefore, clinical efforts are aimed at restoring Treg populations in patients either by infusing new Tregs into the patient or boosting what Tregs remain. For example, adoptive therapy of infused Tregs has been tested in human clinical trials to help prevent graft versus host disease (GVHD) in transplant recipients (147). In this adoptive therapy approach, cord blood was used to derive Tregs *in vitro* for administration to the transplant recipients. Safety and preliminary effectiveness was demonstrated, and rates of GVHD in Treg-infused patients did improve compared to controls (30). However, none of the infused Tregs were detected in the blood after 14 days, which could in part suggest some of the infused T cells died by apoptosis.

This study also underscored the complexity of generating and expanding iTregs *ex vivo*, as there is no 'gold standard' protocol for induction of human Tregs. Since then, a landmark study reported deep molecular profiling of multiple human iTreg differentiation protocols, thus providing a better understanding of the molecular

mechanisms underlying iTreg generation (175). Another method of generating Tregs *ex vivo* involves gene transfer of the transcription factor that is absolutely required for Treg development, forkheadbox P3 (FOXP3). Typically CD4 T cells are retrovirally transduced to express FOXP3 and are then activated with or without other factors to drive Treg differentiation such as TGF-β and IL-2 (89); the outcomes have been somewhat controversial. Generally, FOXP3 expression alone is not sufficient to convert CD4 Tcons into functional Tregs unless FOXP3 expression is unreasonably high (1). However, Passerini *et al* successfully converted naïve CD4 T cells from Treg-deficient patients into functional Tregs with potent suppressive function using lentiviral-mediated FOXP3 gene transfer (148; 149). Unfortunately, the Tregs were not stable upon exposure to inflammatory conditions in a model of GVHD. Despite the challenges and need for improvement in the generation of stable and functionally suppressive Tregs for clinical therapy, it is certainly an exciting time for the field which holds a lot of therapeutic potential. Improvements in Treg stability will likely rely on an improved understanding of FOXP3 and mechanisms of survival in these cells.

Forkhead box P3 (FOXP3)

Null or detrimental mutations of FOXP3 in both mice and humans leads to fatal autoimmune disease. The history of the recognition of this autoimmune disease begins in 1949, when a spontaneous mutation arose in a mouse colony at the Oak Ridge National Laboratory and the mouse strain was named 'scurfy' (167). Scurfy mice had ruffled skin, enlarged spleen and lymph nodes, and died around 3 weeks of age. It was not until the 1990's that the mutation responsible was identified in an X-linked gene that encodes Foxp3. DNA sequencing revealed a 2-bp insertion in the Foxp3 gene resulting in a

truncated Foxp3 protein (167). Studies of the immune phenotype in scurfy mice were instrumental for linking the human FOXP3 gene to a familial X-linked autoimmune disease of undetermined origin, known as immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX). Sequencing of the FOXP3 gene from IPEX patients revealed deleterious mutations consistent with observations from scurfy mice.

IPEX disease in humans almost always involves autoimmune enteropathy, in which the immune system attacks the intestines causing severe diarrhea early in life. This contributes to a failure to thrive. The majority of IPEX patients also test positive for a variety of autoantibodies (12). In addition, dermatitis of the skin, including but not limited to severe eczema often occurs. Patients may also experience endocrine disorders such as Type 1 diabetes mellitus caused by autoimmune reactions of the pancreas. If left untreated, IPEX disease is fatal in humans and therefore requires hematopoietic stem cell transplantation (HSCT) and/or immunosuppression early in life. In a recent retrospective study of 96 IPEX patients from around the world, the overall survival rate after HSCT was 73.2% and after immunosuppression was 65.1% (18). Although IPEX disease is always associated with mutations in FOXP3, at least 70 distinct mutations have been identified, and sometimes identical mutations can yield completely different disease phenotypes in different individuals (12). Hence, functional studies of FOXP3 biology with respect to these mutations are of interest.

Human Tregs are identified by high surface marker expression of the high-affinity IL-2 receptor α (CD25), and low expression of the low-affinity IL-7 receptor (CD127) when compared to other CD4 T cells (178). Although these cell surface markers facilitate fluorescence activated cell sorting (FACS) of Tregs, the most important identifiable

marker of the Treg cell type is expression of the intracellular transcription factor FOXP3. Not only is FOXP3 expression required for Treg development (88), but maintenance of high FOXP3 expression distinguishes functional from non-functional Tregs. Epigenetic regulation of the *FOXP3* locus is necessary to maintain high levels of FOXP3 expression in Tregs; specifically, demethylation of the Treg-specific demethylation region (TSDR) upstream of *FOXP3* (159). This is mediated in part through the action of methyl-CpG-binding domain protein 2 (Md2), which helps recruit histone-modifying and chromatin-remodeling complexes to the TSDR site (210). Interestingly, cytokines may influence the epigenetic architecture of the TSDR; IL-6 mediates acetylation changes that result in less FOXP3 transcription, whereas transforming growth factor β (TGF-β) signaling leads to stabilization of FOXP3 expression (113).

FOXP3 was first described as a repressor of gene transcription, although recent work has described detailed scenarios that allow FOXP3 to act either as a repressor or an activator of gene targets (98). FOXP3 mediates DNA binding via its forkhead domain, although the FOXP3 forkhead domain is thought to have a low affinity for DNA binding sites (169). Therefore, much of FOXP3 transcriptional activity is actually mediated through FOXP3 heterodimers with other transcription factors in larger complexes. Among sequence-specific binding partners are nuclear factor of activated T cells (NFAT), FOXP1, Runx1, and GATA3 (122; 221). Additionally, FOXP3 complexes with histone acetyl transferases (HATs) and histone deacetylases (HDACs) (104). The most well described example is Tat-interacting protein 60kDa (TIP60), which functions as a HAT; upon disruption of FOXP3-TIP60 binding interactions, Tregs lose their ability to

suppress target cells which was demonstrated in IPEX patient-derived Tregs with the most common FOXP3 mutation (p.A384T) (23).

FOXP3 expression in other cell types

The human gene for FOXP3 is located on the X chromosome, highlighting the predominately male IPEX syndrome inheritance pattern. In Tregs, FOXP3 is expressed after TCR and co-stimulatory signaling occurs, at which time the FOXP3 promoter is activated by transcription factors such as NFAT and AP-1 (113). Although FOXP3 has been studied extensively in Tregs, FOXP3 is also expressed transiently in human CD4 and CD8 Tcons after TCR stimulation (62; 237). The purpose of transient FOXP3 expression in Tcons is still largely unknown. A study published in 2012 demonstrated that FOXP3-deficient Tcons proliferated more and produced higher cytokine levels (127). 274 genes were differentially expressed in the presence or absence of FOXP3. FOXP3 was implicated as an intrinsic regulator of T cell activation and proliferation after TCR activation, but there are most likely other roles of FOXP3 in Tcons that have gone unnoticed or unappreciated. FOXP3 expression was also reported in invariant natural killer T (iNKT) cells after exposure to TGF-β, which was associated with regulatory functions that may contribute to immune tolerance (132). A follow-up study observed that rapamycin treatment, an inhibitior of the mechanistic target of rapamycin (mTOR), was required to induce suppressive function, even if TGF-β alone was sufficient to induce FOXP3 expression (134). In conclusion, FOXP3 expression is not restricted to Tregs and likely mediates immune homeostasis in other immune cell subsets. Chapter 3 presents a novel connection between FOXP3 expression in human Tcons and regulation of a programmed cell death pathway needed for immune homeostasis.

PROGRAMMED CELL DEATH

The adaptive immune system contains multiple mechanisms that provide additional layers of immune regulation , ensuring the effective elimination of pathogens while maintaining immune homeostasis. Although Tregs are instrumental in maintaining T cell homeostasis, they embody only one layer of immune regulation in place that ensures effector T cells do not over-accumulate and cause damage to the host. Additional protection from altered immune homeostasis is provided by pre-programmed cell death mechanisms in effector T cells that serve to keep T cell numbers in check (192). Programmed cell death keeps effector T cell numbers in balance, but specific types of cell death can mediate entirely disparate downstream effects on the systemic immune response, and therefore must be considered.

Necroptosis is a form of cell death that may be programmed or accidental, characterized by swelling of organelles followed by cell lysis and a complete collapse of the plasma membrane. The release of cellular contents creates an inflammatory environment due to danger-associated molecular patterns (DAMPs) that activate Toll-like receptors (TLRs) on surrounding cells (163). Pyroptosis is a form of programmed necrosis that occurs following activation of the inflammasome, a multimeric signaling platform that involves members of the nucleotide-binding oligomerization domain (Nod), leucine-rich repeat-containing receptors (NLRs), and pyrin and HIN domain (PYHIN) proteins (56). Cells that undergo pyroptosis swell and rupture due to pores in the plasma membrane; this leads to a strong inflammatory response not only due to release of DAMPs, but also release of pro-inflammatory cytokines IL-1β and IL-18 (46). By contrast, apoptosis is a programmed cell death pathway that generally does not trigger

inflammation and is the primary mechanism of cell death throughout tissue systems in homeostasis and during development. Apoptotic cell death does not involve breakdown or rupture of the plasma membrane; instead, DNA is fragmented and neatly stored into packages with other cytoplasmic contents into 'blebs'. Phagocytes then rapidly recognize the resulting apoptotic bodies and engulf them, avoiding an inflammatory response altogether (105).

Apoptosis

Apoptosis is perhaps the most important type of programmed cell death for T cell homeostasis as it is an essential process for reducing the number of excess T cells reacting to either foreign or self-antigens (188; 230). During a non-pathological T cell response, the majority of clonally expanded effector T cells succumb to apoptosis, leaving only a small number of cells to become memory T cells. The trigger of apoptosis can be 'intrinsic' or 'extrinsic', but is mediated by executioner caspases in either modality. Caspases are constitutively expressed in the cytoplasm of cells as zymogens, inactive precursors of an enzyme. Once proteolytically cleaved, they become activated and carry out protein cleavages at aspartic acid residues on a multitude of cellular proteins leading to apoptosis. Caspases 2, 8, 9, and 10 are considered initiator caspases whereas caspases 3, 6, and 7 are the executioner caspases that ultimately mediate cell death. Whether apoptosis is triggered extrinsically or intrinsically, the pathways converge at the executioner caspases. Although caspases are a hallmark of apoptotic cell death, it is important to note that caspases may also be engaged to execute cell death in the context of programmed necrosis (163). Furthermore, caspase activity does not always trigger cell death. In fact, caspases are also important for T cell activation (141).

Extrinsic pathways of apoptosis are triggered by cell death receptors of the tumor necrosis factor receptor (TNFR) superfamily at the cell surface. Perhaps the best characterized is the Fas receptor (CD95), which is enriched in lipid rafts and binds to Fas ligand (FasL/CD95L) (139). Upon binding of Fas, cytoplasmic tails of CD95 trimers mediate assembly of death-inducing signaling complexes (DISCs). A critical component of the DISC is caspase 8 (and caspase 10 in humans), which becomes recruited to the ligand-receptor complex as a procaspase through death effector domain (DED) interactions. Recruitment of caspase 8 to DISCs is competitively determined by a procaspase 8 mimic, cellular FLICE-inhibitory protein (c-FLIP); c-FLIP may also be recruited to the DISC via its' own DED, preventing procaspase 8 from forming homodimers. Upon sufficient recruitment of procaspase 8, autoproteolytic cleavage allows for activated caspase 8 to leave the complex. Caspase 8 activity can activate executioner caspases directly, or it can cleave Bid, which acts by tipping the balance between pro- and anti-apoptotic proteins at the mitochondrial membrane interface (188).

The importance of extrinsically mediated apoptosis to T cell homeostasis is exemplified by the *lpr/lpr* mouse strain, or autoimmune lymphoproliferative syndrome (ALPS) in humans (193). Lpr mutant mice are defective in Fas protein expression and exhibit a lymphoproliferative syndrome similar to the autoimmune disease lupus erythematosus in humans. Although mature CD4 and CD8 T cells from Lpr mice underwent normal activation and cytokine production, they are defective in apoptosis upon antigen challenge (171). ALPS is more complex genetically, involving loss of function mutations in either FAS, FAS ligand, caspase 10, or caspase 8 (193). Interestingly, Th2 cells are less sensitive to Fas-mediated death than Th1 cells (228),

underscoring how different T cell subsets are often subject to different mechanisms of self-regulation. These subset-specific differences warrant further investigation.

Intrinsic apoptosis is centered on the mitochondrial outer membrane potential (MOMP) and is also referred to as the mitochondrial apoptosis pathway. The MOMP is controlled by a complex balance between pro and anti-apoptotic B cell lymphoma 2 (Bcl-2) family members that share a Bcl-2 homology (BH) motif (either BH1, BH2, BH3, or BH4 motif) (49). BH3 motifs mediate protein-protein interactions, allowing for different family members to directly compete with each other for binding at the mitochondrial membrane. During stressful periods or in response to apoptotic signaling, anti-apoptotic proteins such as Bcl-2, BclxL, and Mcl-1 are overtaken by pro-apoptotic BH3 members Bid and Bim. The activation of BH3-only proteins neutralizes anti-apoptotic family members, but also directly activates Bax and Bak. Normally, Bax and Bak are sequestered away from the mitochondria by anti-apoptotic Bcl-2 proteins, but with a loss of anti-apoptotic Bcl-2 members they become free to oligomerize in the mitochondrial membrane and form pores (121). Upon pore formation, depolarization of the mitochondria releases cytochrome c, apoptosis inducing factor (AIF) and endonuclease G into the cytosol. Procaspase 9, cytochrome c and APAF-1 together form what is called the apoptosome. Activated caspase 9 converges with other apoptotic pathways by the activation of executioner caspases. Ultimately, executioner caspases complete death of the cell by complete proteolytic breakdown of the cytoskeleton and nuclear lamins, and by triggering chromatin condensation.

T cells are specifically programmed to die by apoptosis in multiple situations throughout a beneficial T cell response. First, restimulation-induced cell death (RICD) is

an apoptotic pathway that is executed when previously activated T cells are restimulated with antigen through the TCR in the presence of IL-2 (188). RICD is believed to enforce a limit on the maximum extent of effector T cell expansion. Additionally, the contraction of the specific effector T cells is necessary to make room for the next set of T cells to respond to future infections; the majority of apoptosis during the contraction phase is mediated by CWID. CWID is an intrinsic apoptosis mechanism that becomes activated when IL-2 levels decline following pathogen clearance. It is mediated in part through the action of Tregs, but also occurs naturally when levels of antigen in the microenvironment decline. IL-2 signaling through Ras and ERK activation promotes Bim degradation. Therefore a loss of IL-2 results in Bim accumulation, triggering intrinsic apoptosis (188). Although CWID and RICD both contribute to regulating the T cell response, this dissertation is focused on RICD.

Restimulation-induced cell death (RICD)

RICD, also referred to as activation induced cell death (AICD) was first observed in 1987 when Ashwell *et al* studied cytokine production by T cell hybridomas after TCR stimulation (11). When antigen was added to a mixture of T cells with different antigen specificities, the T cells specific for that antigen died whereas the bystander T cells were unaffected. However, these experiments were conducted with immortalized cells with limitations for understanding accurate RICD conditions necessary in primary T cells. Upon closer examination of cell death kinetics in primary human T cells after TCR restimulation, hallmarks of apoptotic cell death such as cell shrinkage and DNA fragmentation was detected in under 3 hours and 3-5 hours, respectively (215). Lenardo *et al* recognized that prior exposure to IL-2 predisposes T cells to apoptosis; 82% of CD4

T cells died after antigen stimulation if they were pre-treated with IL-2 (102). IL-2 exposure was subsequently shown to induce progression of the cell cycle, and activated T cells that are cycling through S-phase of the cell cycle were found to be susceptible to death by RICD (impacted upon by growth cytokines such as IL-2 and IL-4) (24). Fas-mediated death signaling has been implicated in RICD. Alderson *et al* suggested that FasL may serve to limit the expansion of antigenic-specific T cells by AICD (4). Indeed, restimulation of the TCR induces FasL in an IL-2 dependent manner, thereby enhancing DISC assembly. We now understand that extrinsic apoptotic signals from Fas and intrinsic apoptotic signals mediated by Bim converge to optimize RICD (186; 188). Additionally, the NUR77 and NOR1 orphan nuclear receptors are induced upon TCR restimulation, which also contribute to the intrinsic apoptosis pathway of RICD (43; 170).

RICD sensitivity is also in part dictated by the threshold of the TCR signal in the 'threshold model' of RICD. Specifically, the CD3-associated ζ chains need to be fully activated at their immunoreceptor tyrosine-based activation motifs (ITAMs) (45). ITAMs on the CD3ζ chains are phosphorylated by Lck, creating a recruitment site for the ZAP-70 tyrosine kinase. A critical threshold is the number of ZAP-70 molecules that are recruited to phosphorylated ITAMs. The TCR signal is also amplified by phosphorylation on CD3 ε and γ chains; however, there are also costimulatory and inhibitory molecules that can play a role in tuning TCR signal strength. Furthermore, the phosphorylation status of ITAMs is also controlled by the action of phosphatases. Before ITAMs are phosphorylated, they are pre-bound to inhibitory phosphatases such as SHP-1. Only after displacement of phosphatases can ITAMs receive phosphorylation.

SLAM-associated protein (SAP)

We and others learned that RICD is responsible for controlling the magnitude of the T cell response through close study of primary immunodeficiency patients. X-linked lymphoproliferative disease-1 (XLP) is a rare immunodeficiency caused by loss-of function mutations in *SH2D1A*, which encodes for a small adaptor signaling molecule called signaling lymphocyte activation molecule (SLAM)-associated protein (SAP) (197).

The immune phenotype of XLP patients involves impaired humoral responses due to poor T cell help for B cells, defective CTL cytotoxicity, and poor development of NKT cells. The lymphoproliferative disease manifests primarily in CD8 T cells upon infection. Typically, infection with Epstein-Barr virus (EBV) creates the perfect storm of impaired adhesion of CTLs to infected B cell targets (229) combined with uncontrolled CD8 T cell proliferation. The loss of immune homeostasis during an EBV infection is often fatal in XLP patients due to massive tissue infiltration of CTLs and tissue necrosis in multiple organs (16). However, further study has revealed other immune dysregulation problems independent of EBV infection (145). Treatment for XLP typically requires HSCT, but there is some debate about whether to wait in asymptomatic patients or to perform HSCT pro-actively (196). Thus, alternative treatments for XLP patients are needed. Ultimately, the lymphoproliferative disorder in XLP patients was linked to a specific defect in RICD (185). Therefore, the lymphoproliferative defects demonstrated by patient T cells necessitated further investigation into the molecular mechanisms of SAP in RICD.

SAP is Src homology 2 (SH2)-adaptor protein that functions primarily to propagate TCR signal strength. First, SAP binds to the SLAM family receptor NK, T and

B cell antigen (NTB-A/SLAMF6) to displace the inhibitory phosphatase SHP-1. Displacement of SHP-1 facilitates recruitment and activation of Lck leading to increased signal strength (84). In murine SAP-deficient CTLs, poor killing of B cell targets was observed; loss of SAP lead to increased SHP-1 recruitment to the immunological synapse, and subsequently less Src kinase activation (229). This observation suggests that presence of SAP mediates not only signaling thresholds via SHP-1 displacement, but also regulates synapse organization. Second, SAP inhibits diacylglyercol kinase alpha (DGKα) (14), which normally negatively regulates TCR signaling by decreasing diacylglycerol (DAG) levels. Excitingly, pharmacological inhibition of DGKα in SAP-deficient T cells was able to restore RICD by increasing DAG levels (170). Inhibition of DGKα also protected SAP-deficient mice from excessive CTL expansion during viral infection, representing an example of successful pharmacological intervention to rescue life-threatening immunopathology by manipulating RICD sensitivity.

CELLULAR METABOLISM AND AUTOPHAGY INFLUENCE APOPTOSIS SENSITIVITY

Recently, there has been a robust interest in cellular metabolism of T cell subsets in immunology. Metabolic reprogramming (the cellular switch from mainly oxidative phosphorylation to generate ATP to a more glycolytic program) after T cell activation is required for clonal expansion (82) as well as differentiation (34). Th1, Th2 and Th17 cells are associated with high rates of glycolysis, whereas Tregs generally have low levels of glycolysis and higher rates of lipid oxidation for ATP (130). In addition, memory T cells maintain distinct metabolic states than activated effector T cells to carry out rapid recall responses (33; 117).

Importantly, metabolic reprogramming demonstrated during T cell responses are linked to changes in apoptosis sensitivity. For example, glycolytic metabolism in activated CD8 T cells was linked to enhanced RICD sensitivity (99). Alongside cellular metabolism, macroautophagy (hereby referred to as autophagy) is intimately associated with T cell death sensitivity mainly through its role in mitochondrial health (26; 120; 126; 168). These concepts are reviewed in detail in Chapter 4 (208). Intriguingly, FOXP3 is also linked to changes in cellular metabolism (9). Therefore, studies of RICD and/or FOXP3 mechanisms must consider metabolic programming and autophagy as potential contributors to any observations made.

SUMMARY

The presence of unique T cell subsets allows for flexibility of different types of T cell responses in response to the massive assortment of antigen challenges encountered by the immune system. Whereas Tcons have exceptional proliferative capacity and make suitable responders for acute pathogen clearance, FOXP3+ Tregs serve to protect the boundaries of "self" versus "non-self" and suppress effector T cells as needed to retain balance. The homeostasis of immune cell compartments is intricately controlled by the action of transcription factors, cellular metabolism, and pre-programmed death pathways such as RICD.

FOXP3+ Tregs require IL-2 and experience frequent TCR stimulation, but are highly resistant to RICD (60). FOXP3 is required for RICD resistance in Tregs because a loss in FOXP3 expression leads to increased death sensitivity (214). However, the specific mechanism of FOXP3-mediated resistance in Tregs was unclear. Therefore, our first goal was to elucidate the mechanism of FOXP3-mediated resistance in Tregs, which

is explored in Chapter 2. Furthermore, Tcons are resistant to RICD during activation-induced expansion when FOXP3 is expressed (Figure 2), but the purpose of transient FOXP3 expression in human Tcons remains generally mysterious. We therefore explored whether FOXP3 expression in human CD4 and CD8 Tcons could have an additional role in protecting cells from premature RICD during expansion; these results are discussed in Chapter 3.

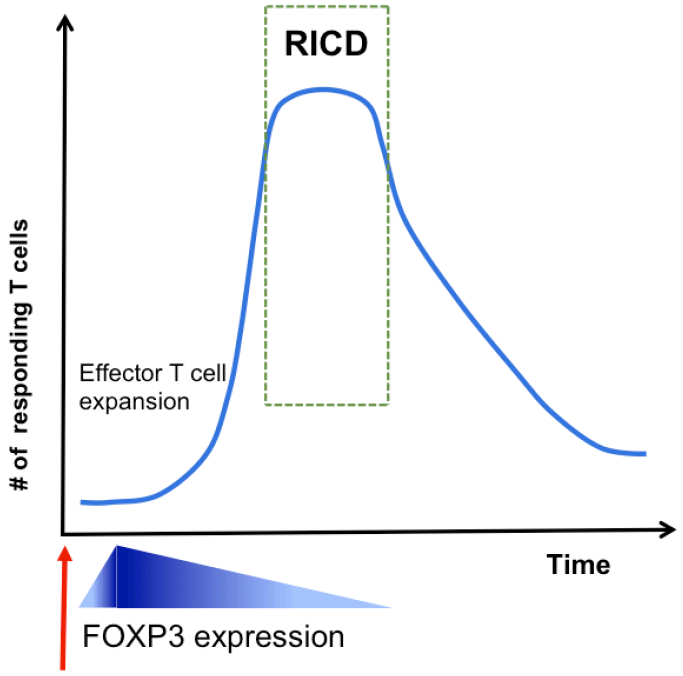


Figure 2. FOXP3 expression in Tcons inversely correlates with RICD sensitivity. In human Tcons, FOXP3 is transiently expressed upon TCR activation (red arrow). After activation, extensive clonal proliferation takes place during which cells are resistant to RICD. As FOXP3 expression wanes, T cells become sensitized to die by RICD.

Hypothesis

The overall hypothesis of this dissertation is that FOXP3 protects Tregs and Tcons from RICD by regulating SAP expression.

Importance

Understanding the molecular determinants of RICD sensitivity during the immune response in different T cell subsets can inform new therapeutic approaches for limiting immunopathology from over-reactive and/or self-reactive T cells. Conversely, therapies that boost T cell responses by protecting cells from RICD could be employed to enhance vaccine efficacy. Although Tregs have unique immunosuppressive capabilities and have been highly studied for clinical uses in recent years, the survival processes that maintain the Treg population remain unclear (180). It is therefore critical that we investigate the regulation of human Treg homeostasis and survival, which includes a mechanistic understanding of apoptosis sensitivity in these cells. Application of this knowledge could enhance the therapeutic potential of these cells for translational therapy in a variety of disease scenarios, including autoimmune disorders, cancer immunotherapy, and transplant rejection.

Using primary human T cells from healthy donors, we have uncovered mechanisms of FOXP3-mediated resistance to RICD in Tregs (Chapter 2) and Tcons (Chapter 3). Furthermore, we consider how cellular metabolism and autophagy contribute to RICD sensitivity (Chapters 3+4) and ultimately shape the T cell response (Chapter 4).

CHAPTER 2: FOXP3 renders activated human regulatory T cells resistant to restimulation-induced cell death by suppressing SAP expression

Published as: Gil Katz*, Kelsey Voss*, Toria F. Yan, Yong Chan Kim, Robert L. Kortum, David W. Scott, and Andrew L. Snow. FOXP3 renders activated human regulatory T cells resistant to restimulation-induced cell death by suppressing SAP expression. *Cellular Immunology* 2018; 327: 54-61

*co-first author

Gil Katz conceptualized the project and formulated the hypothesis. Dr. Katz also conducted preliminary experiments that show low SAP expression and RICD sensitivity in human Tregs. Dr. Katz and Toria F. Yan cloned of the luciferase reporter for the SAP promoter. Yong Chan Kim assisted with the sorting strategy for isolating human Tregs and conducted multiple sorts. Robert L. Kortum assisted in lentivirus preparations. Kelsey Voss repeated preliminary RICD experiments, conducted siRNA knockdown experiments in human Tregs, lentiviral transduction experiments, and chromatin immunoprecipitations. Kelsey Voss wrote the paper and Andrew L. Snow supervised the project and provided edits for the paper.

ABSTRACT

Restimulation-induced cell death (RICD) is an apoptotic program that regulates effector T cell expansion, triggered by repeated stimulation through the T cell receptor (TCR) in the presence of interleukin-2 (IL-2). Although CD4+ regulatory T cells (Tregs) consume IL-2 and experience frequent TCR stimulation, they are highly resistant to RICD. Resistance in Tregs is dependent on the forkhead box P3 (FOXP3) transcription factor, although the mechanism remains unclear. T cells from patients with X-linked lymphoproliferative disease (XLP-1), that lack the adaptor molecule SLAM-associated protein (SAP), are also resistant to RICD. Here we demonstrate that normal Tregs express very low levels of SAP compared to conventional T cells. FOXP3 reduces SAP expression by directly binding to and repressing the SH2D1A (SAP) promoter. Indeed, ectopic SAP expression restores RICD sensitivity in human FOXP3+ Tregs. Our findings illuminate the mechanism behind FOXP3-mediated RICD resistance in Tregs, providing new insight into their long-term persistence.

INTRODUCTION

An efficient adaptive immune response relies on the rapid clonal expansion of effector T cells, followed by the timely disposal of these cells to avoid nonspecific damage to host tissues. The adaptive immune system utilizes specific apoptosis programs to regulate T cell expansion and contraction and maintain homeostasis (192). One of these programs is restimulation-induced cell death (RICD). RICD helps to keep effector T cell expansion in check, triggered by T cell receptor (TCR) restimulation in the presence of interleukin-2 (IL-2) (25; 66; 102; 188). Recent work from our lab has demonstrated that RICD sensitivity can be tuned by alterations in cell metabolism or

diacylglycerol kinase (DGK) activity (99; 170). A better understanding of the underlying mechanisms that influence RICD can therefore lead to novel strategies for controlling the magnitude and potency of a T cell response. However, our knowledge of the molecular signals that govern RICD sensitivity remains inadequate, especially with regard to different T cell populations with variable longevity.

The physiological relevance of RICD is best illustrated in patients with X-linked lymphoproliferative disease (XLP-1), who experience life-threatening, uncontrolled accumulation of $CD8^+$ T cells in response to Epstein-Barr virus (EBV) infection (197). A marked defect in RICD contributes to unconstrained $CD8^+$ T cell expansion in this setting, resulting in severe immunopathology (185). XLP-1 patients harbor null mutations in the signaling adaptor SLAM-associated protein (SAP), which is required for RICD (42; 185). Indeed, work from our lab and others has shown that SAP associates with the signaling lymphocyte activation molecule (SLAM) family receptor NTB-A to potentiate TCR signal strength by displacing SHP-1 and recruiting LCK instead of FYN, which facilitates downstream signaling for apoptosis after TCR re-engagement (72; 84; 229). SAP also represses DGKα to maintain a sufficient pool of diacylglycerol for robust TCR signaling (14; 170), further highlighting SAP as a central player in promoting RICD of effector T cells.

CD4+ regulatory T cells (Tregs) are a specialized subset of T cells that modulate the immune response and suppress the proliferation of lymphocyte effectors. Given their essential role in maintaining immune homeostasis and preventing autoimmunity (157), Tregs are of special interest regarding their therapeutic potential for tolerance induction in a variety of clinical settings (155; 224). However, the processes that ultimately maintain Treg cell survival remain unclear (180). For example, Tregs are extremely

resistant to RICD despite frequent stimulation (often via self-antigen), susceptibility to FAS-induced death, and strict dependency on IL-2 derived from effector T cells (60; 214).

The gene encoding SAP, SH2D1A, was identified as a FOXP3 target in a genome-wide chromatin immunoprecipitation (chIP) study of human Tregs (173). We therefore hypothesized that in human Tregs, FOXP3 restricts RICD by directly suppressing SAP expression. Using primary, activated human Tregs and conventional CD4+ T cells (Tcons) derived from healthy human donors, we confirmed that RICD resistance in Tregs is FOXP3 dependent (214). We now demonstrate that SAP expression is markedly reduced in activated Tregs, and that FOXP3 confers resistance to RICD through repression of the SH2D1A promoter. These findings further elucidate the mechanism of RICD resistance in Tregs, providing new insights into Treg homeostasis.

MATERIALS AND METHODS

Cell isolations and culture conditions

Peripheral blood mononuclear cells (PBMC) were obtained from buffy coats donated by healthy human donors at the National Institutes of Health (NIH) Blood Bank. Access to Blood Bank donors was kindly provided by Dr. Michael Lenardo. $CD4^+$ T cells were purified from PBMC by immunomagnetic negative selection using the EasySep Human CD4+ T cell enrichment kit (Stem Cell Technologies). Cells were then stained on ice for 30 minutes with the following Abs: anti CD4-FITC (clone RPA-T4), anti-CD25-PE-Cy7 (clone BC96), and anti-CD127-PE (clone A019D5) (Tonbo Biosciences). Tregs

and Tcons were sorted on a BD FACSAria cell sorter. The gating strategy is shown in Figure 3, where Tregs were defined as CD4+ CD25hi CD127lo and Tcons were defined as CD4+ CD25lo CD127hi (178).

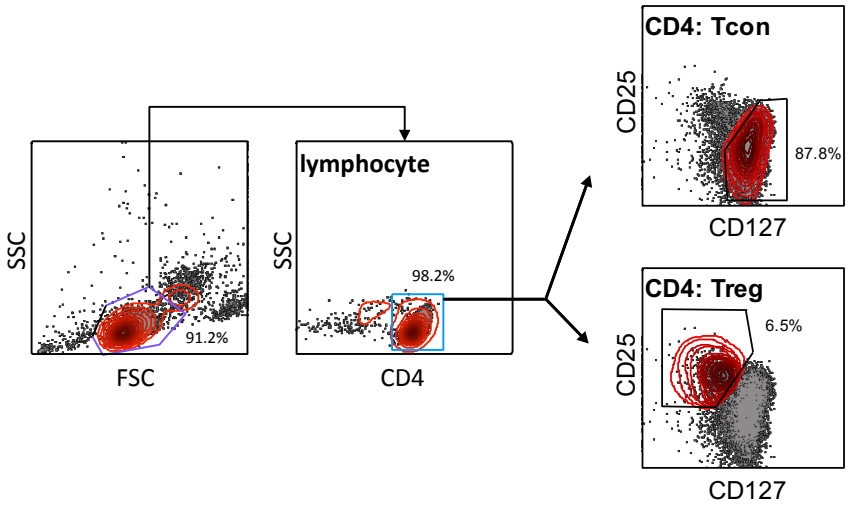


Figure 3. Gating strategy to sort human Tregs and Tcons.
CD4+ T cells were isolated from healthy human blood donors by negative selection and stained with CD4, CD25, and CD127 antibodies before sorting. Lymphocytes were delineated by forward/side scatter gating, and CD4+ cells were further separated as CD25hi CD127lo Tregs or CD25lo CD127hi Tcons.

Sorted cells were activated with anti-CD2/CD3/CD28 antibody-bound biotin beads (Human T cell Activation/Expansion Kit, Miltenyi) in complete RPMI (RPMI 1640 (Life Technologies) + 10% fetal calf serum (FCS) (HyClone) + 1% penicillin/streptomycin (Lonza) + 2 μM ODN (90) for 3 days. Activated T cells were then washed in PBS and subsequently cultured in media as described above with 200 U/mL rIL-2 (PeproTech) and 2 μM ODN at $1x10^6$ cells/mL, replacing the media every 3 days. Jurkat T cells were obtained from the American Type Culture Collection (clone E6.1) and cultured in complete RPMI at 37°C and 5% CO_2.

Flow cytometry and apoptosis assays

RICD assays were performed as described previously (85). Briefly, $1x10^5$ effector T cells were restimulated with 1 μg/ml anti-CD3 mAb (clone OKT3) plus protein A (2 μg/ml) in triplicate wells for 24 hours. Cells were stained with 50nM TO-PRO-3 (Thermo Fisher) to distinguish live and dead cells and analyzed on a BD Accuri C6 flow cytometer. Death was quantified as percent cell loss, based on quantification of viable cells collected under constant time, where % cell loss = (1 – [number of viable cells (treated) / number of viable cells (untreated)]) x 100.

For surface receptor staining, cells were washed in PBS + 1% FBS + 0.01% sodium azide and incubated with antibodies against CD3, CD25, NTB-A, CD95 (FAS) and CD69 (BD Biosciences) on ice for 30 minutes. Intracellular staining was performed using the FOXP3 intracellular staining kit with anti-FOXP3-APC Ab (eBioscience). All flow cytometry analysis was performed with FlowJo version 10.

Western blotting

Cells were lysed in 1% Nonidet P-40 (NP-40) lysis buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 0.5 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 1 mM Na_3VO_4, 1 mM NaF) + complete protease inhibitors (Roche) for 30 minutes on ice. Lysates were cleared by centrifugation and boiled in 2x sample buffer (Laemmli buffer + 50 μM 2-βME) and separated on SDS-PAGE gels (Bio-Rad). Using the Trans-Blot Turbo system (Bio-Rad), proteins were transferred to nitrocellulose membranes and subsequently blocked with 2% Tropix I-Block (Applied Biosystems). Blots were probed with the following antibodies: anti-FOXP3 (Novus Biologicals NB600-245), anti-SAP, anti-LCK (Cell Signaling Technology), anti-β-actin (Sigma-Aldrich). After washing in TBS/0.1%

Tween20, blots were incubated with horseradish peroxidase-conjugated secondary Abs (Southern Biotech), washed again, and developed using enhanced chemiluminescence (SuperSignal, ThermoFisher).

Quantitative RT-PCR

Total RNA was isolated from T cells using QIAshredder and RNeasy Mini Plus columns with DNase digestion (Qiagen). cDNA was prepared using the iScript cDNA kit for RT-qPCR (Bio-Rad), and qPCR was performed with Maxima SYBR Green/ROX qPCR Master Mix (ThermoFisher) using a two-step cycling protocol: 95°C for 1 minute, 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds, followed by a final elongation step at 60°C. The primers used were: SAP forward 5'-tctgtatgaaccctgtgttgg-3', SAP reverse 5'-acaggatgttgtctacttgcc-3', FOXP3 forward 5'-gatggtacagtctctggagc-3', FOXP3 reverse 5'-gggaatgtgctgtttccatgg-3', RPL30 forward 5'-gaatggcatggtcttgaagcc-3', and RPL30 reverse 5'-ggccaccttcttgtgaatgc-3'.

SAP promoter cloning and luciferase assays

The pGL3 Firefly luciferase basic vector (Promega) was digested with KpnI and XhoI in order to insert *SH2D1A* promoter inserts of 1000 bp (maximum activity) versus 100bp (minimal activity) as previously published (144). Segments of the *SH2D1A* promoter were amplified using these primers: SAPprom-F1: 5'atttgcattaatacagtttagcctcaatcgaag-3, SAPprom-F2: 5'-atttgcggtaccgttgttggggtgcttctctc-3', SAPprom-R: 5'-ttactcaccggtggcctggtggactcttgg-3'.

Expression cassettes encoding WT FOXP3 or GFP were PCR amplified and cloned into the pmax vector (Lonza) after cutting with KpnI and XhoI. PCR primers used were: pmaxFOXP3-F 5'-atttgcggtaccgccgccaccatgcccaaccccaggcctg-3', pmaxFOXP3-R

5’-ttactcctcgagtcaggggccaggtgtaggg-3’, The loss of function mutation in the DNA binding domain of FOXP3 (R397W) was introduced by site-directed mutagenesis using DpnI digestion and the following primers for amplification: R397W-F 5’-tgctttgtgTgggtggagagcgagaagg-3’, R397W-R 5’-gctctccacccAcacaaagcacttgtgcagac-3’. For luciferase assays, Jurkat T cells were triple transfected with 5 μg pmax-GFP or FOXP3 expression vector, 5 μg pSAP-GL3 firefly luciferase vector, 0.5 μg pRL-TK renilla luciferase vector, and the pmax vector and assayed after 24 hours. Luciferase activity was quantified using a Dual Luciferase Assay Kit (Promega) and a Synergy H1 microplate reader (Bio-Tek).

Chromatin immunoprecipitation

To perform chromatic immunoprecipitation (chIP) of Jurkat T cells and/or primary T cells, we used the Simple ChIP Plus enzymatic chromatin IP kit (Cell Signaling Technology) with the addition of anti-FOXP3 antibody Clone 206D (Biolegend) or the Mouse IgG1 isotype κ (BD) control antibody. DNA eluted from IPs was amplified by PCR (Apex 2.0 X Taq RED mastermix) with SAP promoter primers SAP-F 5’-aagttattcctggtggcctc-3’, and SAP-R 5’-ttggttcgatcgagcttgcc-3’. Cycling conditions were 95°C for 180 seconds, 32 cycles of 95°C for 10 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, followed by 72°C for 5 minutes. PCR products were then electrophoresed on a 1% agarose gel (1X TAE) with ethidium bromide for visualization.

Lentiviral infections and siRNA transfections

pCLS-YFP-2A-FOXP3 and GFP lentiviral vectors were kindly provided by Dr. James Riley (20). YFP-2A-FOXP3 and GFP expression cassettes were subcloned into the pWPT lentiviral transfer vector using BamHI and SalI. Lentiviruses were produced by co-transfecting the lentiviral plasmid with dR8.2 and VSV-G packaging vectors into 293T cells using calcium-phosphate. Two viral collections were made 48 to 72 h post-transfection, and viral supernatants were pooled and filtered.

For lentiviral transduction of primary T cells, 24 well plates were coated with retronectin overnight at 4°C at 20 μg/ml (Clontech Laboratories). Wells were blocked with 2% BSA in PBS for 30 minutes, and then viral supernatants (or media alone) were added to the plate and centrifuged for 2 hours at 2000 x g at 32°C. Unbound virus was washed from the wells with PBS, and then cells in complete RPMI + 200 U/mL rIL-2 were transferred to the plate and centrifuged at 2000 x g for 15 minutes at 32°C. GFP/YFP expression was measured 24 hours post-infection by flow cytometry to measure transduction efficiency. RICD assays were analyzed on cells counted from gated GFP/YFP+ populations.

T cells were electroporated with siRNAs against FOXP3 (FOXP3HSS121456) and SAP (SH2D1AHSS106218) (ThermoFisher) using the Amaxa Nucleofection 4D system and the P3 Primary Cell kit (Lonza). Stealth RNAi negative control medium GC duplex (ThermoFisher) siRNA was used as a non-specific (NS) control, and all assays were conducted 4 days post electroporation for peak knockdown efficiency.

Statistics

To assess significance in RICD assays, Student t-tests were used in assays comparing two groups, and two-way ANOVA tests were performed for multiple comparisons. For all figures, p-values <0.05 are indicated by *, <0.01 by **. Prism 7 software (GraphPad) was used for all statistical analyses.

RESULTS

Activated human Tregs display minimal SAP expression and are resistant to RICD

Previous work has shown that human Tregs are resistant to RICD, which is dependent on FOXP3 expression (60). However, moderate FOXP3-mediated suppression of FASL alone does not completely account for the profound RICD insensitivity in Tregs (214). To explore this further, we first verified that primary CD25hiCD127lo Tregs sorted from healthy human donors (Figure 3) were resistant to RICD compared to Tcons. As expected, activated Tcons were significantly more sensitive to RICD than Tregs in every donor tested (Figure 4A). However, Tcons and Tregs were equally sensitive to death by direct ligation of the FAS death receptor (Figure 4B). Importantly, apoptosis sensitivity in Tregs could not be explained by alterations in the expression of the T cell receptor (CD3) or FAS, because expression levels were similar in Tcons and Tregs (Figure 4C). The SLAM family receptor NTB-A, which is required for optimal RICD sensitivity (84; 185), also exhibited similar expression in Tregs and Tcons (Figure 4C). As expected, Tregs maintained elevated expression of the high affinity IL-2 receptor CD25 compared to Tcons (Figure 4C).

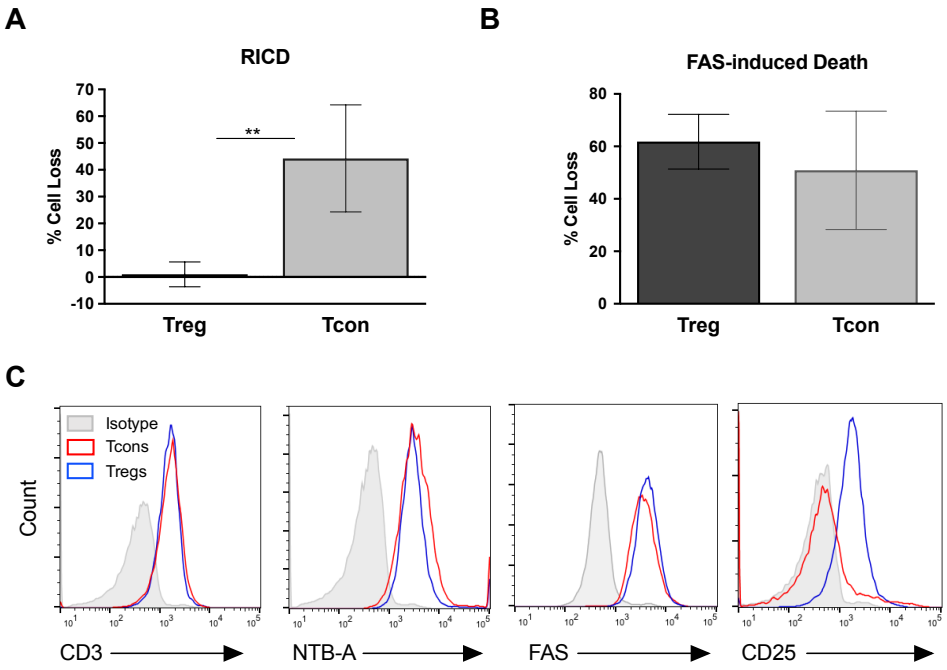


Figure 4. Human Tregs are resistant to RICD, but not FAS-induced death.
(A) Sorted Treg and Tcons were activated with anti-CD2/3/28 beads and expanded in culture with IL-2. Cells were then restimulated with OKT3 antibody (1 μg/ml) plus Protein A (2 μg/ml) for 24 hours in triplicate. Cell death was measured by flow cytometry using TO-PRO-3 live/dead staining. Data are the average ± SD of 4 independent experiments using separate donors. **(B)** Cells cultured as in (A) were treated with 200 ng/ml APO1.3 antibody plus Protein A for 24 hours. Cell death was measured as above; results data are average ± SD of 3 independent experiments. **(C)** Cells were stained with antibodies against CD3, NTB-A, FAS, and CD25, or an isotype control antibody, and analyzed by flow cytometry. Results are representative of 3 independent experiments.

T cells deficient in SAP expression, such as in XLP-1 patients, are highly resistant to RICD (84). We therefore asked whether human Tregs express less SAP compared to Tcons. Indeed, genome-wide ChIP analysis of human Tregs uncovered SH2D1A as a FOXP3 target gene, although this particular finding was not subjected to further validation (173). We therefore hypothesized that FOXP3 confers RICD resistance in activated Tregs by repressing the SH2D1A promoter and reducing SAP expression. We observed that SAP mRNA levels were markedly reduced in activated Tregs compared to

Tcons, exhibiting an inverse relationship with FOXP3 expression (Figure 5A). As expected, this correlated with decreased SAP protein in Treg cell lysates (Figure 5B). Overall, our survey of cells from multiple human donors indicated that activated Tregs contain very little SAP expression compared to Tcons, and are extremely resistant to RICD, similar to XLP-1 patient-derived SAP-deficient T cells.

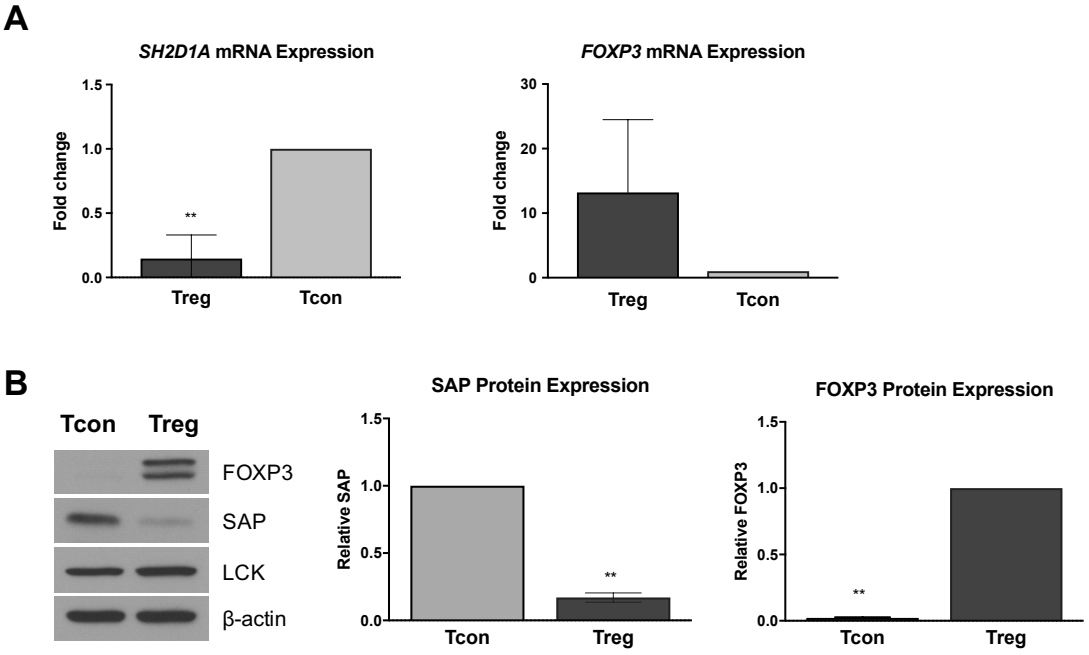


Figure 5. Inverse correlation between FOXP3 and SAP expression in human Tregs. **(A)** RNA was extracted from activated cells, and qRT-PCR was performed with primers specific for *FOXP3* and *SAP* mRNA. *RPL30* was used as a housekeeping gene for normalization. Data are average ± SD of 2 independent experiments. **(B)** Protein lysates were subjected to SDS-PAGE and Western blot analysis for FOXP3, SAP, and LCK. β-actin was used as a loading control. Results are representative of 3 independent experiments.

Expression of FOXP3 suppresses *SH2D1A* promoter activity

We next tested whether ectopic FOXP3 expression can repress *SH2D1A* gene promoter activity. In silico analysis of a ~1000 bp sequence of the proximal *SH2D1A* promoter revealed at least three putative FOXP3 binding sites (144; 173) (Figure 6A). In

order to measure *SH2D1A* promoter activity +/- FOXP3 expression, we employed a luciferase reporter system in Jurkat T cells. Both wild-type (WT) and a loss-of-function mutant of FOXP3 (R397W, defective in DNA binding (15)) were cloned into the pmax expression vector; GFP served as a negative control. The ~1000 bp fragment of the proximal *SH2D1A* promoter was cloned into the pGL3 firefly luciferase vector to create a reporter plasmid of *SH2D1A* promoter activity (~1000bp), with a smaller ~100 bp promoter fragment with minimal activity serving as a negative control. As predicted, the ~1000 bp promoter sequence displayed ~3-fold higher activity than the minimal ~100 bp sequence (Figure 6B). With concomitant transfection of GFP or FOXP3 expression plasmids, we found that robust transient expression of WT FOXP3 substantially reduced luciferase expression driven by the 1000bp *SH2D1A* promoter (Figure 6B). In contrast, FOXP3 R397W failed to repress SAP promoter activity despite comparable overexpression (Figure 6C).

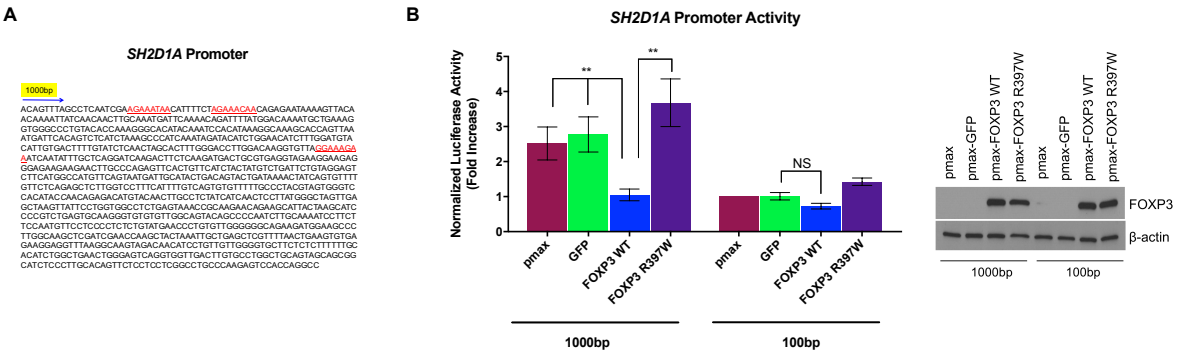


Figure 6. FOXP3 represses *SH2D1A* promoter activity.

(A) Sequence of the proximal *SH2D1A* promoter (~1000 bp) cloned into the pGL3 luciferase vector. Putative FOXP3 binding sites are highlighted in red. **(B)** Jurkat T cells were triple transfected with pmax-GFP or FOXP3 expression vectors, firefly luciferase *SH2D1A* promoter constructs, (1000bp vs. 100bp), and a renilla luciferase control plasmid. Promoter activity was measured using a dual luciferase assay at 24 hours post-transfection. Luciferase activity (firefly/renilla ratio) was normalized to pmax empty vector control cells. **(C)** Expression of

FOXP3 was confirmed by Western blot analysis; β-actin served as a loading control. Data are representative of 3 independent experiments.

FOXP3 directly binds to the *SH2D1A* promoter

The aforementioned results strongly suggested that FOXP3 can directly suppress *SH2D1A* promoter activity through its DNA binding function. To assay for direct FOXP3 binding to the *SH2D1A* gene, we first derived Jurkat T cells stably expressing FOXP3, as opposed to the transient overexpression system described above. Lentiviruses encoding YFP-2A-FOXP3 (allowing for bicistronic expression) or GFP (20) were used to transduce Jurkat T cells. Robust expression of GFP/YFP was maintained over several weeks in culture, indicating stable integration (Figure 7A). FOXP3 protein expression was also readily detected by Western blot (Figure 7B), although at reduced levels compared to transient Jurkat transfections (Figure 6C). As expected, endogenous SAP expression was reduced in Jurkat T cells that stably expressed FOXP3 compared to the GFP control (Figure 7B).

To provide direct evidence of FOXP3 binding to the *SH2D1A* promoter, we lysed Jurkat T cells stably transduced with FOXP3 or GFP and performed chromatin immunoprecipitations (chIP) using an anti-FOXP3 or isotype control antibody. Indeed, we detected an enrichment of *SH2D1A* promoter DNA in FOXP3 IPs relative to an IgG isotype control IP (Figure 7C). Importantly, Jurkat cells expressing GFP alone did not show the same amplification of the *SH2D1A* promoter region after FOXP3 IP. Immunoprecipitation with an anti-histone H3 antibody reliably detected both *SH2D1A* and the housekeeping gene *RPL30*, which confirmed that our ChIP procedure was efficient and selective based on the IP Ab used.

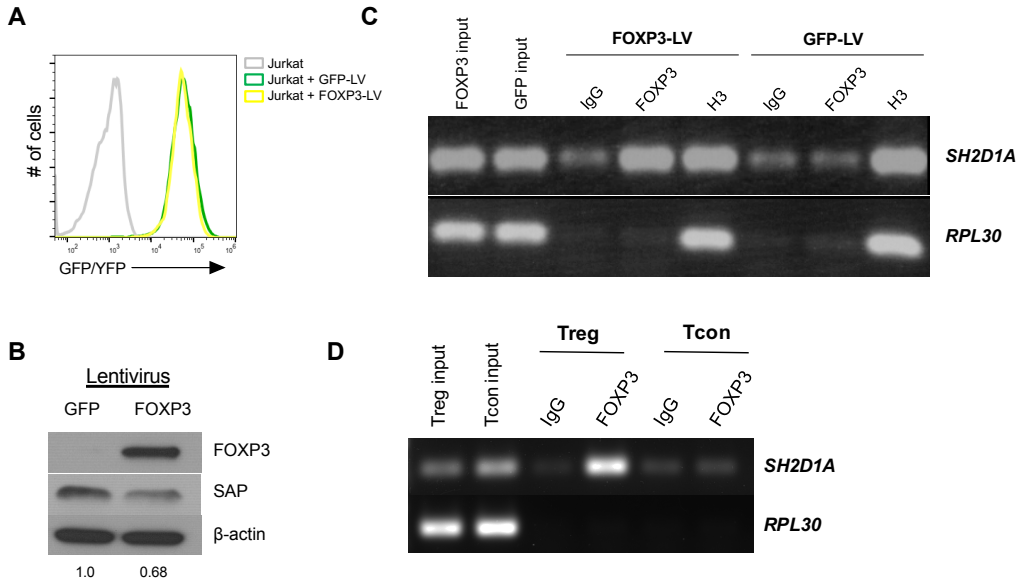


Figure 7. FOXP3 binds directly to the *SH2D1A* promoter.
(A) Jurkat T cells were transduced with GFP or YFP-2A-FOXP3 lentiviral (LV) vectors; stable expression was monitored over time by GFP/YFP fluorescence. **(B)** FOXP3 and SAP expression was measured in LV-transduced Jurkat T cell lysates by Western blot. Numbers represent quantitation of SAP expression by spot densitometry, normalized to LV-GFP transduced cells. **(C)** LV-transduced Jurkat T cells were lysed for chIP using antibodies specific for FOXP3, histone H3, or an isotype IgG control. Immunoprecipitated DNA was then subjected to PCR using primers specific for the *SH2D1A* promoter, or the *RPL30* housekeeping gene. Results are representative of 3 independent experiments. **(D)** ChIP analysis was performed on activated human Tregs and Tcons using FOXP3 or IgG isotype antibodies. Immunoprecipitated DNA was then subjected to PCR using primers specific for the *SH2D1A* promoter, or the *RPL30* housekeeping gene. Data are representative of 2 independent experiments.

To confirm these findings, lysates derived from activated, primary human Tregs and Tcons were also subjected to chIP with FOXP3 and IgG Abs and analyzed for *SH2D1A* promoter binding. As expected, activated Tregs showed enrichment of *SH2D1A* promoter DNA in FOXP3 IPs, whereas Tcons did not (Figure 7D). Taken together, these results demonstrate that FOXP3 can directly bind to the *SH2D1A* promoter, where it is readily detected in activated human Tregs.

FOXP3-mediated RICD resistance in Tregs is dependent on repression of SAP expression

To further explore the mechanism of FOXP3-dependent RICD resistance in human Tregs, we next investigated whether FOXP3-mediated repression of SAP is required for RICD resistance. Freshly isolated Tregs were transfected with non-specific (NS), FOXP3-specific, or FOXP3- plus SAP-specific siRNA and stimulated in vitro. Knockdown of FOXP3 expression sensitized Tregs to RICD, confirming previous reports (214). However, concomitant knockdown of SAP completely blocked the increase in RICD afforded by FOXP3 knockdown (Figure 8A). Reduced target protein expression was confirmed for each siRNA transfection by Western blot analysis, and also revealed a modest increase in SAP expression when FOXP3 was silenced in Tregs (Figure 8B).

If silencing FOXP3 renders Tregs susceptible to RICD by boosting SAP expression, we reasoned that increasing SAP expression alone should also restore RICD sensitivity. To this end, we infected primary activated Tregs with lentiviral vectors expressing YFP-2A-SAP or GFP. At 24 hours post-infection, we restimulated cells with OKT3 and assayed death in the GFP/YFP$^+$ gated population of transduced cells. Ectopic SAP expression dramatically restored RICD sensitivity in YFP$^+$ transduced Tregs, relative to cells transduced with GFP alone (Figure 8C). These results definitively show that RICD resistance in human Tregs is specifically dependent on FOXP3-dependent mediated repression of SAP expression. Furthermore, ectopic expression of SAP alone (using a FOXP3-irrepressible promoter) is sufficient to sensitize activated Tregs to RICD.

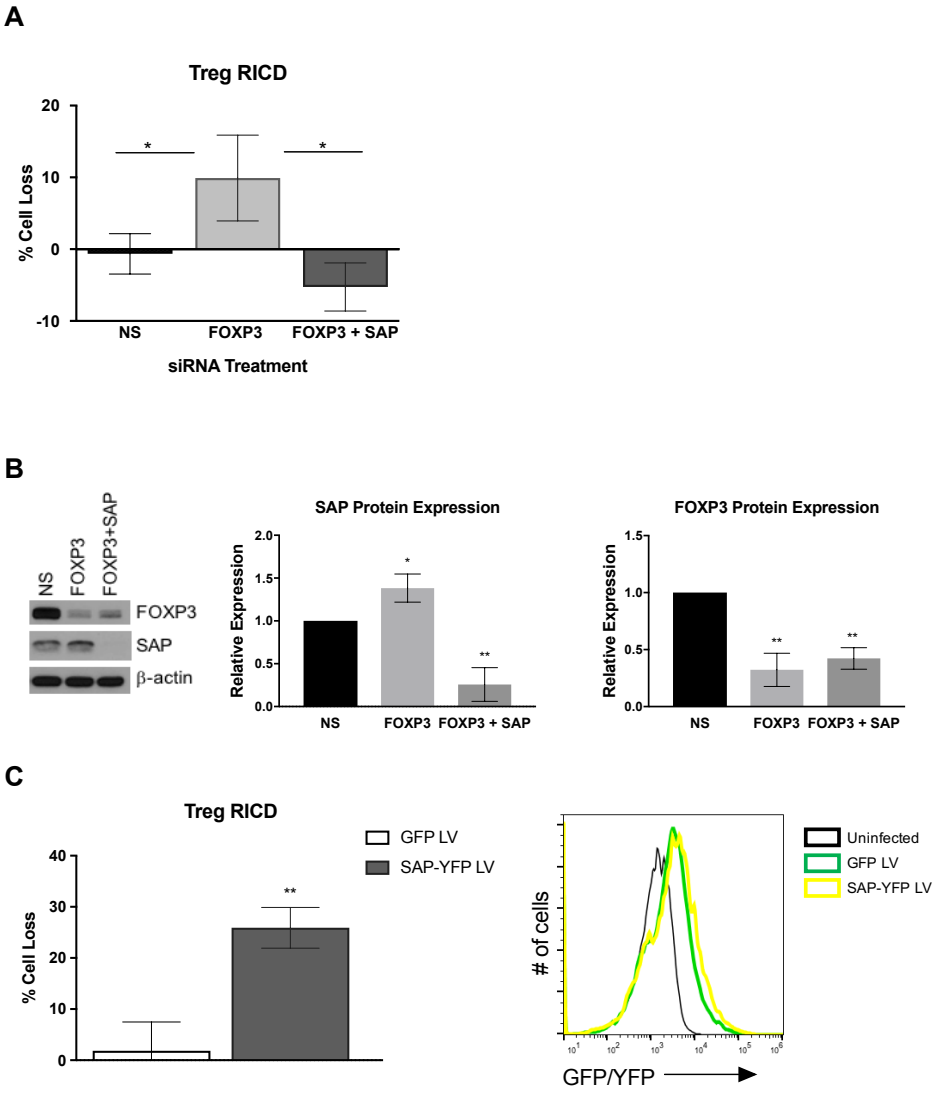


Figure 8. FOXP3 confers RICD resistance in Tregs by a SAP-dependent mechanism. **(A)** Activated Tregs were transfected with non-specific (NS), FOXP3-specific, or SAP-specific siRNAs. At 4 days post-transfection, cells were restimulated with OKT3 antibody (1 µg/ml) plus Protein A (2 µg/ml) for 24 hours in triplicate. Cell death was measured by flow cytometry using TO-PRO-3 live/dead staining. Data are average ± SD of 3 independent experiments using separate donors. **(B)** Knockdown of FOXP3 and/or SAP expression in transfected cells from (A) was assessed by Western blot. β-actin served as a loading control. Data are representative of 3 independent experiments. **(C)** Activated Tregs were transduced with GFP or YFP-2A-SAP LVs during activation-induced expansion. Cells were then restimulated 24 hours later with OKT3 (1 µg/ml) plus Protein A for an additional 24 hours. Cell loss was determined by TO-PRO-3 live/dead staining within GFP/YFP$^+$ gated cell populations. Data are average ± SD of 2 independent experiments. Transduction efficiency of Tregs was assessed 24 hours post infection by flow cytometry compared to uninfected control cells (right).

DISCUSSION

A sufficient CD4+ Treg population must be preserved in order to stave off autoimmunity. A better understanding of apoptosis sensitivity in Tregs is therefore vital to comprehend mechanisms of immune homeostasis and tolerance. Tregs are CD25hi, consume high amounts of IL-2, and experience frequent stimulation from self-antigens (60; 214). Whereas IL-2 consumption and TCR restimulation would typically induce RICD in conventional effector T cells, Tregs have adopted unique strategies to evade apoptosis and persist under these conditions (226). In fact, Treg survival and function is dependent on IL-2 consumption for induction of *FOXP3* mRNA expression and upregulation of the pro-survival protein MCL-1 to combat pro-apoptotic BIM expression (156). Surprisingly, Tregs are able to persist despite experiencing even stronger TCR signals in the periphery (133). We demonstrate here that FOXP3-dependent suppression of SAP renders Tregs resistant to RICD. Although protecting Tregs under these conditions is important for maintaining tolerance, these adaptations to evade RICD could also explain why Tregs are able to persist in the antigen-rich tumor microenvironment, which is likely detrimental to the anti-tumor response (52; 137; 143).

Work from our group and others has illuminated a central role for SAP/SLAM receptor signaling in governing RICD sensitivity in T cells (42; 72; 84). Our results further underscore the importance of SAP-driven signaling in this process, demonstrating that Tregs have evolved to disable this signaling pathway via FOXP3-dependent SAP repression to resist RICD. We posit that other T cell subsets might manipulate SAP-SLAM receptor signaling to elude RICD (e.g. Th17 cells (39)) or adjust RICD sensitivity at distinct phases of the effector T cell response. For example, effector cells that progress

into long-lived memory T cell pools could be shaped in part by SAP-SLAM receptor signaling alterations that promote their persistence. After all, dynamic changes in this pathway shape RICD sensitivity at different phases of the conventional effector T cell response; SAP expression is initially kept at low levels after T cell activation, then gradually increases as effector T cells fully differentiate and become more sensitive to RICD (94; 128). Moreover, transient induction of FOXP3 expression after activation of human Tcons may help preserve these cells from premature RICD during the early clonal expansion phase (127). However, further work is required to determine whether FOXP3 can repress SAP expression in this context.

Our findings confirm previous work showing FOXP3 is necessary for maintaining RICD resistance in Tregs (60; 214). Interestingly, ectopic SAP expression was sufficient to sensitize Tregs to RICD, suggesting SAP can override any direct FOXP3-mediated repression of FASL itself to promote apoptosis. On the other hand, ectopic FOXP3 expression alone was not sufficient to significantly reduce SAP expression in Tcons and confer resistance to RICD (data not shown). Indeed, only considerable overexpression of FOXP3 repressed *SH2D1A* promoter activity in our reporter assays, and reduced endogenous SAP expression by ~30% in stably-transduced Jurkat T cells. We speculate that additional binding partners may be required to stabilize and/or cooperate with FOXP3 to suppress SAP expression, similar to other FOXP3 target genes (169; 221). This idea is also consistent with studies that indicate FOXP3 expression alone is not enough to convert Tcons to Tregs with suppressive function, unless expression levels are artificially high (1; 87). There could also be competition among other transcription factors in Tcons that is not relevant in Tregs. High levels of retrovirally-introduced

FOXP3 in Tcons are necessary to efficiently out-compete endogenous AP-1 for NFAT binding and repression of several activation-induced genes (1). Finally, additional exogenous factors may also aid in FOXP3-dependent SAP suppression. For example, Bhaskaran and colleagues showed that TGF-β1 promotes RICD resistance in FOXP3+ Tregs, perhaps via increased expression of cFLIP (22).

In conclusion, new mechanistic knowledge regarding apoptosis resistance in Tregs could be utilized to manipulate immune responses by changing Treg apoptosis sensitivity. Tuning T cell apoptosis sensitivity to shape immune responses has great potential in attenuating autoimmunity and/or enhancing eradication of pathogens and tumors (188). However, this approach also requires a fuller understanding of how T cell subsets could be differentially impacted. In addition, adoptive Treg cell therapies could be improved by maintaining and/or enhancing RICD resistance. Adoptive transfer of infused Tregs has been used in human clinical trials to help prevent graft versus host disease (GVHD) in transplant recipients (147). Although GVHD rates declined, no infused Tregs were detected in the blood after 14 days, which likely suggests many of the infused Tregs died by apoptosis. Strategies that specifically stabilize FOXP3-dependent repression of the *SH2D1A* promoter could improve clinical usefulness of Tregs by promoting their longevity *in vivo*. Stabilizing RICD resistance could also preserve infused FOXP3-transduced "regulatory" T cells from patients with immune-dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome, which lack natural Tregs due to debilitating *FOXP3* mutations (148). In conclusion, our findings provide new insight into the puzzling TCR-mediated apoptosis resistance demonstrated by CD4+ Tregs, highlighting the importance of SAP suppression in this phenotype.

CHAPTER 3: FOXP3 protects conventional human T cells from premature restimulation-induced cell death

This work was submitted as a research manuscript to *The EMBO Journal* on March 2nd, 2019.

Title: FOXP3 protects conventional human T cells from premature restimulation-induced cell death.

Authors: Kelsey Voss*, Camille Lake, Christopher R. Luthers, Nathaniel M. Lott, Batsukh Dorjbal, Swadhinya Arjunaraja, Bradly M. Bauman, Anthony R. Soltis, Guathaman Sukumar, Clifton L. Dalgard and Andrew L. Snow.

*first-author

Kelsey Voss lead the project, designed the experiments, conducted the majority of the experiments, conducted all data analysis and presentation (except for RNA sequencing data), and wrote the paper.

ABSTRACT

The adaptive immune response relies on specific apoptosis programs to maintain homeostasis. Conventional effector T cell (Tcon) proliferation is constrained by both forkhead box P3 (FOXP3) + regulatory T cells (Tregs) and restimulation-induced cell death (RICD); propriocidal apoptosis triggered by repeated stimulation through the T cell receptor (TCR). Constitutive FOXP3 expression protects Tregs from RICD by suppressing SLAM-associated protein (SAP), a key adaptor protein that amplifies TCR signal strength. The role of transient FOXP3 induction in human Tcons remains

unresolved, but its expression correlates inversely with acquired RICD sensitivity. Here we describe a novel role for FOXP3 in protecting both CD4 and CD8 human Tcons from premature RICD during expansion. Unlike late-stage effector T cells, low levels of RICD in expanding Tcons did not require SAP. Subsequent transcriptome profiling and functional analyses revealed that FOXP3 enhances expression of the SLAM family receptor CD48, which in turn sustains basal autophagy. Our findings suggest that FOXP3 governs a distinct transcriptional program that maintains RICD resistance in early-stage effector Tcons via CD48-dependent protective autophagy.

INTRODUCTION

Clonal T cell expansion is regulated by multiple intrinsic and extrinsic mechanisms that ensure the adaptive immune response is sufficient for pathogen clearance without overtly damaging the host. In order to maintain homeostasis, T cell activation and proliferation must be carefully coordinated with the contraction and disposal of effector T cells. Therefore, pre-programmed cell death programs such as cytokine withdrawal-induced cell death (CWID) and restimulation-induced cell death (RICD, sometimes referred to as activation-induced cell death (AICD)), are critical for balancing robust T cell expansion with efficient contraction and subsequent formation of memory T cells (188; 192). RICD is especially important for constraining excessive T cell responses. For example, massive overaccumulation of RICD-resistant effector T cells in X-linked lymphoproliferative disorder (XLP-1) patients can cause life-threatening immunopathology in certain viral infections (19; 75; 185).

The magnitude of an effector T cell response is a critical factor for controlling certain infections such as viruses (217). Understanding the factors that dictate RICD

sensitivity over the course of the entire T cell response reveals potential targets for tuning T cell death sensitivity, providing an intriguing strategy for boosting or decreasing effector T cell numbers as needed. Activated T cells remain relatively resistant to RICD during initial rounds of clonal expansion. As human effector T cell differentiation progresses over several days, RICD susceptibility emerges and can trigger apoptosis when these cells receive a strong restimulation signal through the T cell receptor (TCR) (45; 179) as they cycle through S-phase (24) and consume interleukin-2 (IL-2) (103). Although changes in Fas-dependent apoptotic signaling may help render effector T cells competent to die by RICD (139; 176), it remains relatively unclear how RICD resistance is enforced in early expanding T cells.

SLAM-associated protein (SAP) is an adaptor that partners with the signaling lymphocyte activation molecule (SLAM) family receptor NK, T, B cell antigen (NTB-A) to amplify TCR signal strength sufficient for triggering RICD (42; 84). XLP-1 patients contain null mutations in the gene encoding SAP (SH2D1A), which results in attenuated TCR signaling upon re-engagement and defective RICD (84). Remarkably, inhibition of diacylglycerol kinase alpha (DGKα), an enzyme that downmodulates TCR signaling with enhanced activity in the absence of SAP, can restore RICD susceptibility in XLP-1 patient T cells and reduce effector CD8 T cell accumulation to limit immunopathology (170). Considering SAP is significantly upregulated in effector T cells relative to naïve or resting T cells (94; 128), these findings collectively suggest SAP expression is a critical molecular determinant that differentiates the TCR restimulation signal for apoptosis.

The importance of SAP for promoting RICD sensitivity was also demonstrated in forkhead box P3 (FOXP3)+ regulatory CD4 T cells (Tregs), which express low levels of

SAP and are extremely resistant to RICD compared to conventional T cells (86). Both human and murine Tregs are very resistant to RICD compared to conventional T cells (Tcons; i.e. non-Tregs), in part via TGF-β1 signaling (22; 60). We and others have previously linked FOXP3 expression to RICD resistance in Tregs (86; 214). In activated human Tregs, we showed FOXP3 muted SAP expression due to direct repression of the SH2D1A promoter. Moreover, ectopic expression of SAP using an independent lentiviral promoter was sufficient to sensitize FOXP3+ Tregs to die by RICD. However, FOXP3 expression is not confined to the Treg compartment alone in humans (62). Human Tcons express FOXP3 transiently after TCR activation, but the function of FOXP3 in this context remains mysterious, considering it does not confer a Treg-like suppressive phenotype (131; 209). Some investigations defined FOXP3 as a negative regulator of cell proliferation and cytokine production in human Tcons (127; 200), but these results have been inconsistent (5; 37). Therefore, it is likely that transient FOXP3 expression serves additional, hitherto unappreciated functions in Tcons. For example, although FOXP3 acts to protect Tregs from RICD by SAP suppression, this possibility has not been studied in Tcons. Indeed, the profile of transient FOXP3 expression in activated CD4+ and CD8+ Tcons correlates inversely with RICD sensitization. We therefore hypothesized that transient FOXP3 expression in activated Tcons helps to protect expanding T cells from RICD by suppressing SAP expression.

In this study, we elucidate a novel intrinsic mechanism of T cell homeostasis by which FOXP3 expression protects newly activated CD4 and CD8 effector T cells from premature RICD during clonal expansion. Surprisingly, FOXP3 did not repress SAP expression in early-stage activated Tcons; in fact, low levels of RICD were induced in a

SAP-independent manner. Instead, we found that FOXP3 promoted increased expression of CD48, a GPI-anchored SLAM-family receptor. Our results suggest this FOXP3-dependent CD48 upregulation in Tcons is required for promoting autophagy, which we and others have demonstrated protects effector T cells from programmed cell death (47; 84). These findings illuminate important differences in factors that govern RICD sensitivity in newly activated T cells versus late stage effectors, indicating FOXP3 can shield both Tregs and Tcons from RICD via distinct mechanisms.

MATERIALS AND METHODS

T cell isolation and culture conditions

Peripheral blood mononuclear cells (PBMC) were obtained from healthy human donors at the National Institutes of Health (NIH) Blood Bank. Access to anonymous Blood Bank donors was kindly provided by Dr. Michael Lenardo. CD4 T cells were purified from PBMC by immunomagnetic negative selection using the EasySep Human CD4 T cell isolation kit (Stem Cell Technologies). CD8 T cells were purified using the Human CD8 T cell isolation kit (Stem Cell Technologies) and naïve CD4 T cells were purified with the Human Naïve CD4 T cell isolation kit II (Stem Cell Technologies).

Primary T cells were activated with either a 2:1 ratio of anti-CD2/CD3/CD28 Ab-bound biotin beads (Human T cell Activation/Expansion Kit, Miltenyi Biotec), or ImmunoCult Human CD3/CD28/CD2 T Cell Activator (Stem Cell Technologies), according to the manufacturer's instructions in complete RPMI (RPMI 1640 (ThermoFisher Scientific) + 10% fetal calf serum (FCS) (HyClone) + 1% penicillin/

streptomycin (Lonza). After 3 days, activated cells were washed with PBS and subsequently cultured in complete RPMI with 200U/mL rIL-2 (PeproTech). Jurkat T cells (ATCC clone E6.1) were also maintained in complete RPMI.

siRNA-mediated knockdowns in primary T cells

T cells were electroporated with siRNAs against FOXP3 (FOXP3HSS121456), SAP (SH2D1AHSS106218), ATG3 (assay ID sc34733), and CD48 (assay ID s2689) (ThermoFisher) using the Amaxa Nucleofection 4D system and the P3 Primary Cell kit (Lonza). Stealth RNAi negative control medium GC duplex siRNA (ThermoFisher) was used as a non-specific (NS) control, and all assays were conducted 4 days post electroporation for peak knockdown efficiency. For day 4 RICD experiments, cells were rested for 1 hour in complete RPMI at 37°C and 5% CO_2 after isolation. Cells were then rested for an additional hour after electroporation before stimulation. Knockdown efficiencies were assessed by flow cytometry or immunoblotting for every experiment.

Restimulation-induced cell death (RICD) assays

RICD assays were conducted as described previously (85). Briefly, 1×10^5 T cells were restimulated with anti-CD3 mAb (clone OKT3) in triplicate wells of a 96 well round-bottom plate for 24 h. Cells were stained with 10 μl of propidium iodide (PI, 1 μg/ml stock) (ThermoFisher) to distinguish live and dead cells and immediately analyzed on a BD Accuri C6 flow cytometer. Death was quantified as percent cell loss, based on quantification of viable cells collected under constant time, where % cell loss = (1 – [number of viable cells (treated)/number of viable cells (untreated)]) × 100. Antibody blockade experiments were conducted by incubating cells with 10 μg/mL of anti-CD48

(Biogems 10511-25), anti-CD2 (Biolegend 309212), anti-CD244 (Invitrogen 16-2449-81), or an isotype IgG1 control (Biogems 44212-25) for 1 hour before restimulation with OKT3. For small molecule inhibitors, T cells were pre-treated for 30 minutes with 100 ng/ml actinomycin D (Sigma Aldrich), 10 μM chloroquine (Sigma Aldrich), 1 μM BEZ-235 (Cayman Chemical), 10 μM E-64d (Cayman Chemical), or a DMSO solvent control prior to OKT3 restimulation.

Flow cytometry

Intracellular staining of FOXP3 was conducted using the Foxp3/Transcription Factor Staining Buffer set (eBioscience) with anti-Foxp3-APC (eBioscience 17-4777-42). For surface receptor staining, cells were washed in PBS + 1% FBS +0.01% sodium azide and incubated with antibodies for 30 minutes on ice. Surface expression of CD48 and its ligands was detected using CD48-FITC (Biolegend 336706) CD244-APC (Biolegend 188549) and CD2-PE (Biolegend 300207). T cell activation markers were assessed with CD25-PE (BD 555432) and CD69-APC (BD 555533). Naïve cells were stained with CD45RA-FITC (Biolegend 304106) and CD62L-PE (Biolegend 304806).

For proliferation assays, day 4 activated T cells were incubated in complete RPMI + IL-2 for 3 hours and then subjected to Click-It Edu Alexa Fluor 488 staining for flow cytometry assays according to the manufacturer's instructions (ThermoFisher). Similarly, CFSE labeling was performed using a CellTrace CFSE Proliferation kit (ThermoFisher). Briefly, $1.5x10^6$ T cells were washed 2x in PBS to remove serum and stained with 1 μM CFSE in PBS for 5 min in the dark at 37°C. Cells were quenched/resuspended in complete RPMI and activated as described above. Cells were activated after CFSE labeling. Cyto-ID staining for autophagic vesicles was conducted according to the

manufacturer's instructions (Enzo Biosciences). Briefly, cells were washed with PBS and then resuspended in Cyto-ID staining solution (without serum) for 30 minutes at 37°C and 5% CO_2. For PI cell cycle analysis, 1-2x10^4 T cells were resuspended in 300 ml PBS and 5 ml ice cold methanol was added dropwise before a 30 minute incubation at -20°C. Fixed cells were resuspended in PBS for at least 30 minutes, pelleted and resuspended in 60 μg/ml PI + 50 μg/ml RNAse A (Sigma) in PBS for 30 minutes at room temperature. Cells in G1, S, and G2/M phases of the cell cycle were gated and quantified by flow cytometry (linear scale). All flow cytometry analysis was conducted using FlowJo v10 software.

Western blotting

For general immunoblotting, cells were lysed in 1% Nonidet P-40 (NP-40) lysis buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 0.5 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 1 mM Na3VO4, 1 mM NaF) + complete protease inhibitors (Roche) for 30min on ice. Lysates were cleared by centrifugation, boiled in 2x sample buffer (Laemmli buffer + 50 μM 2-βME) and separated on 4-20% SDS-PAGE gels (Bio-Rad). Proteins were transferred to nitrocellulose membranes for 7-10 min using the TransBlot Turbo system (Bio-Rad) and subsequently blocked with Odyssey blocking buffer (LI-COR). Blots were probed with the following antibodies: anti-FOXP3 (Novus Biologicals NB600-245), anti-SAP (Cell Signaling Technology #2778), anti-FASL (Ab3, EMD Biosciences), anti-BIM (Enzo Life Sciences), anti-ATG3 (Cell Signaling Technology #3415) anti-β-actin (Sigma-Aldrich). Blots were incubated with IRDye anti-Rabbit or anti-Mouse secondary antibodies (LI-COR) and imaged using the Odyssey CLx instrument.

Nuclear and cytoplasmic fractions were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific). For each sample, 3 x 10^6 cells were washed in PBS and lysed in 0.1mL of CER I lysis buffer. Lamin A/C was used as a marker of nuclear fractions by immunoblotting (4C11 Ab, Cell Signaling Technology).

Transcriptome profiling of primary T lymphocytes

Total RNA was isolated from primary T cells using the RNeasy Plus Mini Kit with on-column DNase digestion (Qiagen). Total RNA integrity was assessed using automated capillary electrophoresis on a Fragment Analyzer (Roche). For all samples RQI > 8.0, a total RNA amount of >75 ng was used as input for library preparation using the TruSeq Stranded mRNA Library Preparation Kit (Illumina, San Diego, CA, USA). Sequencing libraries were quantified by PCR using KAPA Library Quantification Kit for NGS (Kapa, Wilmington, MA, USA) and assessed for size distribution on a Fragment Analyzer. Sequencing libraries were pooled and sequenced on a NextSeq 500 Desktop Sequencer (Illumina) using NextSeq 500 High Output Kit v2 with paired-end reads at 75 bp length. Raw sequencing data were demuxed using bcl2fastq2 conversion software 2.17 before alignment using TopHat Alignment v1.0. Differential transcript expression analysis was performed using Cufflinks Assembly & DE v1.1.0 on BaseSpace Onsite (Illumina). Heatmap visualization of differentially expressed genes were generated using ClustVis software. Transcript abundance quantitation data are deposited in NCBI's Gene Expression Omnibus.

Statistics

Statistical analyses were conducted using GraphPad Prism 8.0.1 software. Error bars in all figures indicate +/- standard deviation unless otherwise specified. All statistical

tests performed are indicated in the Figure Legends. For RICD assays, each data point represents the average % cell loss from an individual donor (the average of triplicate samples).

RESULTS

Activation-induced FOXP3 expression in conventional T cells varies across multiple human donors

TCR-dependent FOXP3 induction in human Tcons has been described by several groups; however, FOXP3 expression varies greatly with different in vitro activation conditions (62; 209; 237). Furthermore, FOXP3 induction in CD8 T cells has been far less studied than in CD4 T cells. Therefore, we first measured FOXP3 expression across multiple healthy human blood donors in both CD4 and CD8 T cells. CD4 and CD8 T cells were isolated from peripheral blood mononuclear cells (PBMC) by negative selection and activated with CD2/3/28 beads. We detected no FOXP3 protein in unstimulated cells, confirming few if any natural Tregs were present. Immunoblotting demonstrated FOXP3 was strongly induced in both CD4 and CD8 T cells between 1-2 days post-activation (Figure EV 1A). Using intracellular flow cytometry, we observed that FOXP3 expression was prominently expressed in a subset of cells, (Figure EV 1B) consistent with others' observations (209). To negate the possibility that this FOXP3+ population originated from contaminating Tregs in our CD4+ Tcon population, we purified naïve CD4 T cells and assessed FOXP3 induction using the same activation conditions. Indeed, FOXP3 induction in the purified naïve T cell population was similar on day 4 post activation (Figure EV 1C).

FOXP3 expression protects newly activated T cells from restimulation-induced cell death

In human T cells, FOXP3 expression is induced upon TCR activation and subsequently diminishes as T cell expansion slows and RICD sensitivity peaks. In order to test whether FOXP3 expression protects expanding T cells from death by RICD, we performed siRNA knockdown (KD) experiments in purified CD4 and CD8 T cells to silence FOXP3 expression during initial activation. Activated T cells were then restimulated through the TCR with anti-CD3 antibody (OKT3) on day 4 post activation, and RICD was measured by propidium iodide (PI) staining 24 hours later (85). Despite variation in FOXP3 expression among different human donors (Figure EV1), FOXP3 KD resulted in significantly higher RICD sensitivity at multiple doses of restimulation in both CD4 and CD8 T cells (Figure 9A). In contrast, FOXP3 KD did not impact RICD at day 17 post activation, when effector Tcons had fully expanded in vitro and reached peak death sensitivity (Figure 9B), correlating with markedly lower levels of FOXP3 expression (Figure 9C). These data suggest transient FOXP3 expression in early expanding Tcons helps to protect them from premature RICD.

Endogenous FOXP3 expression in human Tcons is not sufficient to convert them into suppressive Tregs under physiological conditions due to lower expression levels (209), relating to methylation status of the FOXP3 locus (17). Others have suggested altered cellular localization of FOXP3 may explain this lack of suppressive activity (118). To gain some insight into the function of FOXP3 in early activated Tcons, we asked whether FOXP3 is primarily found in the nucleus or the cytoplasm, and whether restimulation of the TCR could impact FOXP3 localization status. In both expanding (day 4) T cells as well as late stage effectors (day 17) from multiple human donors, we

detected FOXP3 exclusively in the nucleus before and after TCR restimulation (Figure 9C). These data indicate that FOXP3 resides in the nucleus of activated Tcons, likely acting as a functional transcription factor. Furthermore, enhanced RICD in CD4 T cells at day 4 post activation was not due to silencing FOXP3 expression in a small number of contaminating Tregs; naïve CD4 T cells demonstrated a comparable increase in RICD sensitivity on day 4 post activation with FOXP3 KD (Figure 9D). Collectively, these results connect FOXP3 expression to RICD resistance in human Tcons.

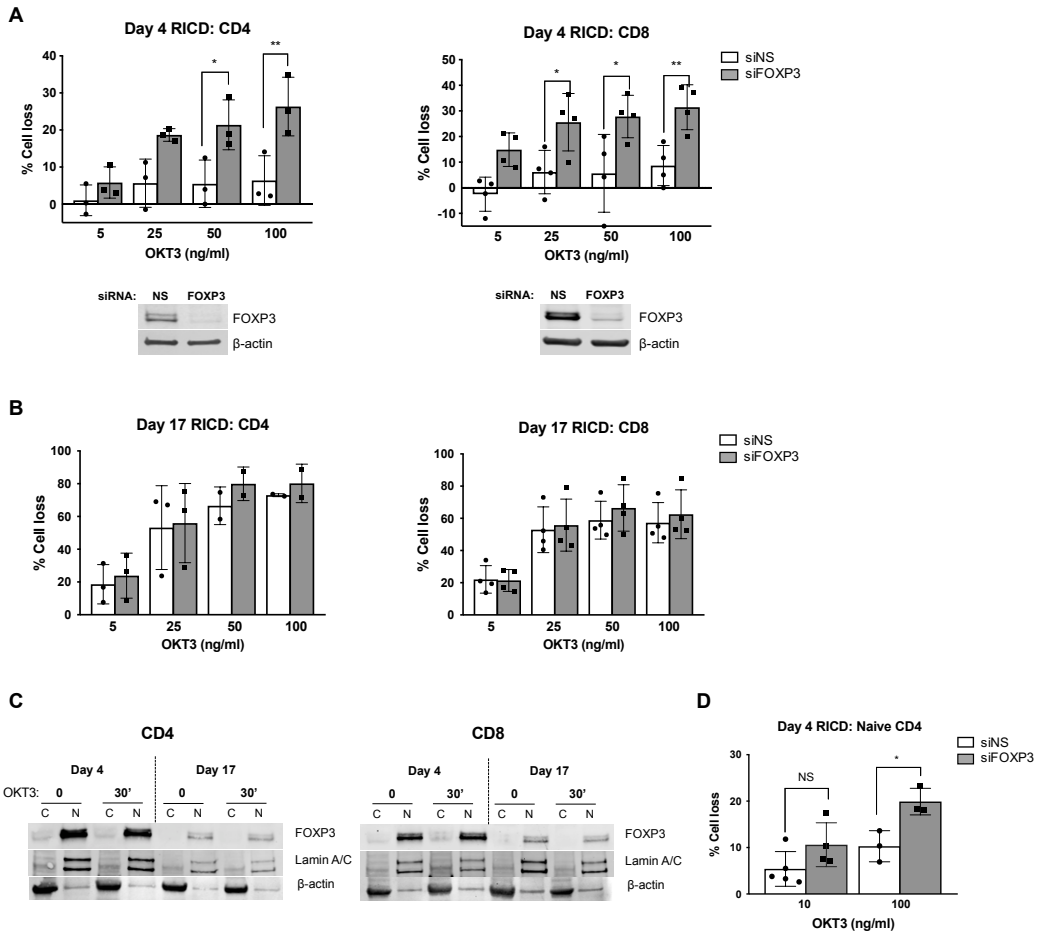


Figure 9. FOXP3 protects expanding T cells from RICD.

A Purified CD4 and CD8 T cells were electroporated with non-specific (NS) scramble siRNA or FOXP3-specific siRNA. Activated cells were then restimulated with OKT3 on day 4 and RICD was measured by PI staining and flow cytometry. Knockdowns were verified by western blot. Statistical significance was tested by one-way ANOVA with Sidak's multiple comparisons

test. CD4: * p=0.017, ** p=0.003. CD8: *p=0.031, * p=0.012, ** p=0.009. Each symbol represents a different human donor.
B Activated CD4 and CD8 T cells were transfected with siRNAs on day 13 post-activation and restimulated for RICD assays on day 17. Statistical significance was assessed by one-way ANOVA; all tests were not significant.
C Day 4 or day 17 activated T cells were restimulated with 500 ng/mL OKT3 for 30 minutes. Cytoplasmic (C) and nuclear (N) fractions were then subjected to SDS-PAGE to assess FOXP3 localization. Lamin A/C was used as a marker for nuclear fractions and β-actin was a marker for cytoplasmic fractions. Data are representative of results from 3 independent experiments.
D Naïve CD4 T cells were purified from 3-5 healthy donors and electroporated with siRNA as in (A). Cells were restimulated with OKT3 on day 4 post-activation to induce RICD. Statistical significance was determined by one-way ANOVA with Sidak's multiple comparisons test * p= 0.021.

FOXP3 knockdown status does not impact T cell proliferation or SAP expression

In order to investigate the mechanism of FOXP3-mediated RICD protection in Tcons, we first assessed whether FOXP3 KD affected T cell proliferation, as FOXP3 has been reported to restrain Tcon proliferation under certain activation conditions (127). However, FOXP3 status did not impact Tcon proliferation in other scenarios (5). We therefore measured the percentage of dividing control or FOXP3 KD cells on day 4 post activation using 5-ethynyl-2'-deoxyuridine (EdU) labeling and flow cytometry (Figure EV 2A). We also assessed cell division by dilution of carboxyfluorescein succinimidyl ester (CFSE) on days 4 and 5 (Figure EV 2B and 2C). Although FOXP3 may act as a negative regulator of proliferation under some conditions, we did not observe any proliferative disparities in our FOXP3 KD cells using either method. Moreover, silencing FOXP3 expression during activation did not alter cell cycle status (Figure EV 2D), which is known to dictate the propensity of effector T cells to die by RICD (24).

We next asked whether traditional pro-apoptotic effector molecules could be responsible for increased RICD in FOXP3 KD cells. We analyzed Fas ligand (FasL)

upregulation, which is intimately associated with RICD execution in late stage effector T cells (39; 204). Intriguingly, TCR restimulation in both CD4+ and CD8+ T cells induced robust FasL expression and cleavage at the plasma membrane, as indicated by the cytoplasmic N-terminal fragment (NTF) (Figure EV 2E). Importantly, FOXP3 KD cells showed no difference in the ability to induce FasL upon restimulation compared to controls. Unlike late stage effector T cells or activated Tregs, we conclude that FasL induction cannot explain increased RICD sensitivity in FOXP3 KD cells. Indeed, FAS receptor engagement in newly activated T cells generally delivers non-apoptotic signals important for cell differentiation (48). We also noted no differences between control and FOXP3 KD cells in expression of BIM (Figure EV 2E), a pro-apoptotic BCL2-family protein that participates in RICD in late-stage effector T cells (187).

We hypothesized that FOXP3 protects expanding Tcons from RICD by suppressing SAP expression, similar to what we have demonstrated in Tregs (86). To address this question, we compared SAP expression in control versus FOXP3 KD lysates. Surprisingly, FOXP3 KD cells did not show any increase in SAP expression (Figure 10A). More fundamentally, we tested whether SAP is required for the low levels of RICD sensitivity observed in early expanding CD4 and CD8 T cells. Although SAP KD reliably decreases RICD sensitivity in late stage effectors (84; 185), reduction of SAP expression was not sufficient to protect cells from RICD at day 4 post activation (Figure 10B), suggesting that RICD susceptibility is regulated differentially between early and late stages of the effector T cell response.

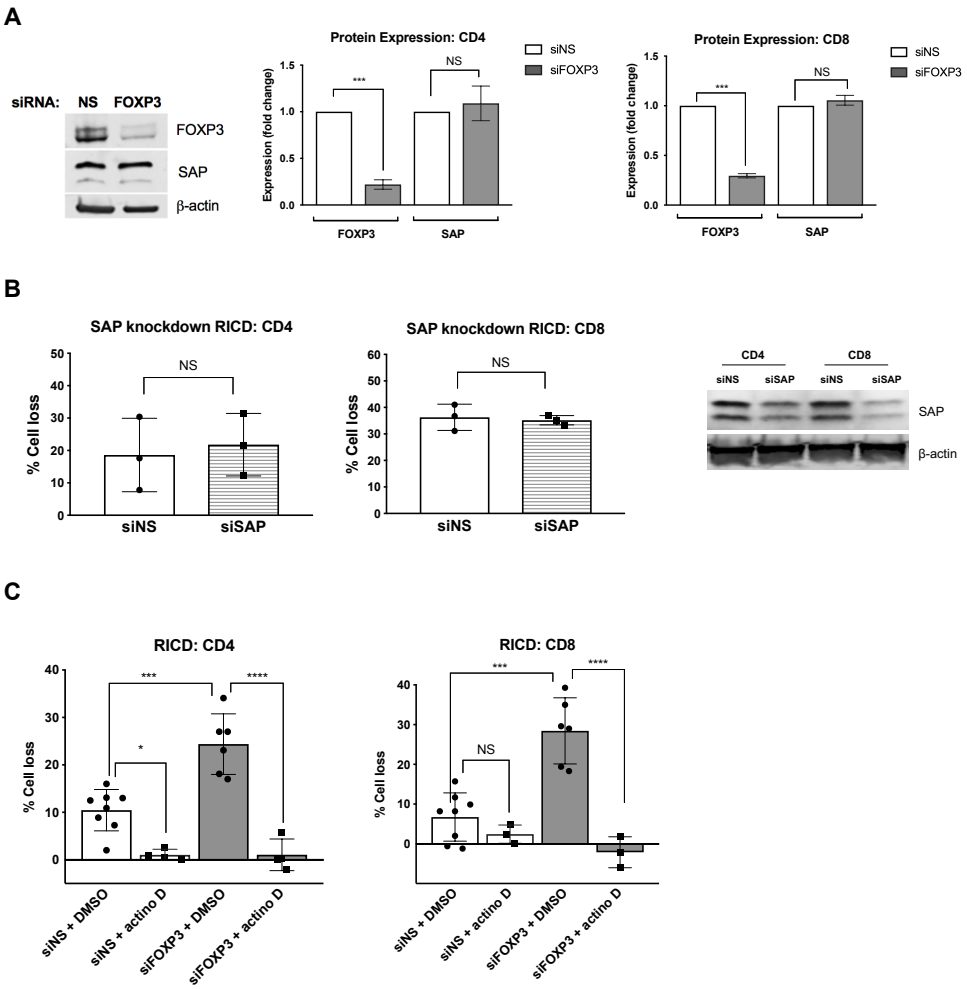


Figure 10. RICD in expanding CD4 and CD8 T cells is SAP-independent, but requires *de novo* transcription.

A Day 4 FOXP3 KD cells were analyzed for FOXP3 and SAP expression by western blot (left panel). Protein expression was quantified after normalization to β-actin (right panels). Data represent 3 independent experiments. Statistical significance was determined by one-way ANOVA with Sidak's multiple comparisons test. CD4: *** $p<0.001$, NS= not significant. CD8: *** $p<0.001$, NS= not significant.

B Purified CD4 and CD8 T cells were electroporated with SAP-specific siRNA or a non-specific (NS) scramble control. Activated cells were then restimulated on day 4 with 100 ng/mL OKT3, and RICD was assessed by PI and flow cytometry. Knockdowns were verified by western blot (right). Statistical significance was assessed by paired t tests, NS= not significant.

C Day 4 KD T cells were pre-treated with 100 ng/mL actinomycin D (actino D) or DMSO solvent control for 30 min before restimulation with 100 ng/mL OKT3. Statistical significance was determined by one-way ANOVA with Sidak's multiple comparisons test. CD4: * $p=0.0198$, *** $p= 0.001$, **** $p<0.0001$. CD8: *** $p=0.001$, ****$p<0.0001$

RICD in expanding T cells is dependent on de novo transcription

Although FOXP3 did not appear to suppress SAP expression, nuclear localization of FOXP3 in expanding Tcons (Figure 9C) implied it likely acts as a functional transcriptional activator or repressor. We determined whether FOXP3-mediated RICD protection was dependent on transcriptional changes by pre-treating control or FOXP3 KD cells with actinomycin D to inhibit *de novo* RNA transcription during TCR restimulation. Surprisingly, actinomycin D treatment almost completely blocked RICD in all T cells, regardless of FOXP3 KD status (Figure 10C). RICD sensitivity in expanding Tcons is therefore entirely dependent on *de novo* transcription, unlike what has been described in late stage effector T cells, which readily succumb to RICD via both pre-formed apoptotic machinery and *de novo* transcription (53).

FOXP3 promotes high CD48 expression in activated T cells

Based on these results, we used an unbiased approach to ascertain global transcriptional changes associated with FOXP3 expression in T cells to identify candidate genes regulating FOXP3-mediated RICD protection. To this end, non-specific siRNA control and FOXP3 KD CD4 and CD8 T cells from 5 healthy human donors were profiled by mRNA sequencing. Comparative gene expression analysis revealed 110 transcripts differentially expressed in either CD4 and CD8 T cells between negative-control and FOXP3 KD treatment groups (Figure 11A). Validation of the knockdown was observed with FOXP3 as the greatest differentially expressed transcript by magnitude (2.93-fold decrease in FPKM value in FOXP3 KD CD4 T cells). Interestingly, the highest abundance transcript that was significantly differentially expressed and decreased by FOXP3 KD in every anonymous human donor sample

profiled was CD48 (SLAMF2), which encodes a protein closely related to the SLAM family of cell surface receptors (Figure 11B) (220). Flow cytometric analysis verified that CD48 protein expression was also significantly decreased in FOXP3 KD T cells (Figure 11C). These data suggest that FOXP3 upregulates CD48 expression after TCR activation. Although typically thought of as a repressor, FOXP3 can also activate of gene transcription in certain contexts (98). Indeed, chromatin immunoprecipitation (ChIP) assays identified CD48 as a target gene for FOXP3 binding in human Tregs (173), and CD48 protein expression is elevated on FOXP3+ Tregs (124).

Transient FOXP3 expression after initial TCR activation helps to enforce RICD resistance during expansion, but effector T cells become sensitized to die as FOXP3 expression wanes over time. We therefore determined whether CD48 expression followed a similar pattern in CD4 and CD8 T cells over 20 days post activation using flow cytometry. We followed CD48 expression on CD4 and CD8 T cells before activation and over 20 days post activation. Indeed, CD48 expression was rapidly induced in activated T cells, peaking at days 4-5 before declining over time (Figure 11E). Although CD48 expression was not strictly dependent on FOXP3 (Figure 11C), our data strongly suggest that FOXP3 enhances CD48 expression on activated Tcons during early expansion.

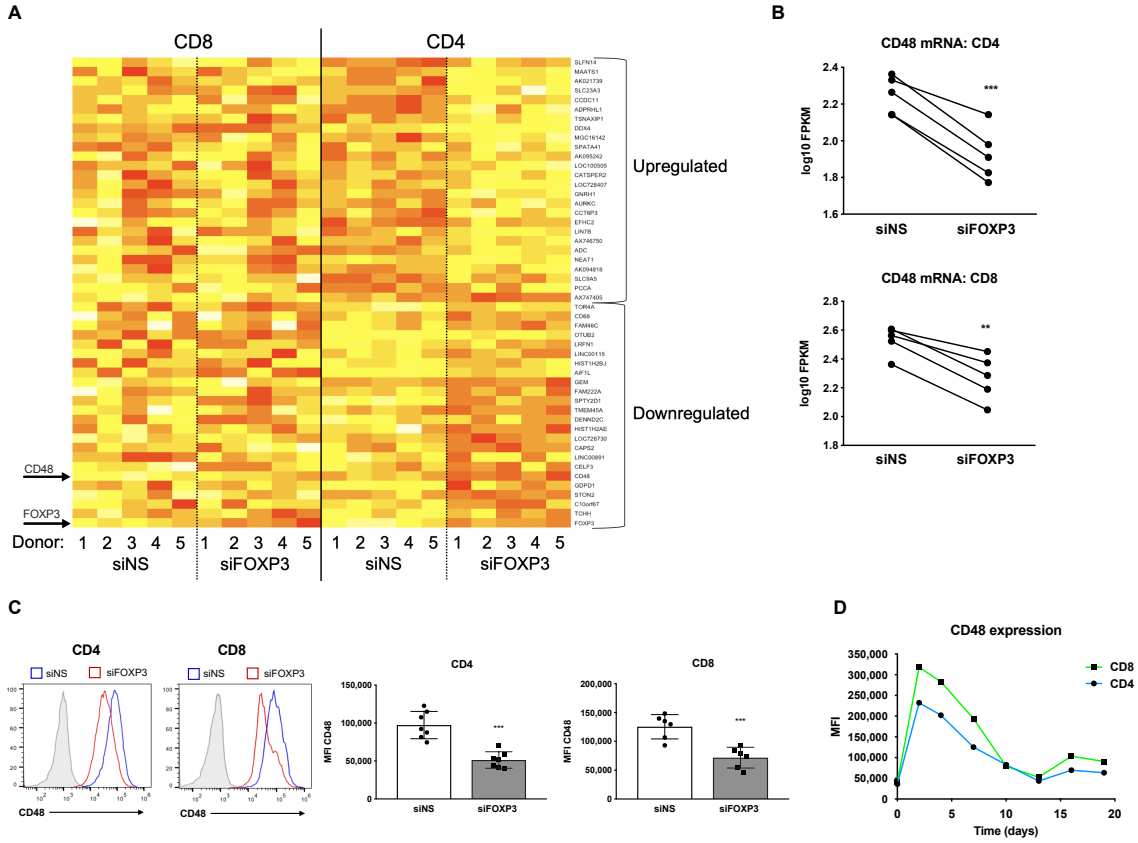


Figure 11. CD48 expression is impaired in FOXP3 KD T cells.

A Day 4 activated CD4 and CD8 KD T cells (NS vs. FOXP3) from 5 anonymous healthy blood donors were subjected to transcriptome profiling by mRNA-Seq. Normalized mean-centered log base 10 FPKM values are used for heatmap visualization of expression levels (yellow to red color scale represents higher to lower expression levels, respectively). Arrows indicate CD48 and FOXP3.

B CD48 transcript levels (fragments per kilobase of transcript per million reads (FPKM)) from each donor are displayed. Statistical significance was determined by paired t test. CD4: ***p=0.0008. CD8: **p=0.0025.

C Cell surface expression of CD48 protein on NS (blue) vs. FOXP3 KD (red) T cells was measured by flow cytometry on day 4 post-activation. Isotype control Ab staining is shown in light gray (left). CD48 expression was quantified by mean fluorescence intensity (MFI) in multiple donors (right panels). Statistical significance was determined using unpaired t tests. CD4: ***p=0.0001 CD8: ***p=0.0008.

D CD48 expression (MFI) was quantified over time on purified CD4 and CD8 T cells before and after activation. Data are representative of 3 separate experiments using different donors.

CD48 protects activated CD4 and CD8 T cells from RICD

To determine if CD48 was involved in enforcing FOXP3-dependent resistance to RICD in early effectors, we conducted siRNA-mediated knockdowns of CD48 in CD4 and CD8 T cells during activation and measured RICD sensitivity. Similar to FOXP3 KD T cells, CD48 KD T cells exhibited analogous increases in RICD sensitivity (Figure 12A). Interestingly, CD48 expression was only partly reduced by both FOXP3 and CD48 siRNA (Figure 12A), further implying that a stable pool of CD48 protein exists independently of FOXP3-mediated transcriptional regulation.

We also asked whether pre-treatment of T cells with a CD48 blocking antibody (Ab) before restimulation would also enhance RICD. Indeed, blockade of CD48 consistently resulted in higher RICD in both Jurkat T cells (Figure 12B) and primary CD4 and CD8 T cells (Figure 12C) at day 4 post-activation. Importantly, CD48 blockade did not boost RICD levels in FOXP3 KD cells (Figure 12C), implying FOXP3 exerts RICD protection through CD48 upregulation in early activated T cells.

CD48 ligand interactions demonstrate variable effects on RICD sensitivity in human donors

CD48 is a GPI-anchored surface receptor that has two known binding partners: CD2 and 2B4 (55). To investigate whether CD48-mediated RICD protection may be dependent on these specific ligand interactions, we first assessed surface expression of CD48, CD2, and 2B4 on control versus FOXP3 KD cells. Although CD48 and CD2 were both expressed at high levels on CD4+ and CD8+ T cells on day 4 post activation, 2B4 expression was relatively low (Figure 12D). Additionally, only CD48 expression was reduced in FOXP3 KD cells, while CD2 and 2B4 expression remained unaffected (Figure 12D). We next employed blocking antibodies specific for CD2 or 2B4 in RICD assays at

day 4 post-activation. Although CD48 blockade consistently increased RICD in both CD4 and CD8 T cells (relative to isotype control Ab), CD2 and 2B4 blockade did not significantly alter death sensitivity (Figure 12E). We noted high variability across donors for RICD levels in this context: for example, half of donors demonstrated increased RICD sensitivity upon 2B4 blockade, whereas other donors showed no change. In some cases CD2 blockade actually reduced RICD sensitivity, whereas other donors showed no change in death compared to controls. To address whether both receptors influenced RICD sensitivity simultaneously, we applied CD2/2B4 blocking antibodies independently or in combination before TCR restimulation for additional donors (Figure 12F). Although donor variability persisted, some trends emerged. For example, application of combined CD2 and 2B4 blockade generally reduced RICD sensitivity compared to 2B4 blockade alone for individual donors (Figure 12F). Collectively, these inconsistent results may reflect the complex, bimodal nature of SLAM receptor signaling (including CD2 and 2B4) (68), which may vary with CD48 abundance and/or unknown CD48 interaction partners. Based on the strong and consistent effects we saw across multiple human donors with CD48 KD or Ab-mediated blockade, we therefore focused on CD48 disruption alone to explore further mechanisms of RICD protection.

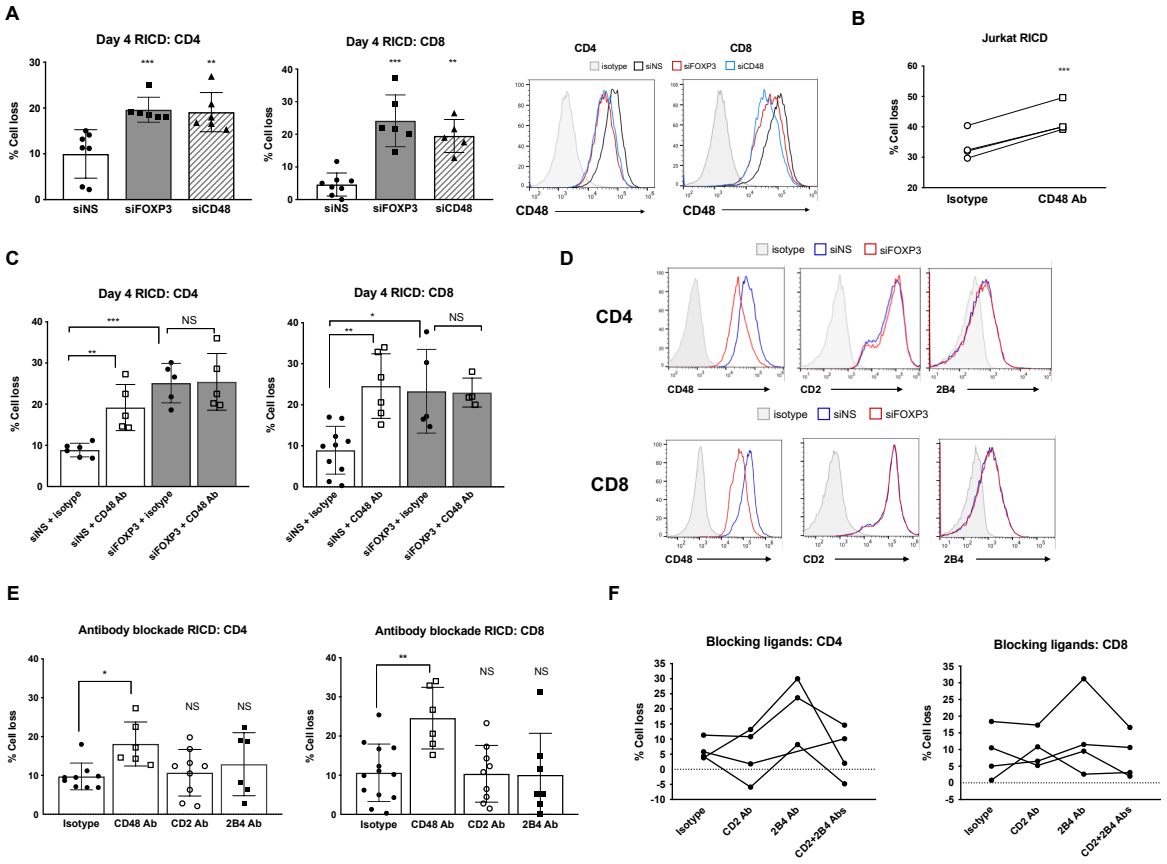


Figure 12. CD48 expression protects expanding CD4 and CD8 T cells from RICD.

A Purified CD4 and CD8 T cells were electroporated with siRNA against FOXP3, CD48, or a non-specific (NS) scramble control. Activated T cells were restimulated with 100 ng/mL OKT3 on day 4 to conduct RICD assays. Statistical significance was determined by one-way ANOVA with Dunnett's multiple comparisons test. CD4: ***p=0.0019, **p=0.003. CD8: ***p= 0.001, **p= 0.0005. Knockdowns were verified by flow cytometry (right).

B Jurkat T cells were incubated with10 µg/mL anti-CD48 blocking Ab or an isotype control Ab for 1 hour before restimulation with 500 ng/mL OKT3 to induce RICD. Statistical significance was determined by paired t test *** p=0.0003

C NS control or FOXP3 KD cells were incubated with 10 µg/mL anti-CD48 blocking Ab or an isotype control Ab for 1 hour and then restimulated with 100 ng/mL OKT3 to induce RICD. Statistical significance was determined by one-way ANOVA with Sidak's multiple comparisons test. CD4: **p=0.0097, ***p=0.0001. CD8: **p=0.0011, *p=0.0042.

D Surface expression of CD48, CD2, and 2B4 was measured by flow cytometry on day 4 activated NS control and FOXP3 KD T cells. Results are representative of at least 3 independent experiments.

E Day 4 activated CD4+ and CD8+ T cells were incubated with 10 µg/mL anti-CD48 blocking Ab, CD2-blocking Ab, 2B4-blocking Ab, or an isotype control Ab for 1 hour and then restimulated with 100 ng/mL OKT3. Statistical

significance was determined by one-way ANOVA with Dunnett's multiple comparisons test. CD4: *p=0.0297 CD8: **p=0.0045.
F Day 4 activated CD4 and CD8 T cells were incubated with 10 μg/mL CD2-blocking Ab, 2B4-blocking Ab, both Abs, or an isotype control Ab for 1 hour. Cells were then restimulated with 100 ng/ml OKT3 to induce RICD. Data from individual donors are displayed with connected lines.

FOXP3 and CD48 expression protect cells from RICD via promotion of autophagy

Our data indicates that FOXP3 protects expanding Tcons from RICD by augmenting CD48 expression. Since this is the first evidence connecting FOXP3 and CD48 expression to relative RICD sensitivity in Tcons, we sought to further elucidate downstream mechanisms of apoptosis protection. Recent reports suggest that autophagy protects late stage effector T cells from RICD (47), and that elevated autophagy in Tregs maintains their survival and functional integrity (213). We first asked whether autophagy protects early expanding T cells from RICD at day 4 post activation. Indeed, cells pre-treated with chloroquine (CHQ) or E-64d to inhibit autophagy showed increased death following TCR restimulation at day 4 (Figure 13A). We then asked if siRNA-mediated knockdown of FOXP3 or CD48 would alter basal autophagy in early activated T cells, as detected by Cyto-ID, a highly specific, quantitative stain for autophagic vesicles (67). Remarkably, both FOXP3 and CD48 KD T cells exhibited a small but significant decrease in autophagy relative to control KD T cells (Figure 13B). To specifically interrogate the importance of autophagy in FOXP3+ Tcons, we transfected activated T cells with siRNA specific for FOXP3, ATG3, or combined. ATG3 is an E2-like ubiquitin carrier protein that is essential for autophagosome maturation (77). Neither FOXP3 nor ATG3 KD altered TCR-induced CD25 and CD69 expression (Figure EV 3), indicating T

cell activation remained intact under these conditions. Congruent with pharmacological inhibition of autophagy, ATG3 KD enhanced RICD sensitivity in both CD4 and CD8 T cells, similar to FOXP3 KD cells (Figure 13C). However, simultaneous silencing of FOXP3 and ATG3 expression did not further increase RICD, suggesting the protective effect of FOXP3 is dependent on autophagy. Finally, we pre-treated cells with the dual PI-3K/mTOR inhibitor dactolisib (BEZ235) (119) to promote more autophagy before TCR restimulation (Figure 13D). BEZ235 pre-treatment significantly protected only FOXP3 KD cells from death, suggesting enforced elevation of autophagy could rescue FOXP3-depleted Tcons from RICD.

Since CD48 KD T cells also displayed less autophagy than controls (Figure 13B), we next tested whether CD48 blockade would also decrease basal autophagy. We first noted a reduction in Cyto-ID staining in Jurkat T cells treated with CD48 blocking Ab versus isotype control (Figure 13E), indicative of decreased autophagy. In primary Tcons, we also detected a decrease in Cyto-ID staining with CD48 blockade in control cells, which was lost in FOXP3 KD cells that already showed decreased Cyto-ID fluorescence (Figure 13F). Taken together, our results illuminate a novel pathway whereby transient FOXP3 protects early expanding Tcons from premature RICD by promoting a CD48-dependent autophagy program.

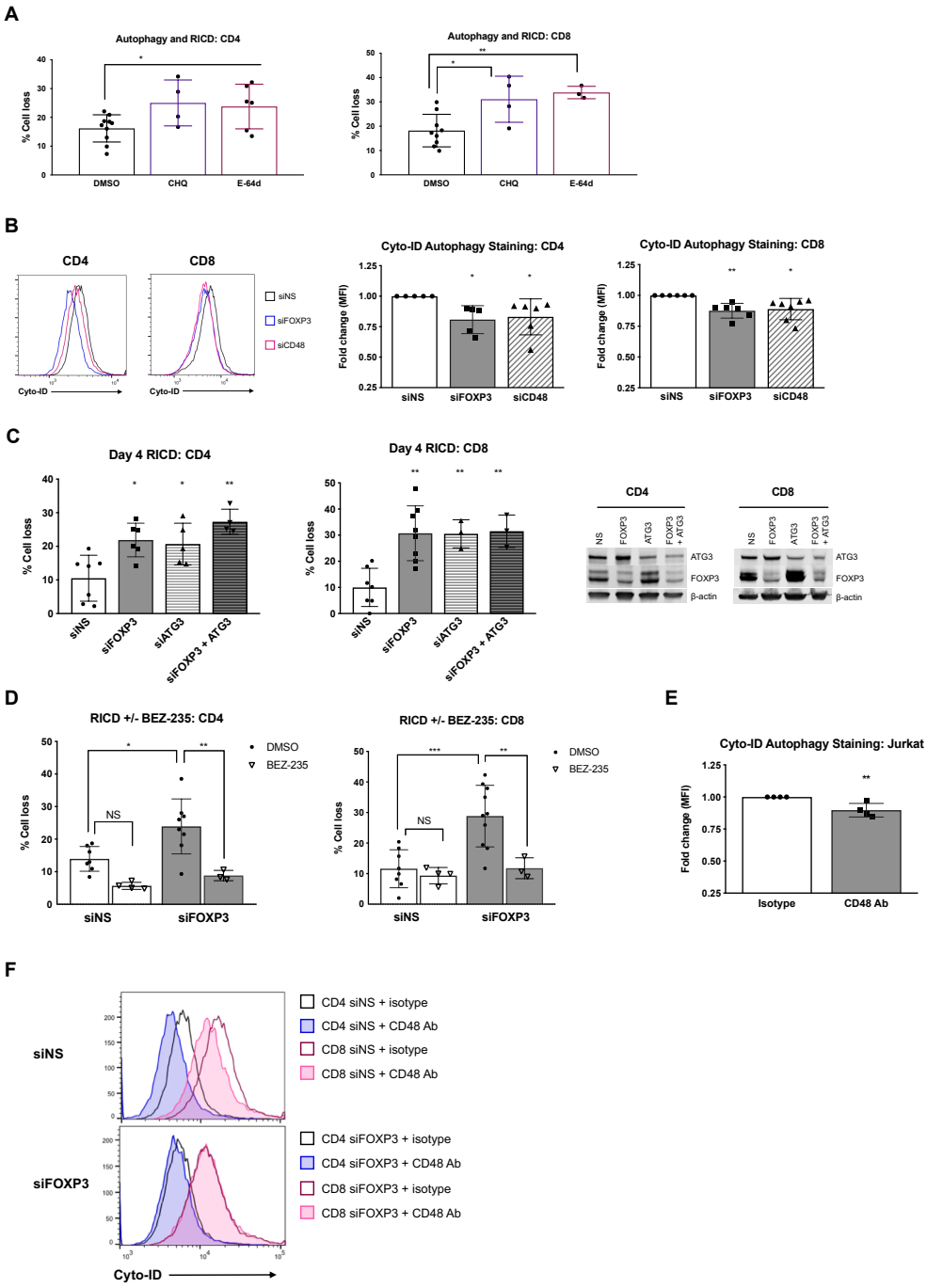


Figure 13. FOXP3 and CD48 expression promote autophagy to protect T cells from RICD.

A Activated CD4 and CD8 T cells were treated with 10 μM chloroquine (CHQ), 10 μM E-64d, or DMSO solvent control for 1 hour before restimulation with OKT3 to induce RICD. Statistical significance was determined by one-way ANOVA with Bonferroni's multiple comparisons test. CD4: *p=0.0222, *p=0.0244 E-64d. CD8: *p=0.0108, **p=0.0051.

B Day 4 activated FOXP3 and CD48 KD T cells were stained with Cyto-ID to quantify autophagic vesicles. Cyto-ID MFI was normalized to NS control KD cells. Each data point represents a value from an individual donor (right panels). Statistical significance was determined by one-way ANOVA with Dunnett's multiple comparisons test. CD4: *p=0.0309 siFOXP3 *p=0.0462 siCD48. CD8: **p=0.0064, *p=0.0109.

C Purified CD4+ and CD8+ T cells were electroporated with siRNA against FOXP3, ATG3, FOXP3 + ATG3 combined, or non-specific (NS) control. Activated T cells were restimulated with 100 ng/mL OKT3 on day 4 to conduct RICD assays. Statistical significance was determined by one-way ANOVA with Sidak's multiple comparisons test. CD4: *p=0.0115 siFOXP3, *p=0.0349 siATG3, **p=0.0011. CD8: ***p=0.006, **p=0.0082 siATG3, **p=0.0056 siFOXP3+ATG3. Knockdowns were verified by western blot analysis (right).

D Day 4 activated CD4 and CD8 KD T cells (NS vs. FOXP3) were pre-treated with 1μM BEZ235 or DMSO for 30 minutes before restimulation with 100 ng/mL OKT3 to induce RICD. Statistical significance was determined by one-way ANOVA with Sidak's multiple comparisons test. CD4: *p=0.0104, **p=0.0032. CD8: **p=0.0083, ***p=0.0003.

E Jurkat T cells were treated with 10 μg/mL anti-CD48 blocking Ab or an isotype control Ab for 4 hours and then subjected to Cyto-ID autophagy staining. Fold change in Cyto-ID MFI (normalized to NS) is shown at right for 4 independent experiments. Statistical significance was determined by unpaired t test. ** p= 0.0081.

F Day 4 activated CD4 and CD8 KD T cells (NS vs. FOXP3) were incubated with 10 μg/mL anti-CD48 blocking Ab or an isotype control Ab for 6 hours and stained with Cyto-ID. Data are representative of 3 independent experiments.

EXPANDED VIEW (EV) FIGURES

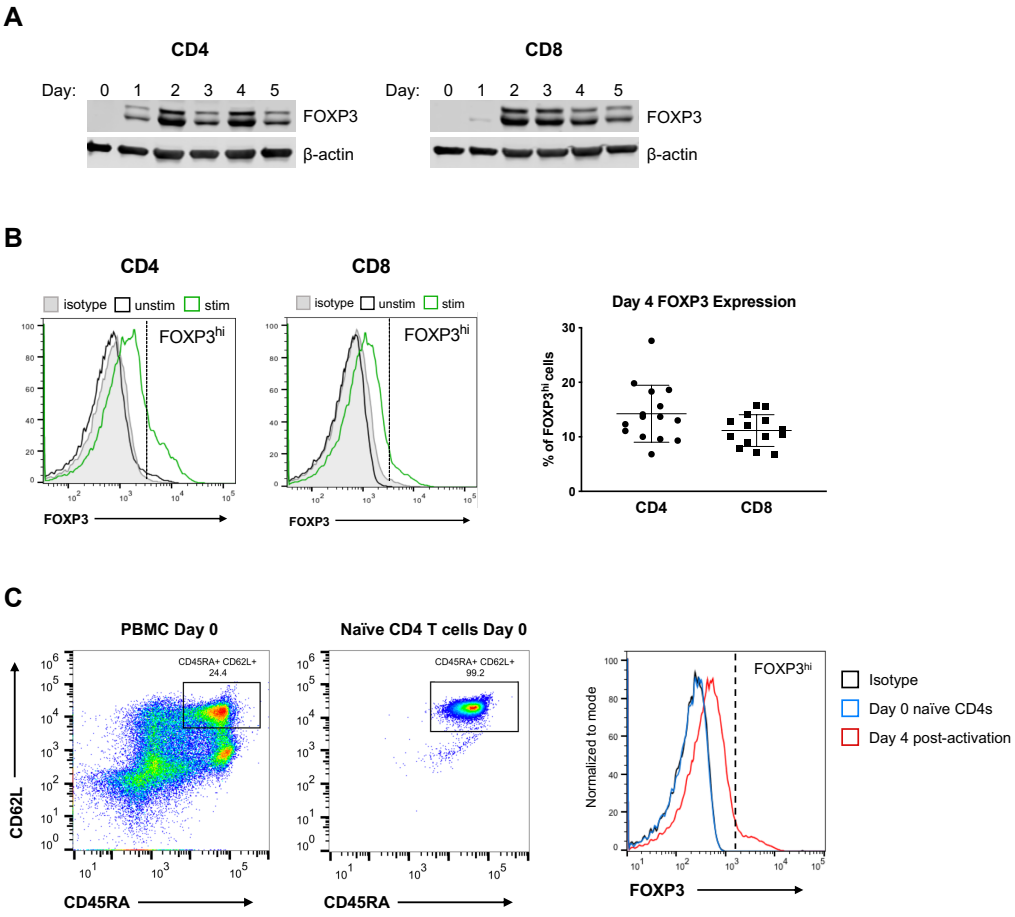


Figure EV 1. FOXP3 expression in multiple human donors.

A Primary CD4 and CD8 T cells were isolated by negative selection from healthy human donors. Cells were then activated with α-CD2/3/28 beads on day 0 and exogenous IL-2 was added to the media on day 3. Whole cell lysates were subjected to SDS-PAGE and immunoblot analysis. Data are representative of 3 independent experiments.

B Single-cell analysis of FOXP3 expression by intracellular flow cytometry (ICFC). Purified CD4 and CD8 T cells were activated as in (A). FOXP3 expression was measured by ICFC on day 4. Unstimulated cells were included as a negative control. Cells were defined as FOXP3hi as shown (left), and quantified across multiple donors (right).

C Naïve CD4 T cells were purified from healthy donor PBMCs. Purity was validated by surface staining of CD45RA and CD62L. Naïve cells were then activated with Immunocult and FOXP3 expression was assessed on day 4 post-activation as in (B).

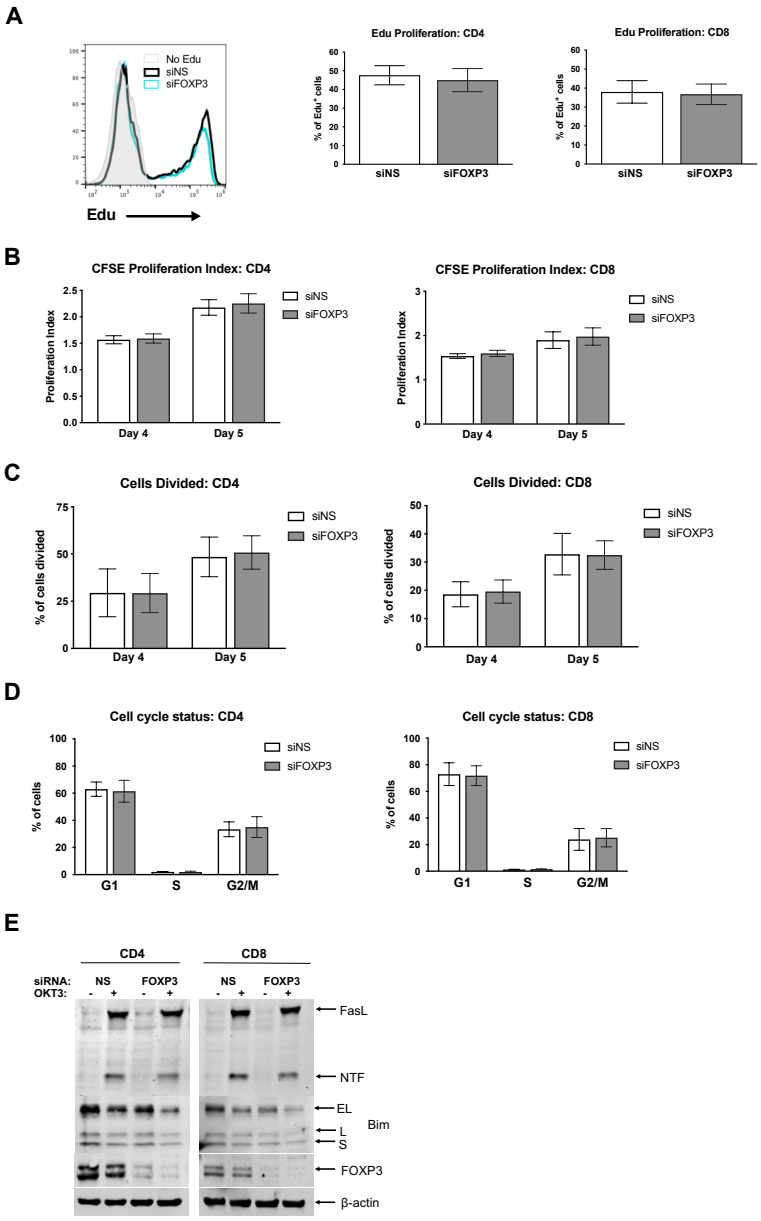


Figure EV 2. FOXP3 knockdown does not impact T cell proliferation, cell cycle status, or Fas ligand and BIM expression.

A Day 4 CD4 and CD8 KD cells were subjected to Click-iT Edu staining to quantify the percentage of dividing cells. Cells incubated with no Edu-FITC served as a negative control shown in gray (left). Data are the combination of 3 independent experiments.

B Carboxyfluorescein succinimidyl ester (CFSE) labeling was conducted on CD4 and CD8 FOXP3 KD cells on day 0 before activation. CFSE was then analyzed by flow cytometry on days 4 and 5 post-activation. The proliferation index was calculated by the total number of divisions by the number of cells that went into that division. Data from 3 independent experiments are plotted.

C CFSE labeling was conducted on KD cells as in (B). The % of cells that had undergone at least one cell division were determined by flow cytometry. Data from 3 independent experiments are plotted.

D Day 4 CD4 and CD8 FOXP3 KD cells were subjected to cell cycle analysis by propidium iodide staining and flow cytometric analysis. Data are the combination of 3 independent experiments.

E Activated CD4 and CD8 KD cells were restimulated with 500ng/mL OKT3 for 4 hours on day 4 post-activation. Lysates were subjected to SDS-PAGE and immunoblotting for Fas ligand (FasL) and its' cytoplasmic N-terminal fragment (NTF) cleavage product. BIM immunoblotting consisted of 3 BIM transcripts: extra-long (EL), long (L) and short (S). Figure is representative of data from 3 independent experiments.

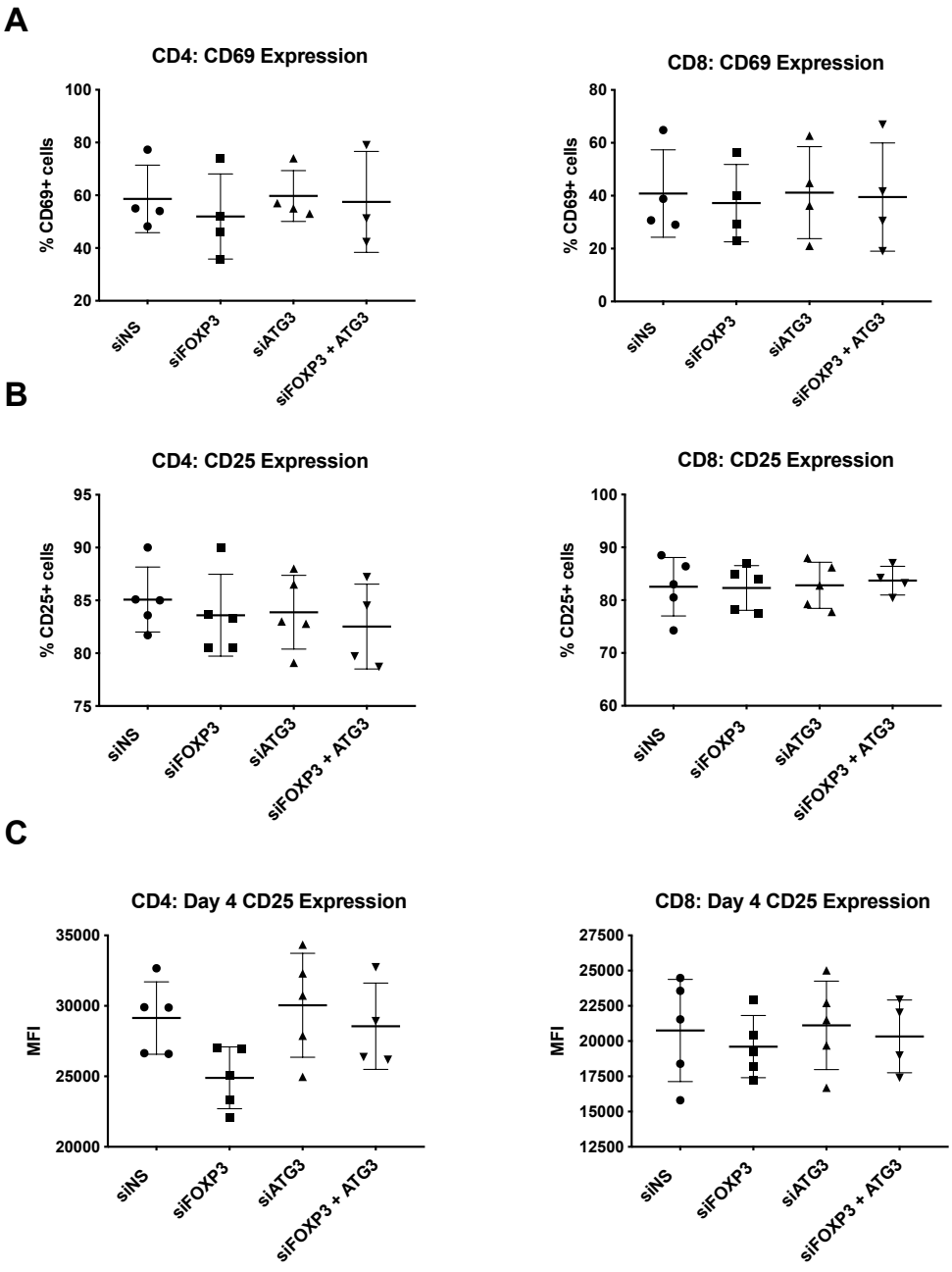


Figure EV 3. FOXP3 and ATG3 silencing on T cell activation markers.

Purified CD4 and CD8 T cells were electroporated with siRNA against FOXP3, ATG3, FOXP3 and ATG3 combined, or a non-specific (NS) scramble control. Cells were then activated with Immunocult and subjected to cell surface staining on day 4 post-activation.

A CD69 expression was measured by flow cytometry on day 4.

B CD25 expression was measured by flow cytometry on day 4.

C CD25 mean fluorescence intensity (MFI) on day 4.

DISCUSSION

The overwhelming majority of literature concerning the function of FOXP3 in T cell immunity has focused on how FOXP3 governs the transcriptional program of Tregs. Indeed, null and loss of function *FOXP3* mutations cause immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome in humans due to loss of Treg development, thus warranting the attention. Aside from this, although transient TCR-induced FOXP3 expression in human (but not murine) Tcons after TCR activation has been described by many groups, its function has never been fully elucidated. Studies to date suggest that FOXP3 in human Tcons may serve independent functions, compared to its role in Tregs. For example, FOXP3 was implicated as an intrinsic regulator of Th17 cells by suppressing IFN-γ production and up-regulating CCR4 (127). Th17 cells have higher FOXP3 expression than Th1 cells, and intriguingly Th17 cells are more resistant to RICD than Th1 cells (38; 183). Oddly enough, FOXP3 acts as a negative regulator of cytokine production in equine T cells as well (37), which may represent a conserved function of FOXP3 in Tcons more analogous to the human immune system than mice. In the present study, we asked whether transient FOXP3 upregulation in protects newly-activated Tcons from RICD, similar to CD4 FOXP3+ Tregs (86; 214). Indeed, our results show for the first time that FOXP3 also modulates RICD sensitivity in both CD4+ and CD8 human Tcons.

Although transient FOXP3 expression protected a proportion of both CD4 and CD8 T cells from premature RICD, this protection was SAP-independent. Silencing of FOXP3 did not noticeably alter SAP expression, and silencing of SAP itself had no impact on RICD sensitivity in early expanding T cells. These data therefore implied a

FOXP3 could elicit RICD resistance in different T cell compartments through distinct mechanisms. Indeed, Tcons and Tregs are subjected to unique challenges that likely require differential regulation of apoptosis. For example, Tregs require IL-2 from effector T cells and experience frequent TCR restimulation via self-antigen, both during thymic development and in the periphery (133). Therefore, it is logical that FOXP3 in Tregs acts to suppress SAP in order to lower TCR signal strength threshold and evade RICD. Nevertheless, our previous findings indicated that ectopic expression of FOXP3 alone in Tcons was not sufficient to effectively suppress SAP and enforce RICD resistance (86). Downstream FOXP3-dependent transcriptional effects may be restricted by the availability of certain interacting partners in Tregs versus Tcons, such as NFAT and Helios (169; 201). For example, Helios is an important binding partner that is present only in natural Tregs and required for FOXP3 to mediate certain suppressive features (74; 87; 177). More recent studies have only revealed additional complexity in the variety of co-factors FOXP3 interacts with to mediate transcriptional repression or induction in Tregs (98). Further studies will be required to dissect which FOXP3-containing complexes are responsible for suppression of SAP in Tregs versus induction of CD48 and other molecules in early Tcons.

Our results showing differential RICD sensitivity in early (day 4) versus late stage (day 17) CD4 and CD8 T cells emphasize the importance of temporal apoptosis regulation. Induced FOXP3 expression helped to enforce RICD resistance during early T cell expansion, but not during later stages when FOXP3 levels are considerably diminished. Conversely, SAP had no impact on RICD sensitivity in early activated Tcons despite its critical role in boosting RICD sensitivity in late stage effectors, when both

SAP and NTB-A expression are at peak levels. Our results highlight some important differences in how RICD is regulated in newly activated T cells. For example, we found that low levels of RICD measured at day 4 post-activation were completely dependent on *de novo* transcription. Additionally, Fas ligand (FASL) and BIM expression were unaffected by FOXP3 KD in early Tcons, and likely do not contribute substantially to the execution of RICD in this context compared to late stage effectors (186; 212). Dynamic changes in costimulatory and coinhibitory receptor signaling also modify TCR signaling and metabolism to influence RICD sensitivity (208). Metabolic reprogramming is emerging as an important governor of T cell apoptosis; indeed, we discovered that glycolysis facilitates RICD in late stage effectors by licensing optimal FASL induction (99), but newly activated cells likely differ considering they resist direct FAS-induced killing (129). Clearly, RICD sensitivity is carefully calibrated temporally via different mechanisms throughout the life of a T cell, although AICD/RICD literature has focused primarily on thymocyte selection and late stages of effector T cell differentiation.

Our RNA-Seq analyses and FOXP3 KD results suggest that FOXP3 directly upregulates CD48 expression, consistent with the presence of FOXP3 binding motifs in the CD48 promoter, as well as genome-wide ChIP-Seq paper identifying CD48 as a FOXP3 target gene in human Tregs (173). Higher CD48 expression was also noted on FOXP3+ Tregs compared to Tcons in mice (124). More importantly, our findings illuminate a novel role for CD48 in protecting CD4 and CD8 T cells from premature RICD. Knockdown or blockade of CD48 consistently increased RICD sensitivity, despite variable results obtained with blockade of its two known interaction partners, CD2 and 2B4. These observations underscore the complexity of SLAM receptor signaling and will

require further clarification to determine whether (a) CD2/2B4 compete for CD48 ligation with differential signaling outcomes, (b) CD48 binds to hitherto unknown ligands on T cells, or (c) whether CD48 signals in a ligand-independent manner. Indeed, activating versus inhibitory signaling through 2B4 ligation on natural killer cells may depend on CD48 abundance on target cells (220). CD48 is a glycosylphosphatidylinositol (GPI)-linked protein that localizes to cholesterol-rich lipid rafts and can influence TCR signal transduction from within the immunological synapse (55; 138). Regardless, the novel role we describe here for CD48 in promoting apoptosis resistance may shed new light on phenotypes noted in previous murine T cell studies. Purified CD4 T cells from CD48-deficient mice showed a profound defect in proliferation after activation *in vitro* (65). Given that the absolute cell number (measured solely by tritiated thymidine incorporation) reflects both the rate of proliferation as well as cell death, the decreased number of CD48-deficient CD4 T cells noted in this study could be indicative of enhanced RICD sensitivity.

Our data suggest CD48 impacts RICD sensitivity in part by promoting autophagy. A wealth of emerging evidence points to macroautophagy as a critical homeostatic process in T cells that regulates their proliferation, metabolism, differentiation, and survival (54). Our results are consistent with the model recently proposed by Corrado *et al* by which macroautophagy protects T cells from RICD via clearance of damaged mitochondria upon TCR restimulation (47). Indeed, key autophagy proteins like ATG12 and ATG3 have been directly implicated in regulating mitochondrial homeostasis and intrinsic cell death (166). Utilizing both pharmacological interventions and ATG3 KD, we found autophagy protected early activated CD4+ and CD8+ T cells from RICD, and

that FOXP3 or CD48 KD resulted in less basal autophagy. Blockade of CD48 also reduced autophagy in early Tcons, suggesting that some feature of tonic CD48 signaling at higher expression levels enhances basal autophagy. The signaling mechanisms that connect CD48 and autophagy regulation remain unclear, but this novel connection warrants further investigation, especially in light of other differentially expressed genes regulated by FOXP3 expression.

Given that CD48 can "tune" apoptosis sensitivity of CD4 and CD8 T cells, our results highlight a potentially attractive opportunity for CD48-targeted immunotherapies, as others have suggested (2; 55; 125). Unlike various costimulatory and coinhibitory receptors with defined cytoplasmic signaling domains and cell-specific expression patterns, CD48 is GPI-anchored protein ubiquitously expressed on many hematopoietic cell types. Additionally, there may be some functional differences in CD48 signaling and apoptosis sensitivity between CD4 and CD8 subsets that our experimental approaches could not decipher. Despite these uncertainties, the potential for CD48-targeted therapies is appealing. For example, anti-CD48 antibody administration ameliorated experimental autoimmune encephalomyelitis (EAE) disease severity in mice, specifically by limiting the number of pathogenic CD4 T cells (124). We posit this effect could be explained in part because CD48 blockade constrains expansion of activated pathogenic cells via increased RICD sensitivity. In conclusion, our results describe a novel function for FOXP3 expression in shielding human Tcons from premature RICD during expansion by upregulating CD48 and protective autophagy. Although the precise protective mechanisms linking FOXP3, CD48, and macroautophagy remain to be completely defined, we believe this nascent signaling program presents a potential therapeutic target

for novel immunotherapies that can hinder or boost human T cell responses as needed by tuning RICD susceptibility during clonal expansion.

ACKNOWLEDGEMENTS

We thank Michael Lenardo for generously providing access to anonymous healthy donor buffy coats from the NIH Blood Bank. We also thank Robert Kortum, Brian Schaefer, Chou-Zen Giam, Edward Mitre, and Jason Lees for helpful discussions. We thank Kateryna Lund and Kheem Bhist for flow cytometry assistance and support.

This work was funded by grants from the National Institutes of Health (1R01GM105821) and Uniformed Services University.

AUTHOR CONTRIBUTIONS

K.V. and A.L.S. conceptualized the project. K.V. designed and conducted the experiments, analyzed the results, graphed and visualized the data, and wrote the manuscript. N.M.L., C.L.D. A.R.S. and G.S. conducted RNA-Seq experiments and analyses. C.L., C.R.L., B.D., S.A., B.M.B. assisted with various experiments. A.L.S. and C.L.D. reviewed and edited the paper and supervised the project.

CHAPTER 4: Metabolic reprogramming and apoptosis sensitivity: defining the contours of a T cell response

Published as: Kelsey Voss*, Sasha E. Larsen, and Andrew L. Snow. Metabolic reprogramming and apoptosis sensitivity: defining the contours of a T cell response. *Cancer Letters* 2017; 408:190-196.

*first-author

This work was published as a review article. Kelsey Voss wrote all sections of the paper, except for the tumor microenvironment section which was predominantly written by Andrew L. Snow. Sasha E. Larsen contributed to sections on glycolytic metabolism and RICD as well as general edits.

ABSTRACT

An effective adaptive immune response hinges on the rapid clonal expansion of T cells in response to antigen. The sensitivity of these T cells to programmed cell death (i.e. apoptosis) is carefully calibrated at various stages to ensure a robust yet measured reaction that resolves without inflicting unintended damage to host tissues. To meet bioenergetic demands associated with vigorous proliferation, acquisition of effector functions, and memory formation, T cells also undergo dynamic changes in their metabolism at every stage of this response. In this review, we focus on relatively recent studies that illuminate intimate links between metabolic programs and apoptosis sensitivity in T cells. We then examine how these connections ultimately influence T cell survival and function within the metabolically taxing environs of the tumor microenvironment.

INTRODUCTION

The adaptive immune response is intricately linked to the cellular metabolism of lymphocytes. Many recent studies have underscored the importance of dynamic metabolic reprogramming over the course of the immune response (34; 153). Basic properties associated with effector T and B cells, including proliferation, differentiation, migration, and effector functions (e.g. cytokine production), have different metabolic demands and therefore require flexibility in metabolic programming. This metabolic adaptability is also vital for responding T cells that encounter challenging anatomical niches, including the tumor microenvironment or certain sites of infection such as *Mycobacterium tuberculosis (Mtb)*-induced granulomas.

Although advances in our understanding of both immunology and cancer biology have empowered progress in immunometabolism research, the extent to which changes in metabolic programs shape the T cell memory response requires further elucidation. Moreover, the magnitude and potency of any T cell response is also ultimately shaped by the sensitivity of those T cells to programmed cell death at various phases. However, the question of how cellular metabolism contributes to T cell apoptosis during the immune response has only just started to be investigated.

As the adaptive immune response unfolds, responding T cells become sensitized to various apoptotic signals in order to maintain immune homeostasis. Following activation and several days of clonal expansion, effector T cells become increasingly sensitive to apoptosis in the presence of cytokines (24; 102). Specifically, at the peak of an adaptive immune response when antigen is still abundant, T cells are subject to a "negative feedback" apoptotic mechanism mediated by restimulation of the T cell

receptor (TCR) (172). This TCR-induced apoptosis, also known as restimulation-induced cell death (RICD), constrains the magnitude of effector T cell proliferation to prevent damaging immunopathology (188). As pathogens and/or transformed cells are eliminated from the system, antigens are cleared and cytokines such as interleukin-2 (IL-2) that promote T cell expansion also decline. Waning cytokines and growth factors induce an intrinsic apoptosis program known as cytokine withdrawal-induced death (CWID), also referred to as activated cell autonomous death (ACAD) (28; 192). CWID culls most of the effector T cell population during this contraction phase, leaving only a small pool of T cells that persist as memory T cells. Both RICD and CWID are critical for maintaining immune homeostasis; therefore, this review will focus on how these death programs are influenced by T cell metabolism (Figure 14).

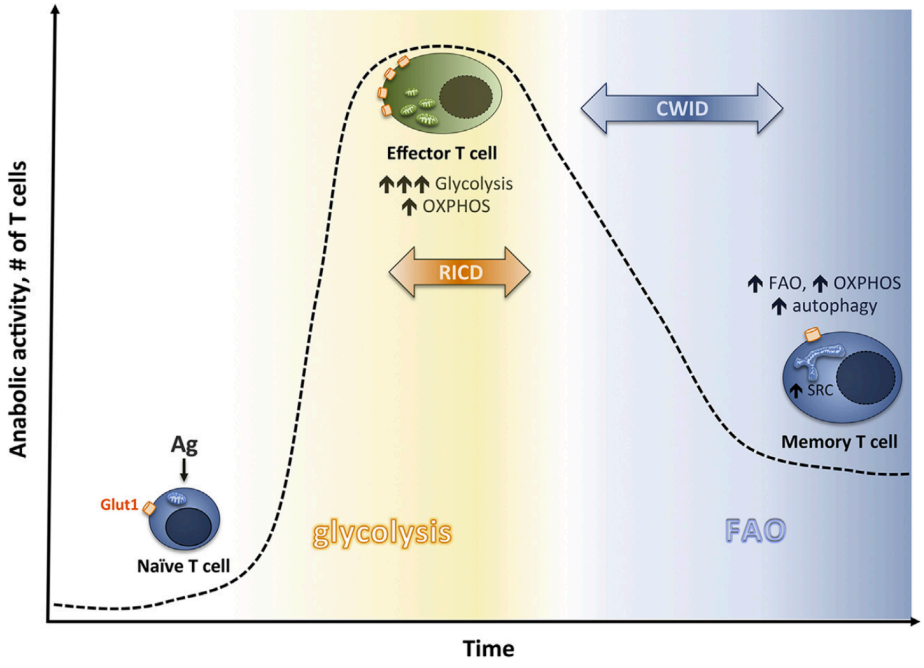


Figure 14. Sensitivity to critical apoptosis pathways correlates with metabolic reprogramming over the course of the T cell response.
Naïve T cells exist in a metabolically quiescent state until antigen (Ag stimulation), which triggers increased Glut1-mediated glucose uptake and an abrupt increase in aerobic glycolysis (as well as OXPHOS). Enhanced

glycolysis sensitizes effector T cells to RICD by enabling TCR-induced FasL induction upon Ag re-encounter. As Ag is cleared, most effector T cells are culled through CWID. Those T cells that persist into the memory pool likely escape both RICD and CWID by inducing protective autophagy and turning off glycolysis in favor of FAO-direct OXPHOS. This metabolic switch returns memory T cells to a more quiescent state, "primed" for rapid recall responses via increased spare respiratory capacity.

CHANGES IN T CELL METABOLISM IMPACT APOPTOSIS SENSITIVITY

Intricate links between metabolism and apoptosis have been detailed in various eukaryotic cell types, often with mitochondria as the central players. Studies establishing connections between apoptosis and glycolysis, the tricarboxylic acid cycle (TCA cycle), and the pentose phosphate pathway (PPP) have been explored in the context of cancer, diabetes, and neurodegenerative disorders (7). With the recent explosion of research into immunometabolism, particularly in T cells, links to apoptosis sensitivity are just now garnering attention.

Apoptosis is a particularly important regulator of immune homeostasis because either a paucity or overaccumulation of lymphocytes can have detrimental consequences, giving rise to immunodeficiency or autoimmunity, respectively. If control is lost through apoptotic defects, an overactive immune response to pathogens can pose danger to the host in the form of lymphoproliferation and immunopathological damage (188; 193). Furthermore, chronically activated T and B lymphocytes undergoing rapid proliferation are at increased risk of sustaining mutations, which can contribute to the development of lymphoma (192). Alternatively, premature or excessive apoptosis could result in a lackluster T cell response that leaves the host susceptible to infectious diseases and/or cancer. Overall, T cell apoptosis sensitivity is carefully calibrated over the course of an adaptive immune response to ensure both effective clearance of pathogen and disposal of

activated cells that are no longer needed and potentially dangerous to the host if left unchecked.

Naïve T cells are metabolically quiescent, relying largely on oxidative phosphorylation (OXPHOS) for ATP generation prior to antigen engagement (32). Upon TCR/CD28 stimulation, activated T cells upregulate their anabolic metabolism to meet higher demands required for energy production, rapid cell proliferation and acquisition of effector functions. This burst of activity is associated with rapid uptake of nutrients like glucose and amino acids that jumpstart a profound increase in both glycolysis and OXPHOS, although the increase in glycolysis is much larger (82; 115). Activated T cells upregulate cell surface expression of the glucose transporter Glut1, which is dependent on CD28 costimulation (80; 115). Glut1-mediated glucose import and subsequent processing through the glycolytic machinery provides an abundant carbon source for building macromolecules for clonal expansion, as well as ATP. This shift to glucose dependency can also protect T cells from intrinsic apoptotic stimuli, such as osmotic stress in mouse T cell lymphomas (27). Similarly, the viability of proliferating Jurkat and primary human T cells in culture decreases as glucose availability is limited, highlighting the importance of glucose as a metabolic substrate for activated T cell survival (115) (6). Increased T cell death during glucose deprivation was linked to mitochondrial integrity, which is normally controlled by a balance of pro- and anti-apoptotic B cell lymphoma 2 (Bcl-2) family proteins. Specifically, increased expression of pro-apoptotic Bcl-2 domain homology 3 (BH3)-only proteins such as Bim, Puma and Noxa can initiate programmed cell death by binding and counteracting the function of anti-apoptotic Bcl-2, Bcl-xL, and Mcl-1 proteins (164). In fact, knockdown of Noxa improved survival of activated T cells

experiencing glucose limitation (6), establishing a specific role for Noxa in eliminating glucose-starved T cells. The pro-survival effect of cytokines like IL-2 and IL-7 for expanding T cells is also linked to enhanced glucose influx. Specifically, IL-7-mediated survival conferred by STAT5-dependent Akt activation was partly dependent on increased Glut-1 expression and glucose uptake; IL-7 alone was insufficient to rescue cells from apoptosis in glucose-limiting conditions (219). Moreover, DNA synthesis inhibitors like 6-mercaptopurine block Jurkat T cell proliferation via inhibition of glycolytic (and glutaminolytic) flux (58). Therefore, glucose not only provides a primary fuel source for activated T cells, but also ensures their persistence as the adaptive immune response takes off.

As glucose is processed through the glycolytic machinery, several molecular intermediates are shuttled into biosynthetic pathways (e.g. PPP, serine biosynthesis) that contribute to DNA replication and proliferation [1]. Pyruvate is ultimately formed as a key intermediate at the crossroads of several metabolic pathways. During clonal T cell expansion, excess pyruvate is fermented into lactate and secreted despite abundant oxygen (i.e. aerobic glycolysis) – a common feature of rapidly dividing cells (153). However, pyruvate continues to enter the TCA cycle as acetyl-CoA to drive both lipid synthesis and OXPHOS – the most efficient pathway for ATP production. Increased influx of essential amino acids (e.g. glutamine) triggered by T cell activation also provides substrates for the TCA cycle to maintain OXPHOS. Although enhanced glycolysis clearly impacts cell death sensitivity in expanding effector T cells (see next section), recent research suggests OXPHOS constitutes a critical T cell survival signal as well. In studying patients with mitochondrial disease resulting from genetic defects in

cytochrome *c* oxidase (COX), OXPHOS dysfunction was linked to increased apoptosis after activation, which contributed to immunodeficiency (198). To ascertain the significance of COX function in T cells, the gene encoding COX10, a critical assembly factor for complex IV, was conditionally deleted in murine CD4+ T cells. The resulting COX-deficient T cells demonstrated an increase in both dead and actively dying cells at 72 hours post activation *in vitro* compared to wild type cells. The enhanced apoptosis sensitivity did not appear to be due to differences in Fas ligand (FasL) expression nor differential oxidative stress. Although the mechanism linking OXPHOS deficiency to increased apoptosis remains unclear, these studies suggest a vital role for COX activity and OXPHOS in effector T cell survival.

Another major component of T cell metabolism is fatty acid synthesis and/or oxidation. Importantly, fatty acids are not only nutrients for energy production, but also can act as signaling molecules in T cells. In addition, the composition of fatty acids that make up cell membrane phospholipids contribute to the physical properties of the membrane and its signaling components (35). Literature connecting fatty acid metabolism to T cell differentiation and memory development has been reviewed previously (109). However, nascent exploration of links between fatty acid metabolism and apoptosis sensitivity in T cells has yielded interesting insights. For example, palmitic acid exposure induced apoptosis in a dose-dependent manner in the Jurkat T-cell line and primary human T cells (195). However, activated CD4+ T cells succumb to apoptosis when fatty acid synthesis or fatty acid uptake is inhibited *in vitro* (8). Furthermore, fatty acid oxidation (FAO) plays a particularly important role in the formation and persistence of memory T cells. Unlike effector T cells, memory T cells utilize FAO to fuel OXPHOS

instead of glycolysis to meet their energy demands (116; 153). Indeed, limiting glycolysis via inhibition with rapamycin or RNAi-mediated knockdown of mTORC1 enhances memory T cell development (154; 160). The FAO-rich metabolism of memory T cells is associated with a higher mitochondrial mass and therefore a higher spare respiratory capacity (SRC) than effector T cells (202), which is critical for surviving energetically stressful periods (142). Interestingly, this difference can be reduced to dynamic remodeling of the mitochondrial architecture, which is required for optimal OXPHOS efficiency (31). Ultimately, memory T cells are much longer-lived than effector T cells. Therefore, it appears that adapting a different type of metabolism can result in survival advantages, and likely impacts secondary effector T cell function during a recall response (203).

THE SIZE OF THE RESPONDING EFFECTOR T CELL POOL IS SHAPED BY THE INTERPLAY BETWEEN METABOLIC REPROGRAMMING AND SPECIFIC APOPTOSIS PATHWAYS

Restimulation-induced cell death (RICD), previously known as activation-induced cell death (AICD), is a critical pre-programmed death pathway that constrains the size of a responding effector T cell pool (188). This apoptosis pathway occurs in cycling effector T cells that are strongly restimulated through the TCR in the presence of IL-2. Defects in RICD perturb immune homeostasis by allowing uncontrolled T cell expansion and extensive immunopathology, as illustrated by X-linked lymphoproliferative disease (XLP-1). In the absence of SAP, a small adaptor molecule required for signaling lymphocyte activation molecule (SLAM) family receptor signaling, attenuated TCR signal strength in XLP-1 patient T cells manifests in poor induction of pro-apoptotic molecules like FasL, Bim, Nur77 and NOR-1, and ultimately impaired RICD (185). Defining the molecular determinants of RICD can therefore provide valuable therapeutic

targets for controlling T cell responses by adjusting RICD sensitivity – a paradigm we recently demonstrated in the context of XLP-1 (170). RICD can even be further exploited as a mechanism for peripheral tolerance induction via precise clonal deletion of autoreactive T cells (231).

Lipid metabolism, primarily of fatty acids, has also been coupled to changes in RICD sensitivity. Indeed, T cell hybridomas treated with myriocin, an inhibitor of serine palmitoyl-CoA transferase, exhibited a reduction in RICD sensitivity (189). The inhibition of sphingolipid synthesis seemingly impacted apoptosis through modulation of CD95 death receptor signaling and caspase activation. However, preliminary data from our lab has shown that myriocin treatment on primary human CD8+ T cells does not impact RICD. On the other hand, inhibition of fatty acid synthase (FASN) with cerulenin or C75 decreased RICD significantly (unpublished data), suggesting different nodes in lipid metabolism are indeed critical for this apoptosis pathway and require further study.

In the context of cancer, cell growth has previously been linked to an inverse correlation between apoptosis sensitivity and nutrient-dependent metabolic activity (123). Although effector T cells follow a parallel path by relying upon aerobic glycolysis for accelerated proliferation, new research suggests that enhanced glycolytic flux also renders these T cells more prone to apoptosis as clonal expansion peaks. Specifically, we recently demonstrated a direct correlation between RICD sensitivity and glycolytic flux in human CD8+ T cells (99). Reducing either glycolytic activity or exogenous glucose availability rendered effector T cells significantly less sensitive to RICD. Interestingly, this study demonstrated that active glycolysis specifically facilitates the induction of pro-apoptotic FasL upon TCR restimulation, accounting for enhanced RICD sensitivity in

highly glycolytic T cells. Collectively, these data indicate that RICD susceptibility is linked to metabolic reprogramming and represents an elegant mechanism of maintaining immune homeostasis, such that only activated effector T cells undergoing aerobic glycolysis are licensed to die through this pathway. Further studies are needed to identify other temporal factors required to render effector T cells competent to die via RICD well after initial clonal expansion has occurred. It also has not been determined whether switching back to a more metabolically quiescent state helps to shield T cells from RICD as they transition into the memory pool.

T CELL METABOLISM AND DEATH SENSITIVITY CAN INFLUENCE IMMUNOLOGICAL MEMORY

The size of the responding effector T cell pool is not only important for the acute adaptive response, but also has implications for memory formation. Indeed, the number of effector CD8+ T cells that expand in response to infection directly correlates with the subsequent size of the memory T cell compartment (13). Similarly, the dynamics of cytokine withdrawal and ensuing apoptosis impacts the size of the memory T cell pool. T cells entering the memory pool must curtail glycolysis (perhaps to escape RICD) and return to a more catabolic state, largely dependent on FAO to fuel OXPHOS, with enhanced SRC for rapid recall responses. In fact, genetic and pharmacological interventions that augment this switch (e.g. deoxyglucose, rapamycin, etc.) also enhance memory T cell formation (10; 161; 194). However, glucose uptake is still required for memory T cell survival in a manner dependent on Notch signaling (117).

During the contraction phase of the T cell response triggered by cytokine withdrawal, T cells also increase autophagic activity to cope with the stress of growth factor deprivation (100; 114). Macroautophagy, referred to here as autophagy, is a

catabolic process by which cellular proteins and organelles are degraded to provide free amino acids and allows T cells to maintain ATP production. Importantly, the link between autophagy and changes in metabolism may induce or protect cells from apoptosis during stressful periods, depending on the context (26; 120; 126; 168; 184). However, autophagy is clearly required for T cell activation, and plays a critical role in the survival and differentiation of memory and regulatory T cells (126; 213; 222). Inhibition of NOTCH1 signaling induces metabolic shutdown and triggers autophagy for survival in T cell acute lymphoblastic leukemia (T-ALL); co-inhibition of autophagy synergistically enhanced anti-NOTCH treatment efficacy in eliminating T-ALL cells (73). Additionally, we recently demonstrated that secondary effector T cells derived from sorted human memory CD8+ T cell subsets (central and effector memory) are differentially sensitive to CWID (100). Effectors derived from the central memory (CM) pool are more resistant to RICD than their effector memory (EM)-derived counterparts, due to more sustained protective autophagy and reduced BIM expression (100). Hence, disparate rates of autophagy and subsequent apoptosis in memory T cell subsets likely influence the magnitude of a secondary immune response, which is an important consideration for adoptive T cell transfer strategies.

THE TUMOR MICROENVIRONMENT ALTERS T LYMPHOCYTE METABOLISM AND THE ANTI-TUMOR RESPONSE BY TUNING APOPTOSIS SENSITIVITY

Tumor infiltrating lymphocytes (TIL) advance into extremely hostile territory upon entering the tumor microenvironment. Cancer cells evade T cell immunosurveillance by both escaping antigen recognition altogether (“immunoediting”) (206), and maintaining an immunosuppressive microenvironment that severely compromises T cell effector functions. Although recent efforts for improving anti-tumor

immunity have rightly focused on restoring residual, functionally hobbled TIL via immune checkpoint blockade therapies (50; 152), many effector T cells simply die on the battlefield (112). In some cases, self-antigen bearing immature or tolerogenic APCs found in tumor draining lymph nodes, or within the tumor microenvironment, can contribute to suboptimal $CD8^+$ T cell activation, anergy, and apoptosis –traditionally termed "cross-tolerance" (97). This form of T cell depletion is likely BIM-dependent (51), suggesting apoptosis is triggered by a dearth of critical nutrients and growth factors. On the other hand, robustly activated effector Th1 CD4+ and CD8+ T cells can succumb to death in response to chronic antigen stimulation, bolstered by toxic metabolites (e.g. lactate) and tumor-derived effector molecules such as galectins and FasL itself (165; 218). Ultimately those T cells that survive despite growth factor withdrawal and/or chronic antigen exposure exhibit a transcriptionally distinct "exhausted" state (216). T cell exhaustion is associated with increased expression of programmed death-1 (PD-1) and other co-inhibitory receptors (e.g. CTLA-4, LAG3) that render T cells hyporesponsive to antigen stimulation and therefore ineffective at eliminating tumor cells.

So how might the distinct metabolic environment within tumors, often distinguished by hypoxia and aerobic glycolysis (a.k.a. the "Warburg effect"), influence apoptosis sensitivity of responding T cells? Recent studies demonstrate that fierce competition by tumor cells for oxygen, glucose, and other key metabolites directly hobbles T cell expansion and effector functions by hindering their own metabolic needs, forcing TIL to adopt an "exhausted" phenotype (34; 40; 76). In fact, co-inhibitory receptors like PD-1 enforce exhaustion by suppressing glycolysis and promoting fatty

acid oxidation (FAO), which increases harmful reactive oxygen species (ROS) that can impact T cell survival (199). Components of the "cancer secretome" may also directly hinder TIL function, including high concentrations of lactate and tryptophan metabolites (34). However, the effects of many tumor-secreted factors on immunometabolism and apoptosis sensitivity require further exploration. Interestingly, regulatory T cells (Tregs) adapt a metabolic phenotype reliant on OXPHOS and FOXP3-dependent transcriptional regulation in order to function in low glucose, lactate-rich environments (9). Indeed, progressive depletion of lipid reserves via FAO and induction of autophagy may also help FOXP3- TIL persist for a while under these conditions (151). Over time, however, many of those TIL likely perish in response to prolonged metabolic stress, with apoptosis eventually triggered in response to severe deprivation of glucose, essential amino acids (e.g. tryptophan, glutamine, etc.) and/or autocrine/paracrine IL-2 (112). Mitochondrial dysfunction and buildup of reactive oxygen species likely contribute to CWID/ACAD as PD-1 expression increases on exhausted T cells (21). At the same time, those exhausted TIL that remain alive but functionally crippled within the tumor may also escape RICD by modulating TCR signal strength and glycolysis. Work from our group and others has demonstrated that optimal RICD sensitivity requires strong TCR restimulation, IL-2 driven cell cycling, and glycolysis; the magnitude of glycolytic flux directly correlates with FasL induction and RICD sensitivity in human $CD8^+$ effector T cells (99; 188). Thus it appears that although decreased metabolic drive in exhausted TIL dampens anti-tumor effector functions, it can promote survival in the short term by deterring those apoptosis pathways normally responsible for constraining (RICD) or contracting (CWID) the effector T cell pool in any adaptive immune response. We propose that while "rationing"

of available fuels via catabolic metabolism spares TIL from acute growth factor withdrawal, modulation of both TCR signal strength and glycolytic metabolism via inhibitory receptors protects against death from chronic TCR restimulation (Figure 15).

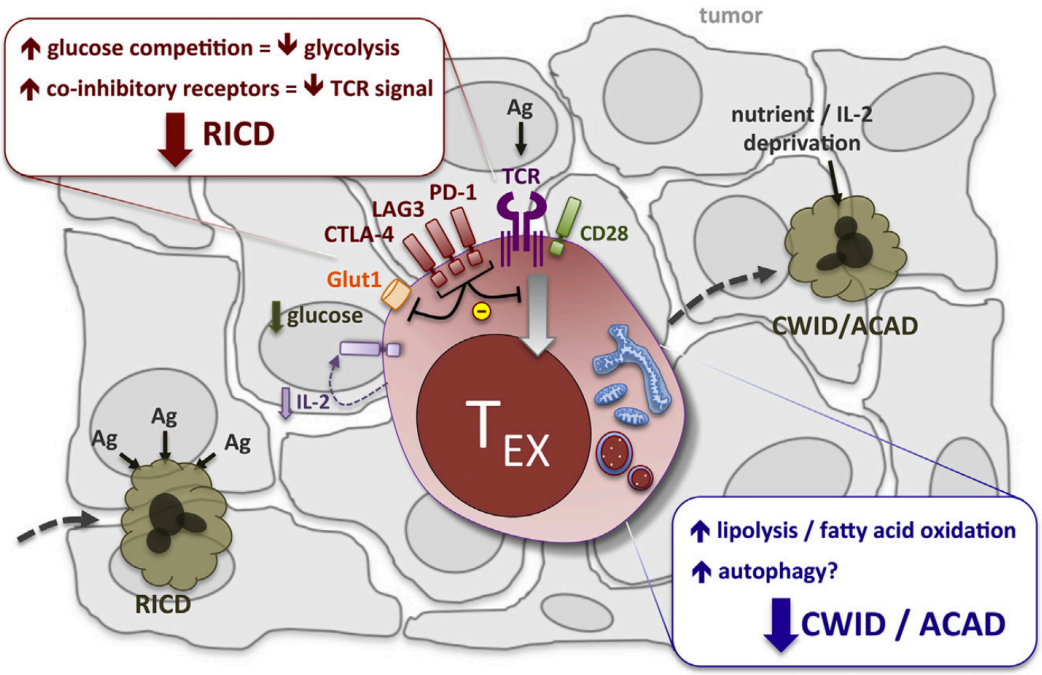


Figure 15. Exhausted TIL (Tex) escape apoptosis via altered metabolism. Highly glycolytic T cells that infiltrate a solid tumor may be susceptible to RICD immediately upon repeated Ag encounter. However, the upregulation of co-inhibitory receptors like PD-1 attenuate TCR signal strength and promote FAO over glycolysis. This metabolic switch likely helps exhausted TEX escape RICD despite chronic Ag exposure, and survive despite fierce completion for glucose, amino acids, and other nutrients inside the harsh tumor microenvironment. Eventually, severely exhausted T cells exhibit profound metabolic stress and likely become susceptible to CWID/ACAD in response to prolonged nutrient and cytokine deprivation.

Checkpoint blockade therapy, including anti-PD-1/PD-L1 and anti-CTLA-4 blocking antibodies, rescue TIL effector functions in part by restoring glucose availability in the tumor microenvironment and reinvigorating their anabolic drive (40). However, not all TIL respond to these interventions. “Severely” exhausted TIL exhibiting a more

terminally-differentiated state (e.g. PD-1hi Tim3+) do not respond to PD-1/PD-L1 checkpoint blockade (78; 81), perhaps because they are already committed to an apoptotic fate (83). Indeed, engagement of Tim3 by pro-apoptotic ligands (e.g. galectin-9) may promote death via RICD ((234), unpublished observations). This is consistent with the general idea that improved survival and self-renewal of less terminally-differentiated T cells provide superior protection against tumors (91) and even persistent infections like Mtb (106). Those TIL that do respond to checkpoint blockade are re-energized in the short term to eliminate tumor cells via enhanced cytotoxicity and effector functions, but paradoxically may be rendered more sensitive to RICD as glycolysis ramps up. In the context of viral infection, the small fraction of effector T cells that end up populating the long-lived memory pool are those that downregulate glycolysis and maintain FAO and protective autophagy (34; 222). The fate of such "memory precursor" cells may be determined as early as the first asymmetric division of a newly stimulated T cell, during which mTORC1, c-Myc, and other drivers of glycolysis are selectively partitioned into the daughter cell destined for rapid clonal expansion and enhanced but short-lived effector function (162; 205). It is tempting to speculate that those T cells capable of tempering glycolytic drive and shifting to a more quiescent metabolic state survive to become memory T cells in part by evading both RICD and CWID. If true, we posit that evolving strategies for the optimization checkpoint blockade immunotherapies should be mindful of tuning relative apoptosis sensitivity of tumor-specific T cells via metabolic manipulations (71). Optimized TIL responses must strike a temporal balance between acute restoration of glycolytic flux for short-term effector function, followed by a gradual switch to FAO and/or autophagy that ushers cells into the

long-lived memory pool, maintained by healthy mitochondrial architecture for robust OXPHOS. Such strategies are currently under investigation and yielding promising results (150; 194). The importance of apoptosis sensitivity extends to adoptive T cell therapies for tumor eradication, for which the selection of appropriate T cell subsets for expansion and autologous transfer is imperative for sustained anti-tumor responses. Our finding that human effector $CD8^+$ T cells derived from the CM vs. EM subset survive better after IL-2 withdrawal may help to explain their superior expansion and protective capacity *in vivo* (100) and may help explain why CM T cells give rise to longer-lived, more robust and efficacious effector T cell responses. Furthermore, RICD sensitization must also be considered carefully when engineering such T cells for precision targeting using chimeric antigen receptors (CARs). CARs with the strongest activating potential *in vitro* often compromise the anti-tumor potency of those T cells *in vivo* due to enhanced susceptibility to FasL-dependent RICD (95), which correlates with enhanced glycolysis. Hence the targeting of specific T cell subsets and/or design of CARs that enforce balanced metabolic reprogramming could promote stronger and more sustained anti-tumor responses by recalibrating T cell apoptosis sensitivity.

CONCLUSION

In summary, immunometabolism plays a critical role in governing the scale and efficiency of effector and memory T cell responses, in part by calibrating apoptosis sensitivity properly over the duration of the immune response. Further elucidation of these mechanistic conditions will inform and improve translational applications that rely on a robust but regulated T cell attack, particularly in stressful niches like the tumor microenvironment.

CHAPTER 5: Discussion

The goal of this dissertation was to elucidate mechanisms of RICD sensitivity in primary human T cells. Previous observations of RICD resistance in FOXP3+ Tregs was puzzling; how can a specialized subset of T cells be resistant to RICD and yet meet all the known requirements for death sensitization? This warranted the investigation of FOXP3-mediated RICD resistance in Tregs as discussed in Chapter 2. Given the ability of FOXP3 to protect Tregs from RICD, we hypothesized that transient FOXP3 expression in human Tcons could serve a similar function. In Chapter 3, we described a novel role for FOXP3 in CD4 and CD8 Tcons in protecting activated T cells from premature RICD during expansion. Strikingly, the mechanisms of FOXP3-mediated protection from RICD were completely different in Tregs and Tcons, cementing the idea that different subsets of T cells are subject to different homeostasis challenges and have evolved unique mechanisms of survival in addition to their functional differences.

In addition to FOXP3-mediated mechanisms of RICD sensitivity, we investigated T cell metabolism and tuning of death sensitivity because of the intricate mesh that exists between metabolic activity and apoptosis sensitivity as discussed in Chapter 4. This section will discuss the implications of our findings in Chapters 2 and 3, as well as the potential contributions of cell metabolism that must be considered when interpreting these findings. Additional unpublished data is introduced during this discussion in order to grasp a complete understanding of our findings in a broader context; these figures can be found in Appendix A.

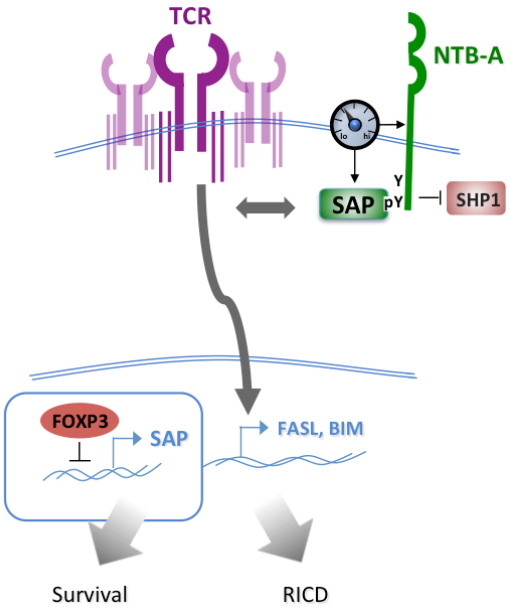


Figure 16. Model of FOXP3-mediated resistance to RICD in Tregs.
FOXP3 directly suppresses SAP expression at the promoter. SAP expression displaces the inhibitory phosphatase SHP1 from the NTB-A receptor in order to boost proximal TCR signaling. Upon TCR restimulation with antigen, SAP levels in human Tregs are not sufficient to boost the strength of the TCR signal to induce pro-apoptotic molecules (such as FASL and BIM) needed to execute RICD. Figure adapted from Andrew L. Snow.

We have shown in Chapter 2 that Tregs evade RICD by FOXP3-mediated SAP suppression, despite meeting the traditional 'requirements' for sensitization (active cell cycling, IL-2 consumption, and restimulation through the TCR). Our model of RICD resistance in Tregs is centered on decreased SAP expression, which supports the 'threshold model' of RICD sensitivity (Figure 16). Indeed, an adaptation (SAP suppression) that conceivably occurred to handle frequent TCR restimulation is suitable for Tregs, considering they experience strong TCR signaling both in the thymus and the periphery (133).

Clearly, the NTB-A/SAP signaling axis is central to RICD across the immune system. Not only is this the signaling axis with characterized dysfunction in XLP patient T cells, but our results in healthy human donor Tregs represent another evolutionary adaptation to manipulate RICD sensitivity by specifically targeting SAP signaling (presumably also mediated through NTB-A). Moreover, altered SAP signaling and RICD disruption contributed to *Mycobacterium tuberculosis* (*Mtb*) infection outcomes in humans; T cells from low responder (LR) Tuberculosis patients were more resistant to RICD via abrogated NTB-A signaling (72). The unfavorable Th2 phenotype (in the case of *Mtb* infection) was directly influenced by NTB-A/SAP signaling leading to qualitative differences in the immune response of infected individuals. These important observations underscore the power of perturbations in this signaling axis- not only in the clear immunodeficiency setting seen by XLP disease, but also in determining the quality of the T cell response to infectious agents in otherwise healthy individuals.

Our findings of RICD sensitivity have important implications for clinical Treg therapies. As discussed in Chapter 1, infusion of Tregs has shown great potential in the clinic for autoimmune diseases, but some therapies have been stunted by a loss of infused Tregs during treatment. Only with an improved understanding of the molecular mechanisms of apoptosis sensitivity in these cells can progress be made to improve the longevity of Treg therapies (182). It is not as simple as infusing a higher number of Tregs if the quality of the Tregs is insubstantial (147). Not only do Tregs need to possess extraordinary immunosuppressive function, but they also must persist in the host long enough to sustain the benefits of renewed immune homeostasis. Therefore, targeted

approaches to stabilize FOXP3-mediated suppression of SAP have the potential to improve the efficaciousness of Treg-based therapies.

Unanswered questions and future directions

FOXP3 depends heavily on co-factors to mediate transcriptional changes, and we have not identified the co-factor required for FOXP3-mediated SAP suppression in Tregs. Importantly, ectopic FOXP3 expression was not sufficient to suppress SAP in Tcons (data not shown), suggesting the co-factor required is specific to Tregs. An example of a co-factor could be a transcription factor such as Helios, which is expressed in Tregs and is important for some of their suppressive functions (177). However, not all Tregs have the same expression pattern of FOXP3 binding partners. For example, Helios expression is heterogenous in Tregs, as Helios+ and Helios- cells are both present within the nTreg population (74). Therefore, the binding partner(s) required for SAP suppression should be identified. This could greatly inform basic Treg biology, especially with respect to their survival. Is it possible that some subsets of Tregs have 'factor X' and some not? Would that mean those Tregs are longer-lived due to improved evasion of RICD? If Tregs with 'factor X' do survive longer due to enhanced RICD resistance, efforts could be made to generate specific Treg types for therapeutic use.

Generally, a setback of the amazing immunoregulatory power of Tregs is maintaining their suppressive capacity when faced with inflammatory environments. In a case of an IPEX patient mutation A384T, altered DNA-binding specificity of FOXP3 was specifically associated with a defect in Tregs to combat inflammation in non-lymphoid tissues (232). Additionally, destabilization of FOXP3 expression itself can occur when Tregs encounter the inflammatory milieu, resulting in pathogenic 'ex-Tregs' (233). In

addition to destabilization of FOXP3, it is also possible that inflammatory environments alter the availability of FOXP3 binding partners, leading to a loss of SAP suppression and ultimately increased RICD sensitivity.

Tregs have a very distinct metabolic profile from effector T cells, which could certainly contribute to their RICD resistance. As discussed in Chapter 4, Tregs have relatively low levels of glycolysis, and glycolytic metabolism enhances RICD sensitivity in CD8 T cells (99). However, the metabolic influences of RICD has not been explored in Tregs and could be easily tested; Would forcing Tregs to switch to a more glycolytic metabolism program sensitize them to die by RICD? On one hand, FOXP3 is upstream of metabolic programming in Tregs because it suppresses expression of Myc (glycolysis) and enhances OXPHOS (9). However, the metabolic state of the cell must feedback on FOXP3 expression itself, because development of Tregs relies on inhibition of mTORC1 (44). Additionally, mice with precursor cell expressed, developmentally downregulated 4 (Nedd4) family interacting protein (Ndfip1) deletion have decreased numbers of Tregs, especially in the small bowel (101). Tregs from Ndfip1-deficient mice had increased rates of glycolysis, and restimulated Ndfip1-deficient Tregs had an even higher glycolytic rate when compared to restimulated WT Tregs. Therefore, it is an easy assumption that maintaining a metabolic program with low in glycolysis would be important for maintaining RICD resistance in Tregs, which could be due in part to stabilizing FOXP3 expression.

In Chapter 3, we describe a novel role for CD48 in protection from RICD in Tcons. CD48 expression promoted higher basal autophagy levels. Indeed, Tregs have been documented to have high CD48 expression relative to Tcons (124) and autophagy is

integral to their functional stability (213). However, whether high CD48 expression in Tregs also contributes to RICD resistance has not been tested.

CONVENTIONAL T CELLS

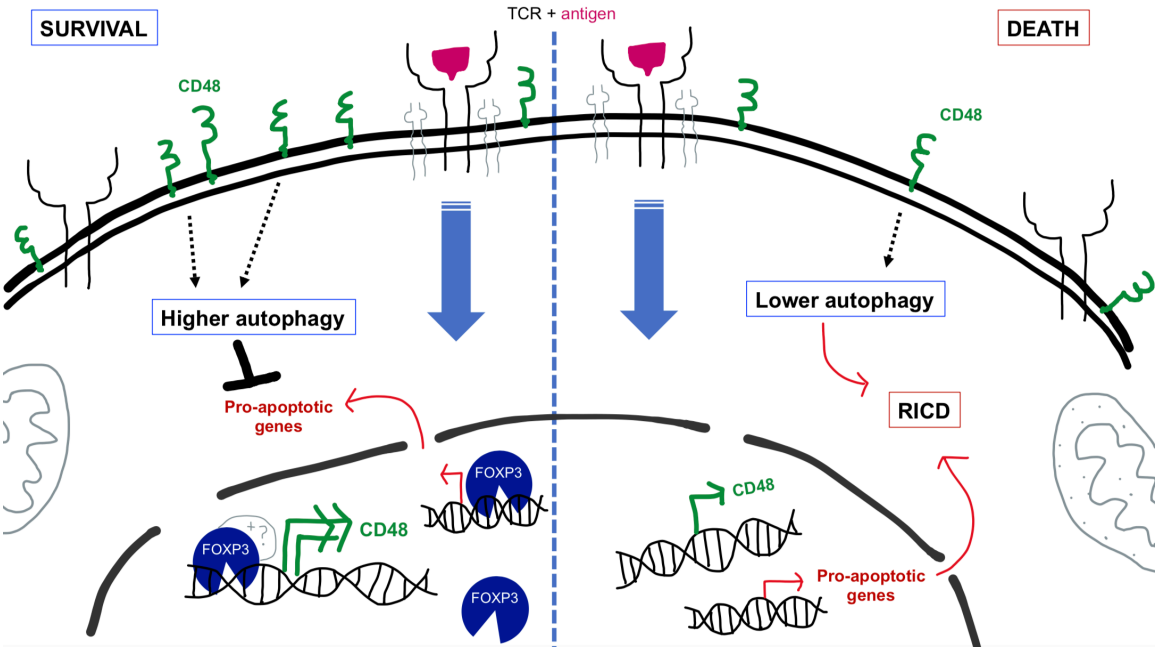


Figure 17. Model of FOXP3-mediated resistance to RICD in expanding Tcons. Upon TCR activation of CD4 and CD8 T cells, FOXP3 and CD48 is induced. Although all cells upregulate CD48 expression, only some cells are FOXP3+, and they have higher levels of CD48 expressed on the cell surface. Basal autophagy levels in FOXP3+ T cells are higher and protect T cells from TCR restimulation-induced transcription of pro-apoptotic genes.

In Chapter 3, we describe a completely novel signaling axis in activated human Tcons that involves FOXP3, CD48, and autophagy. Although FOXP3 has been widely studied in Tregs and described as a protective factor required for RICD resistance, FOXP3 expression has also been reported in other immune cells (113). CD48 is also present on other hematopoietic cells similar to other SLAM family receptors. Therefore, although this signaling axis has just been identified, it's possible that it has impacts on

immune function beyond RICD protection in expanding Tcons. It is important to note that although all FOXP3+ cells were CD48+, not all CD48+ cells were FOXP3+, meaning that there are other factors shaping CD48 expression. Nonetheless, there could have been observations related to FOXP3/CD48/autophagy signaling perturbations that have gone unreported or unappreciated due a lack of knowledge.

Now that we have created awareness and provided a basic description of this novel signaling axis, others may begin to appreciate how it impacts human health in their respective research fields. For example, polymorphisms in CD48 have been linked to susceptibility to multiple sclerosis (MS), and pathogenic CD4 T cells were CD48hi in an EAE mouse model of MS (124). Administration of anti-CD48 antibody during EAE was therapeutic, specifically due to reducing CD4 T cell numbers. Could inducing RICD in pathogenic Tcons via CD48 blockade explain the therapeutic results? The signaling pathway we have described should also be considered beyond basic T cell biology; particularly in asthma research. Membrane-bound levels of CD48 expression on B, T, NK cells and monocytes were significantly higher in asthma patients with the most disease severity (61). Interestingly, CD48 also exists as a soluble form (sCD48) and showed the opposite correlation in patient serum; lower sCD48 levels were associated with severe asthma. Upon further study, sCD48 levels were elevated in nonallergic asthma patients compared to allergic asthma patients (29). Clearly, CD48 signaling on T cells and other immune cells could be underlying some of these associations.

In Chapter 1 we discussed the importance of kinetics in T cell responses and homeostasis. Our model of transient FOXP3 expression in Tcons supports this notion. The expression pattern of FOXP3 correlates with inversely with RICD sensitivity (Figure

2), and this was consistent the kinetics of CD48 expression (Figure 11D). A more direct test of this relationship was conducted by silencing FOXP3 expression at different time points throughout the T cell response. FOXP3 KD T cells were significantly protected from RICD at day 4-post activation (Figure 9A), and intermediately (although not statistically significant) protected at day 7 post-activation (Appendix A- Figure 18); By day 17 post-activation we observed no effect of FOXP3 expression on RICD sensitivity (Figure 9B). To conclude, the kinetics of factors that govern apoptosis sensitivity should not be overlooked in future studies, as further supported by our findings of FOXP3 and CD48 expression in human Tcons. Our work also helps to explain why expanding activated Tcons are resistant to RICD, which was previously unknown.

Autophagy was a clear part of our model for FOXP3/CD48-mediated protection from RICD (Figure 17). Indeed, disrupting autophagy by ATG3 silencing or small molecule inhibitors increased RICD sensitivity while promoting autophagy decreased RICD (Figure 13). Although this data clearly demonstrates that autophagy is protective in expanding Tcons, we have assumed that autophagy protects T cells by the same mechanisms proposed in the model by Corrado *et al* in late stage effectors (47). In their model, TCR restimulation induces a loss of protective autophagy. In our expanding Tcons at day 4, restimulation with OKT3 also reduced Cyto-ID autophagy staining, with a lesser extent in FOXP3 KD T cells (Appendix A- Figure 19). However, the details of how autophagy specifically protects expanding T cells from RICD remain to be confirmed. Corrado *et al* reported that lower levels of autophagy after TCR restimulation renders T cells susceptible to apoptosis due to fragmented mitochondria, which fail to be cleared via mitophagy. Interestingly, FOXP3 KD T cells exhibited decreased

mitochondrial membrane potential (Appendix A- Figure 20). Although differences were modest, they were consistent among many donors and could indicate that cells with decreased FOXP3 and/or CD48 expression are at a baseline state of poor mitochondrial health, making it easier for TCR restimulation to tip them over the edge to apoptosis.

Unanswered questions and future directions

The significance of FOXP3 in immunology is most clearly represented by its' deficiency in scurfy mice and IPEX patients. Although the disease is inherently associated with autoimmunity and Treg dysfunction, it is possible that IPEX patients also have enhanced RICD sensitivity in Tcons. In addition, FOXP3-mediated cytokine regulation could be impaired, skewing the T cell response. These observations would of course be overshadowed by the more severe autoimmune manifestations. However, IPEX mutations have a range of disease severity and some FOXP3 mutations are relatively mild. Would these patient Tcons have lower CD48 expression than healthy controls? Is it possible that they have less robust T cell responses due to premature RICD? Would a less robust T cell response subsequently create a poor memory T cell pool? If the above predictions were found to be true, efforts could be made to boost CD48 expression in order to restore sufficient T cell numbers and enhance memory formation.

Knowledge of FOXP3-dependent mechanisms of RICD resistance could potentially guide a multitude of clinical applications. For example, genetically engineering T cells to be resistant to death signals enhanced adoptive cell transfer therapy for cancer (223). T cells could also be engineered to resist RICD signals by sustaining stable and high CD48 expression levels. Additionally, strategies that boost FOXP3 and/or CD48 expression in Tcons would be advantageous for eliciting improved vaccine

responses. The extent of effector T cell proliferative expansion is proportionately associated with the amount of memory T cells that form after the contraction phase (217). Therefore, protecting more expanding T cells from premature RICD has the potential to form a more robust T cell response resulting in a larger memory pool. To attack this problem from a FOXP3-centered view would require a better understanding of the factors that are upstream of FOXP3 expression in Tcons. Some clues may arise from studies of T cell metabolism. For example, T cells deprived of glutamine exhibited higher FOXP3 expression *in vitro* (93) whereas α-ketoglutarate (α-KG) treatment of Tregs inhibits Treg differentiation (unpublished data, Naomi Taylor).

As discussed previously, metabolism clearly regulates FOXP3 expression in Tregs. We therefore also investigated whether FOXP3 silencing in Tcons altered T cell metabolism (Appendix A- Figure 21). Day 4 FOXP3 KD T cells had increased L-lactate in the media, suggesting that FOXP3 suppresses glycolytic metabolism in Tcons similar to Tregs (130) (Appendix A- Figure 21A). Tregs display very low levels of the glucose transporter Glut-1 compared to Tcons (63). However, FOXP3 KD T cells demonstrated an unexpected decrease in Glut-1 expression (Appendix A- Figure 21B). However, the change in glucose transporter expression had no impact on the actual flux of glucose into the cell as demonstrated by 2-NDBG flux assays (Appendix A- Figure 21C). Ultimately, blocking glycolysis with 2-deoxyglucose (2-DG) did protect T cells from RICD (Appendix A- Figure 21D), but whether glycolysis is truly involved in tuning RICD sensitivity in expanding cells is still unclear. It's possible that acute 2-DG treatment in highly active T cells simply resulted in a general metabolic 'slow-down', and perhaps cell cycle arrest, explaining the decreased RICD sensitivity. Given that the proportion of

FOXP3+ cells in day 4 CD4 and CD8 T cells is quite small (~10-15% on average), the sensitivity of our assays to global changes in metabolism and RICD are limited. However, our preliminary data suggests that FOXP3 in Tcons could also contribute to metabolic programming and should be considered for future study.

Our novel results connecting CD48 and RICD sensitivity validate CD48 as a target for immunotherapy. Blockade or silencing of CD48 consistently increased RICD sensitivity across multiple human donors. However, whether CD48 differentially mediates RICD in different subsets of T cells needs to be addressed. Furthermore, we don't know the mechanism by which CD48 expression promotes autophagy. Although autophagy is central to our model of protecting Tcons from RICD, CD48 could be protecting cells by additional mechanisms. For example, CD8 T cell lines treated with CD48-blocking antibody demonstrated downregulation of basic leucine zipper transcriptional factor ATF-like (BATF) transcripts (107). Importantly, in a mouse model of BATF overexpression, mice develop a lymphoproliferative disorder specifically due to a lack of apoptosis in T cells (110). Conversely, BATF-deficient CD8 T cells exhibited dramatic defects in clonal expansion and underwent 'proliferative catastrophe' upon antigen encounter (96). These data likely represent a key function for BATF in protecting expanding Tcons from premature RICD. In Tregs, FOXP3 is upstream of BATF induction because the IPEX patient mutation A384T results in a dysregulation of BATF expression (70). Considering all of these observations, it would appear that FOXP3 KD cells have decreased CD48 expression as well as decreased BATF expression, which protects cells from RICD. Indeed, our preliminary data show that BATF protein expression is significantly reduced in both FOXP3 and CD48 KD T cells (Appendix A-

Figure 22). The potential role of BATF in RICD sensitivity and within the FOXP3/CD48/autophagy signaling axis should therefore be investigated.

FOXP3 in Tcons: the million-dollar question

Although we have uncovered a novel role for transient FOXP3 expression in Tcons, it is important to remember that FOXP3 is only expressed in a proportion of activated T cells. Therefore, the ultimate intellectual question of this field is: What determines which Tcons express FOXP3, and what is the ultimate fate of these cells? This question has not been answered whatsoever, although we can generate multiple hypotheses given recent work from our lab and others.

A simple hypothesis is that T cells that express FOXP3 at higher levels after activation are destined to become Th17-like cells. This would be consistent with the observations that Th17 cells express higher levels of FOXP3 than other subsets (127). Additionally, Th17 cells are highly resistant to RICD (183). Experimental designs have been limited by lack of a FOXP3 reporter gene on the cell surface to facilitate FACS of live T cells. However, ICFC could be conducted to identify FOXP3+ and FOXP3- cell populations in combination with IL-17 staining to look for associations. Alternatively, FOXP3 silencing could be conducted on CD4 and CD8 T cells during activation; Days later, cells would be restimulated to assess cytokine production. We would expect to see a decrease in the proportion of cells that synthesize IL-17 in the FOXP3 KD cultures.

A second hypothesis is that FOXP3+ Tcons are destined to become memory T cells. Indeed, it is feasible that programming for different fates occurs at very early time point in the immune response, similar to what was described by Badovinac *et al* in regard to programmed T cell death (13). This hypothesis is supported by the metabolic profile of

FOXP3+ cells. Given that FOXP3 expression dampens glycolysis (in Tregs and also in our preliminary results in Tcons), perhaps FOXP3+ Tcons are more metabolically suited to convert into memory T cells which also display low glycolysis and high OXPHOS. Furthermore, autophagy is critical for memory formation in both CD4+ and CD8+ T cells (140; 222), which is consistent with this hypothesis. Would FOXP3 KD T cells treated with cytokines to drive memory formation (e.g. IL-7, IL-15) *in vitro* result in less memory formation? If so, could this be corrected with metabolic intervention?

Finally, a third hypothesis is that FOXP3+ Tcons are an intermediate T cell phenotype that haven't been described. Specifically, this intermediate phenotype would represent T cells within the effector population that are activated and yet semi-suppressive (Treg-like) to provide an additional layer of immune homeostasis during a T cell response. This hypothesis is supported by the debate in FOXP3 literature over whether expression of FOXP3 in conventional T cells confers suppressive functions. Interpretation of these opposing findings largely depends on the sensitivity of the assays being used, as well as which specific type of 'suppressive' assay is being conducted (1; 209; 237). There is probably truth to both sides of the argument. In a subset of T cells that are FOXP3hi (relative to other Tcons), there could be some suppressive function. Whereas in the majority of Tcons with little to no FOXP3 expression, there would be no apparent suppressive capabilities.

One cutting-edge technique has the potential to address many of these hypotheses: single-cell (sc) RNA sequencing (scRNA-Seq). This technology has the power to collect transcriptome data from hundreds to thousands of T cells in a dynamic and heterogenous sample, while allowing for a single-cell breakdown of gene transcripts. We recently

activated naïve CD4 T cells from a healthy human donor and subjected them to scRNA-Seq with our collaborators on day 4 post-activation. As seen in Appendix A- Figure 23, what began as a presumably homogenous population of naïve CD4+ T cells displayed multiple distinct clusters based on transcriptome relatedness by day 4 post-activation, highlighting the heterogenous nature of activated T cells. Specifically, 8 distinct clusters were identified by unbiased t-distributed stochastic neighborhood embedding (t-SNE) cluster analysis. The limitation in this particular experiment was the efficiency of FOXP3 transcript detection among the entire sample set (Appendix A- Figure 24). The predicted percentage of FOXP3+ cells as determined by ICFC was ~9%. A total of 5,185 cells were sequenced. Unfortunately, the percentage of FOXP3 detected by sequencing was only 3.99%, constraining downstream analyses to 207 cells. Despite low FOXP3 detection that does not necessarily represent true FOXP3 expression levels in the sample, our preliminary data suggests that FOXP3+ cells are not localized to a specific cluster of closely related cells (Appendix A- Figure 24). Nonetheless, with technical improvements in library saturation and thereby capturing the true FOXP3 transcript landscape, we will soon be able to determine what gene transcripts are significantly different in FOXP3+ compared to FOXP3- cells. Ultimately, scRNA-Seq could uncover the identity of the subset of FOXP3+ Tcons and is part of our future directions.

RICD: WHO LIVES AND WHO DIES?

This dissertation work focused on FOXP3 as a key player in RICD sensitivity of human T cells. We have additionally linked cell metabolism, CD48 expression, and autophagy to this equation. However, there are certainly other factors that determine whether a T cell lives or dies after TCR restimulation. While conducting hundreds of

RICD assays in our lab, we have noticed that upon restimulation with high doses of OKT3 and presumably saturating the T cell system, 100% cell loss has never been achieved in activated T cells. Therefore, the ultimate question for RICD sensitivity is what determines the few cells that survive versus the rest of the T cell culture that dies? There could be a temporal component to this, but apoptosis in late stage effectors should occur within the 24 hour window of our RICD assays. With the advent of scRNA-Seq, it would be possible to address this important question. Cells restimulated with high doses of OKT3 could be subjected to sequencing as part of a time course. Cells that upregulate signature apoptotic transcripts associated with RICD could be analyzed apart from the survivors. This unbiased approach has the power to open our viewpoint on factors that mediate RICD sensitivity. For example, it's possible that all T cells in the culture do upregulate apoptotic genes, and yet some cells evade death by other mechanisms we haven't considered. Indeed, some cells can recover from experiencing transient engagement of cell death machinery, termed 'revival'(64). Eventually, single-cell analysis could allow us to predict which cells are destined to live or die. However, we must be open-minded in the cell death field when we label and characterize cells as live or dead, given that traditional apoptosis markers may not reliably define all cells destined to die.

Appendix A: Supplementary data

INTRODUCTION

The purpose of this appendix is to include additional unpublished data that was collected during preparation of the manuscript detailed in Chapter 3. Ultimately, this section is meant to provide supplemental figures for an improved understanding and discussion of FOXP3 in Tcons, as well as preliminary data relevant for future directions. The methodology of each experiment is included in the figure legends.

FIGURES

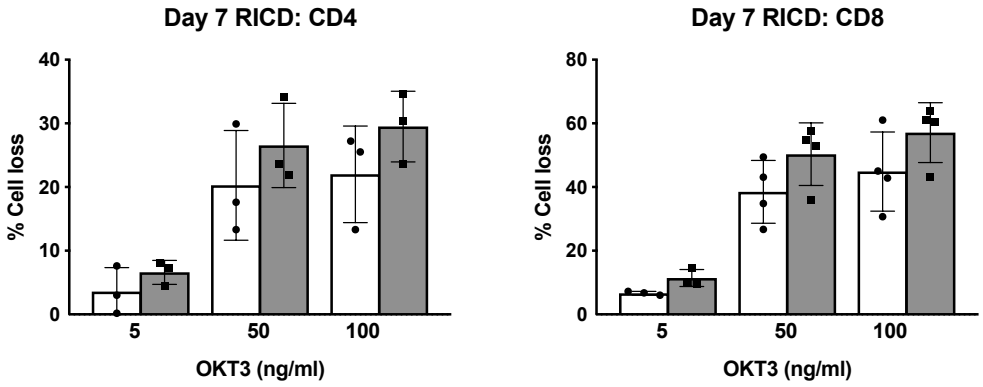


Figure 18. Day 7 FOXP3 knockdown T cells show an intermediate phenotype of RICD protection.
Purified CD4 (left) and CD8 (right) T cells were activated with anti-CD3/28/2 beads. On day 3, cells were electroporated with non-specific control siRNA (white bars) or FOXP3-specific siRNA (gray bars). On day 7, cells were restimulated with OKT3 to induce RICD. Each data point represents the average cell loss from an individual donor. Knockdowns were verified by Western blot (not shown).

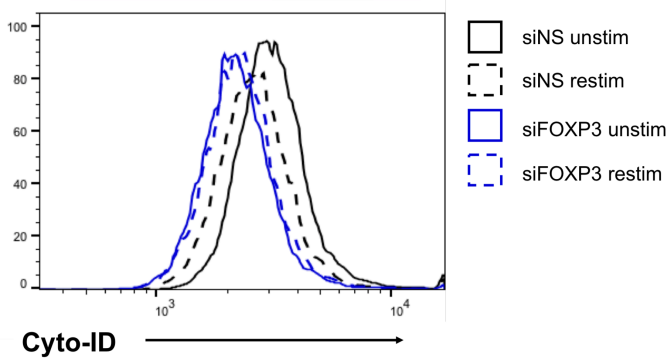


Figure 19. TCR restimulation decreases autophagy.

Day 4 KD CD4 T cells were either left unstimulated, or restimulated with 100ng/ml OKT3 for 6 hours. Cells were washed with PBS and stained with Cyto-ID autophagy reagent and subjected to flow cytometry. Figure shows one representative experiment of at least 3 independent experiments. Similar results were obtained in day 4 CD8 KD T cells.

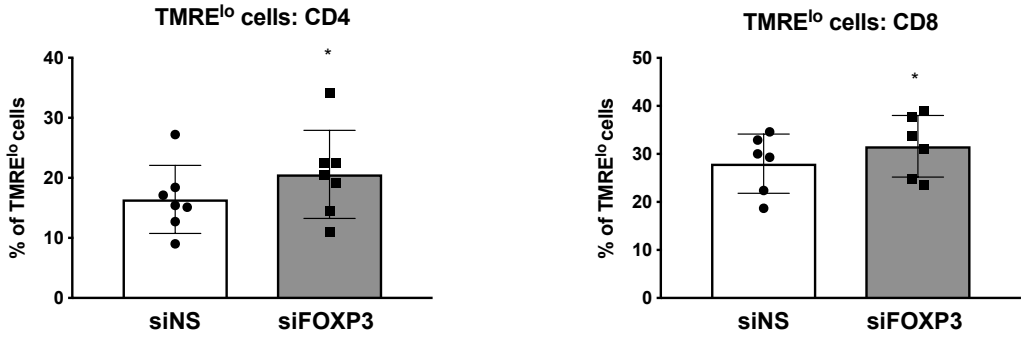


Figure 20. FOXP3 knockdown T cells exhibit slightly decreased mitochondrial membrane potential.

Day 4 KD T cells were stained with tetramethylrhodamine, ethyl ester (TMRE) for 30 minutes and washed with PBS twice. The percentage of TMRElo cells was quantified by flow cytometry. A decrease in the fluorescence intensity as seen by a histogram indicated the percentage of cells with a lower mitochondrial membrane potential. *$p<0.05$ by paired t test.

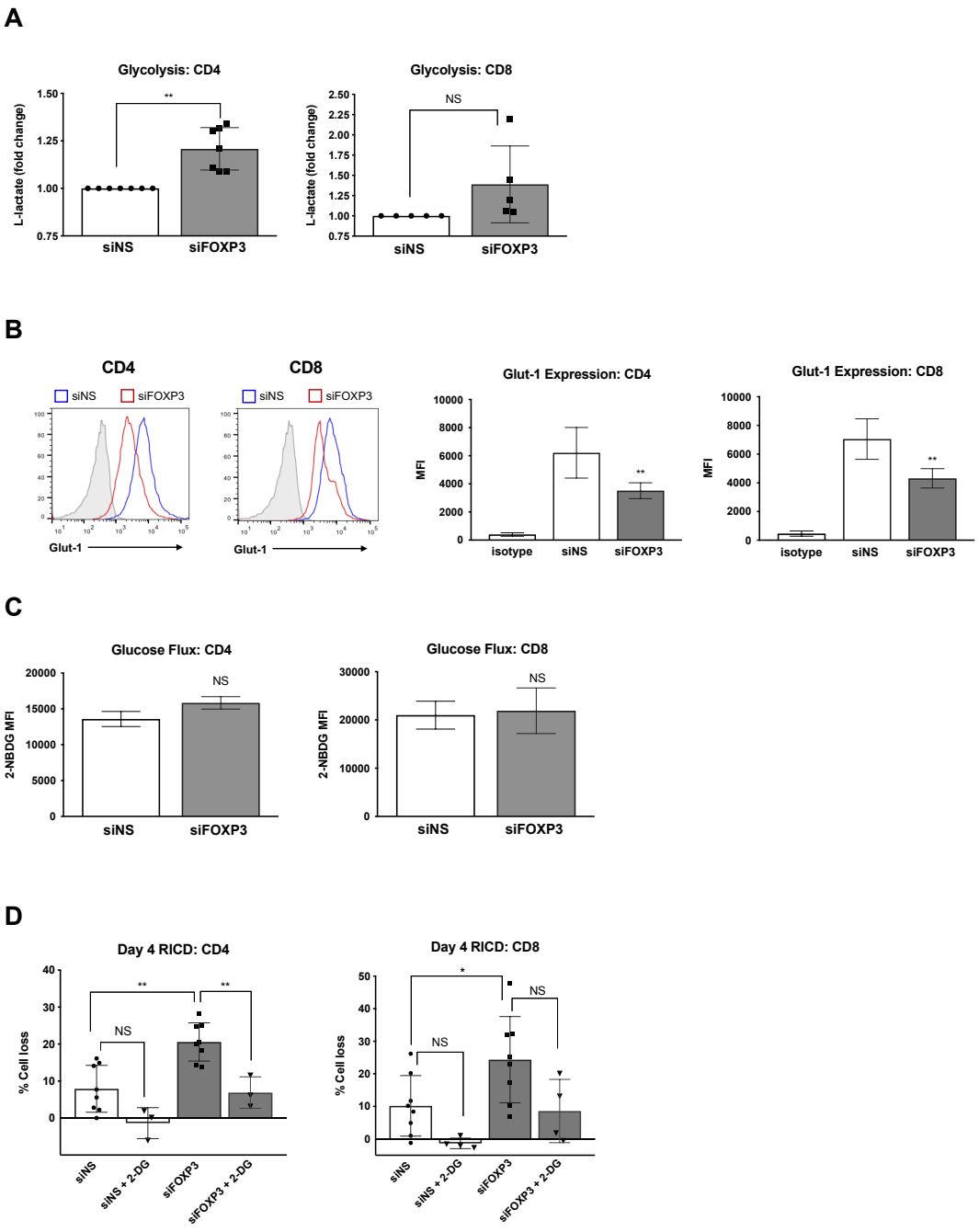


Figure 21. FOXP3 knockdown and glycolysis.

A) Purified CD4 and CD8 T cells were electroporated with non-specific (NS) or FOXP3-specific siRNA and activated with Immunocult. After 4 days of activation in complete RMPI with dialyzed serum, viable cells were counted by trypan blue exclusion and supernatants were subjected to L-lactate assay in triplicate (company name). L-lactate was normalized to the number of live cells at the time of collection (per million cells). Each data point represents the average value from an individual donor.

B) Day 4 KD T cells were stained for surface Glut-1 expression with Glut1-PE (company). The mean fluorescence intensity (MFI) was measured by flow cytometry. Graphs are the average of at least 4 independent experiments.
C) Day 4 KD T cells were incubated with 2-NBDG for 20 minutes in rich RPMI media and washed twice with PBS. The mean fluorescence intensity (MFI) of 2-NBDG was measured by flow cytometry. Figures are the average of 2 independent experiments.
D) Day 4 KD T cells were incubated with 2-DG for 30 minutes to inhibit glycolysis. Cells were then restimulated with 100ng/mL OKT3 to induce RICD. *p<0.05, **p<0.01 by one-way ANOVA.

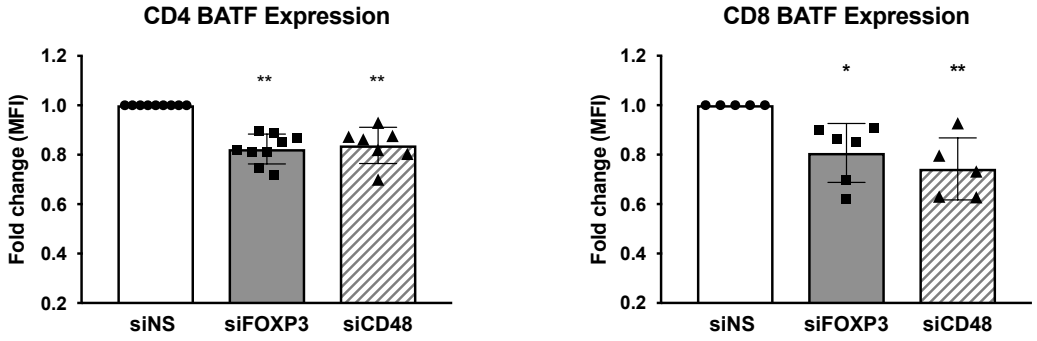


Figure 22. FOXP3 and CD48 knockdown T cells have reduced BATF expression. Day 4 CD4 (left) and CD8 (right) KD cells were analyzed for BATF expression by intracellular flow cytometry. The mean fluorescence intensity (MFI) of BATF was collected by flow cytometry and normalized to the control non-specific (siNS) cells. Each data point represents the value from an independent donor. **p<0.01, *p<0.01 by one-way ANOVA.

Single-cell (sc) RNA-Seq

This experiment was conducted as a collaboration with Dr. Clifton Dalgard. Naïve CD4 T cells were isolated from a healthy human donor and activated with Immunocult (Stem Cell Technologies). On day 3, cells were washed with PBS and resuspended in fresh complete RPMI with rIL-2 (100U/mL). Cells were prepared for sequencing on day 4 post-activation. Single cell preparations were conducted according to the 10xGenomics protocol. T cells were 98% viable before library preparation as determined by trypan blue staining. We targeted 9,000 cells for partitioning, and ultimately to be sequenced. Data analysis was conducted entirely by Nathaniel Lott, according to the Seurat R analysis pipeline (Satija lab, New York Genome Center).

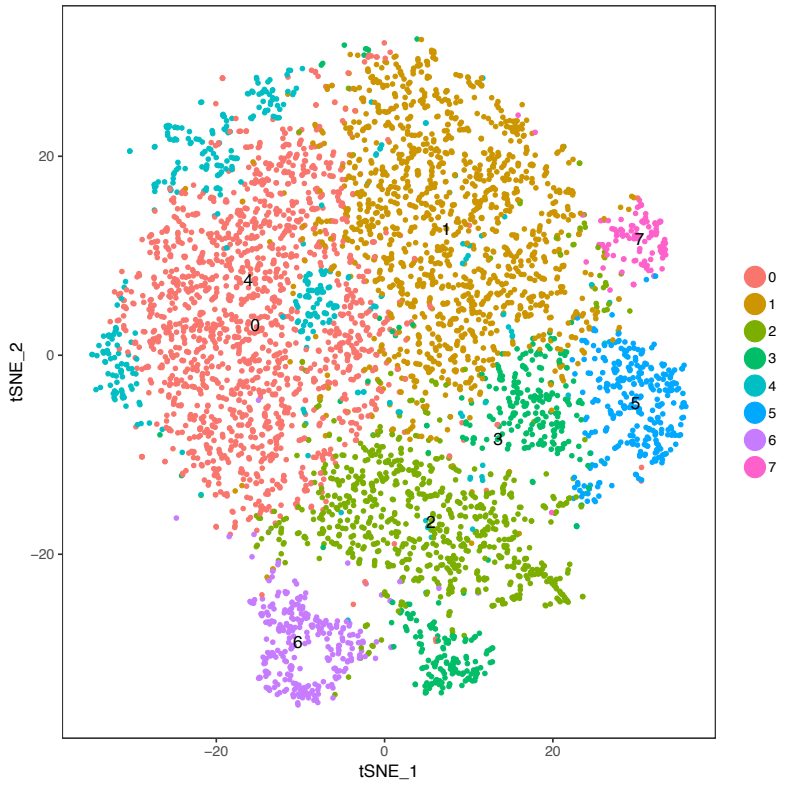


Figure 23. t-distributed stochastic neighborhood embedding (t-SNE) cluster analysis. Every dot represents a cell that was sequenced. Non-biased clustering analysis determined 8 distinct clusters based on transcriptomic relatedness (labeled 0-7 and displayed in unique colors).

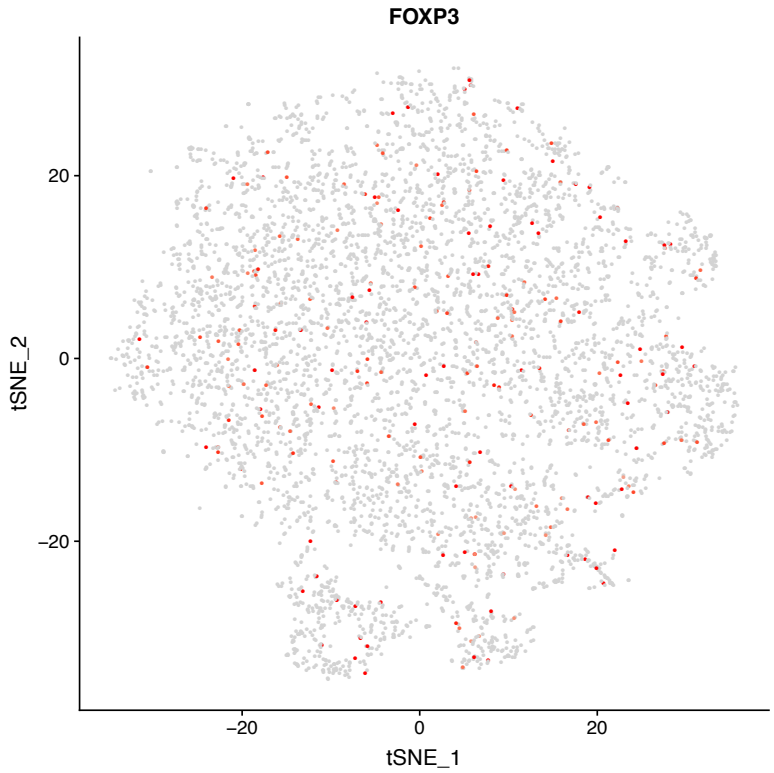


Figure 24. FOXP3 detection in scRNA-Seq dataset.
Each sequenced cell is represented by a gray dot in t-distributed stochastic neighborhood embedding (t-SNE). Cells with detectable FOXP3 transcripts are indicated in red. A total of 207 cells were characterized as FOXP3+ by Seurat R analysis.

REFERENCES

1. Aarts-Riemens T, Emmelot ME, Verdonck LF, Mutis T. 2008. Forced overexpression of either of the two common human Foxp3 isoforms can induce regulatory T cells from CD4(+)CD25(-) cells. *Eur J Immunol* 38:1381-90
2. Abadia-Molina AC, Ji H, Faubion WA, Julien A, Latchman Y, et al. 2006. CD48 controls T-cell and antigen-presenting cell functions in experimental colitis. *Gastroenterology* 130:424-34
3. Albert MH, Anasetti C, Yu XZ. 2006. T regulatory cells as an immunotherapy for transplantation. *Expert Opin Biol Ther* 6:315-24
4. Alderson MR, Tough TW, Davis-Smith T, Braddy S, Falk B, et al. 1995. Fas ligand mediates activation-induced cell death in human T lymphocytes. *J Exp Med* 181:71-7
5. Allan SE, Crome SQ, Crellin NK, Passerini L, Steiner TS, et al. 2007. Activation-induced FOXP3 in human T effector cells does not suppress proliferation or cytokine production. *Int Immunol* 19:345-54
6. Alves NL, Derks IA, Berk E, Spijker R, van Lier RA, Eldering E. 2006. The Noxa/Mcl-1 axis regulates susceptibility to apoptosis under glucose limitation in dividing T cells. *Immunity* 24:703-16
7. Andersen JL, Kornbluth S. 2013. The tangled circuitry of metabolism and apoptosis. *Mol Cell* 49:399-410
8. Angela M, Endo Y, Asou HK, Yamamoto T, Tumes DJ, et al. 2016. Fatty acid metabolic reprogramming via mTOR-mediated inductions of PPARgamma directs early activation of T cells. *Nat Commun* 7:13683
9. Angelin A, Gil-de-Gomez L, Dahiya S, Jiao J, Guo L, et al. 2017. Foxp3 Reprograms T Cell Metabolism to Function in Low-Glucose, High-Lactate Environments. *Cell Metab* 25:1282-93 e7
10. Araki K, Turner AP, Shaffer VO, Gangappa S, Keller SA, et al. 2009. mTOR regulates memory CD8 T-cell differentiation. *Nature* 460:108-12
11. Ashwell JD, Cunningham RE, Noguchi PD, Hernandez D. 1987. Cell growth cycle block of T cell hybridomas upon activation with antigen. *The Journal of experimental medicine* 165:173-94
12. Bacchetta R, Barzaghi F, Roncarolo MG. 2018. From IPEX syndrome to FOXP3 mutation: a lesson on immune dysregulation. *Ann N Y Acad Sci* 1417:5-22
13. Badovinac VP, Porter BB, Harty JT. 2002. Programmed contraction of CD8(+) T cells after infection. *Nat Immunol* 3:619-26
14. Baldanzi G, Pighini A, Bettio V, Rainero E, Traini S, et al. 2011. SAP-mediated inhibition of diacylglycerol kinase alpha regulates TCR-induced diacylglycerol signaling. *J Immunol* 187:5941-51
15. Banerjee-Basu S, Baxevanis AD. 2004. Structural analysis of disease-causing mutations in the P-subfamily of forkhead transcription factors. *Proteins* 54:639-47
16. Bar RS, DeLor CJ, Clausen KP, Hurtubise P, Henle W, Hewetson JF. 1974. Fatal infectious mononucleosis in a family. *N Engl J Med* 290:363-7

17. Baron U, Floess S, Wieczorek G, Baumann K, Grutzkau A, et al. 2007. DNA demethylation in the human FOXP3 locus discriminates regulatory T cells from activated FOXP3(+) conventional T cells. *Eur J Immunol* 37:2378-89
18. Barzaghi F, Amaya Hernandez LC, Neven B, Ricci S, Kucuk ZY, et al. 2018. Long-term follow-up of IPEX syndrome patients after different therapeutic strategies: An international multicenter retrospective study. *J Allergy Clin Immunol* 141:1036-49 e5
19. Bassiri H, Janice Yeo WC, Rothman J, Koretzky GA, Nichols KE. 2008. X-linked lymphoproliferative disease (XLP): a model of impaired anti-viral, anti-tumor and humoral immune responses. *Immunol Res* 42:145-59
20. Basu S, Golovina T, Mikheeva T, June CH, Riley JL. 2008. Cutting edge: Foxp3-mediated induction of pim 2 allows human T regulatory cells to preferentially expand in rapamycin. *J Immunol* 180:5794-8
21. Bengsch B, Johnson AL, Kurachi M, Odorizzi PM, Pauken KE, et al. 2016. Bioenergetic Insufficiencies Due to Metabolic Alterations Regulated by the Inhibitory Receptor PD-1 Are an Early Driver of CD8(+) T Cell Exhaustion. *Immunity* 45:358-73
22. Bhaskaran N, Quigley C, Weinberg A, Huang A, Popkin D, Pandiyan P. 2016. Transforming growth factor-beta1 sustains the survival of Foxp3(+) regulatory cells during late phase of oropharyngeal candidiasis infection. *Mucosal Immunol* 9:1015-26
23. Bin Dhuban K, d'Hennezel E, Nagai Y, Xiao Y, Shao S, et al. 2017. Suppression by human FOXP3+ regulatory T cells requires FOXP3-TIP60 interactions. *Sci Immunol* 2
24. Boehme SA, Lenardo MJ. 1993. Propriocidal apoptosis of mature T lymphocytes occurs at S phase of the cell cycle. *European journal of immunology* 23:1552-60
25. Boehme SA, Lenardo MJ. 1993. Propriocidal apoptosis of mature T lymphocytes occurs at S phase of the cell cycle. *Eur J Immunol* 23:1552-60
26. Booth LA, Tavallai S, Hamed HA, Cruickshanks N, Dent P. 2014. The role of cell signalling in the crosstalk between autophagy and apoptosis. *Cell Signal* 26:549-55
27. Bortner CD, Scoltock AB, Cain DW, Cidlowski JA. 2016. T-cell development of resistance to apoptosis is driven by a metabolic shift in carbon source and altered activation of death pathways. *Cell Death Differ* 23:889-902
28. Brenner D, Krammer PH, Arnold R. 2008. Concepts of activated T cell death. *Crit Rev Oncol Hematol* 66:52-64
29. Breuer O, Gangwar RS, Seaf M, Barhoum A, Kerem E, Levi-Schaffer F. 2018. Evaluation of Soluble CD48 Levels in Patients with Allergic and Nonallergic Asthma in Relation to Markers of Type 2 and Non-Type 2 Immunity: An Observational Study. *J Immunol Res* 2018:4236263
30. Brunstein CG, Miller JS, McKenna DH, Hippen KL, DeFor TE, et al. 2016. Umbilical cord blood-derived T regulatory cells to prevent GVHD: kinetics, toxicity profile, and clinical effect. *Blood* 127:1044-51
31. Buck MD, O'Sullivan D, Klein Geltink RI, Curtis JD, Chang CH, et al. 2016. Mitochondrial Dynamics Controls T Cell Fate through Metabolic Programming. *Cell* 166:63-76

32. Buck MD, O'Sullivan D, Pearce EL. 2015. T cell metabolism drives immunity. *J Exp. Med*. 212:1345-60
33. Buck MD, O'Sullivan D, Pearce EL. 2015. T cell metabolism drives immunity. *J Exp Med* 212:1345-60
34. Buck MD, Sowell RT, Kaech SM, Pearce EL. 2017. Metabolic Instruction of Immunity. *Cell* 169:570-86
35. Calder PC. 2008. The relationship between the fatty acid composition of immune cells and their function. *Prostaglandins Leukot Essent Fatty Acids* 79:101-8
36. Cao X, Cai SF, Fehniger TA, Song J, Collins LI, et al. 2007. Granzyme B and perforin are important for regulatory T cell-mediated suppression of tumor clearance. *Immunity* 27:635-46
37. Cavatorta DJ, Erb HN, Felippe MJ. 2012. Activation-induced FoxP3 expression regulates cytokine production in conventional T cells stimulated with autologous dendritic cells. *Clin Vaccine Immunol* 19:1583-92
38. Cencioni MT, Santini S, Ruocco G, Borsellino G, De Bardi M, et al. 2015. FAS-ligand regulates differential activation-induced cell death of human T-helper 1 and 17 cells in healthy donors and multiple sclerosis patients. *Cell Death Dis* 6:e1741
39. Cencioni MT, Santini S, Ruocco G, Borsellino G, De Bardi M, et al. 2015. FAS-ligand regulates differential activation-induced cell death of human T-helper 1 and 17 cells in healthy donors and multiple sclerosis patients. *Cell Death Dis* 6:e1785
40. Chang CH, Qiu J, O'Sullivan D, Buck MD, Noguchi T, et al. 2015. Metabolic Competition in the Tumor Microenvironment Is a Driver of Cancer Progression. *Cell* 162:1229-41
41. Chen Y, Zander R, Khatun A, Schauder DM, Cui W. 2018. Transcriptional and Epigenetic Regulation of Effector and Memory CD8 T Cell Differentiation. *Front Immunol* 9:2826
42. Chen ZH, Liu ZH, Yu C, Ji DX, Li LS. 2007. Endothelial dysfunction in patients with severe acute pancreatitis: improved by continuous blood purification therapy. *Int J Artif Organs* 30:393-400
43. Cheng LE, Chan FK, Cado D, Winoto A. 1997. Functional redundancy of the Nur77 and Nor-1 orphan steroid receptors in T-cell apoptosis. *EMBO J* 16:1865-75
44. Coe DJ, Kishore M, Marelli-Berg F. 2014. Metabolic regulation of regulatory T cell development and function. *Front Immunol* 5:590
45. Combadiere B, Freedman M, Chen L, Shores EW, Love P, Lenardo MJ. 1996. Qualitative and quantitative contributions of the T cell receptor zeta chain to mature T cell apoptosis. *J Exp Med* 183:2109-17
46. Cookson BT, Brennan MA. 2001. Pro-inflammatory programmed cell death. *Trends Microbiol* 9:113-4
47. Corrado M, Mariotti FR, Trapani L, Taraborrelli L, Nazio F, et al. 2016. Macroautophagy inhibition maintains fragmented mitochondria to foster T cell receptor-dependent apoptosis. *EMBO J* 35:1793-809

48. Cruz AC, Ramaswamy M, Ouyang C, Klebanoff CA, Sengupta P, et al. 2016. Fas/CD95 prevents autoimmunity independently of lipid raft localization and efficient apoptosis induction. *Nat Commun* 7:13895
49. Cui J, Placzek WJ. 2018. Post-Transcriptional Regulation of Anti-Apoptotic BCL2 Family Members. *Int J Mol Sci* 19
50. Dammeijer F, Lau SP, van Eijck CHJ, van der Burg SH, Aerts J. 2017. Rationally combining immunotherapies to improve efficacy of immune checkpoint blockade in solid tumors. *Cytokine Growth Factor Rev*
51. Davey GM, Kurts C, Miller JF, Bouillet P, Strasser A, et al. 2002. Peripheral deletion of autoreactive CD8 T cells by cross presentation of self-antigen occurs by a Bcl-2-inhibitable pathway mediated by Bim. *J Exp Med* 196:947-55
52. de Rezende LC, Silva IV, Rangel LB, Guimaraes MC. 2010. Regulatory T cell as a target for cancer therapy. *Arch Immunol Ther Exp (Warsz)* 58:179-90
53. Dhein J, Walczak H, Baumler C, Debatin KM, Krammer PH. 1995. Autocrine T-cell suicide mediated by APO-1/(Fas/CD95). *Nature* 373:438-41
54. Dowling SD, Macian F. 2018. Autophagy and T cell metabolism. *Cancer Lett* 419:20-6
55. Elishmereni M, Levi-Schaffer F. 2011. CD48: A co-stimulatory receptor of immunity. *Int J Biochem Cell Biol* 43:25-8
56. Evavold CL, Kagan JC. 2018. How Inflammasomes Inform Adaptive Immunity. *J Mol Biol* 430:217-37
57. Feng LL, Wang X. 2010. Targeting Foxp3+ regulatory T cells-related immunosuppression for cancer immunotherapy. *Chin Med J (Engl)* 123:3334-42
58. Fernandez-Ramos AA, Marchetti-Laurent C, Poindessous V, Antonio S, Laurent-Puig P, et al. 2017. 6-mercaptopurine promotes energetic failure in proliferating T cells. *Oncotarget* 8:43048-60
59. Foster PS, Maltby S, Rosenberg HF, Tay HL, Hogan SP, et al. 2017. Modeling TH 2 responses and airway inflammation to understand fundamental mechanisms regulating the pathogenesis of asthma. *Immunol Rev* 278:20-40
60. Fritzsching B, Oberle N, Eberhardt N, Quick S, Haas J, et al. 2005. In contrast to effector T cells, CD4+CD25+FoxP3+ regulatory T cells are highly susceptible to CD95 ligand- but not to TCR-mediated cell death. *J Immunol* 175:32-6
61. Gangwar RS, Minai-Fleminger Y, Seaf M, Gutgold A, Shikotra A, et al. 2017. CD48 on blood leukocytes and in serum of asthma patients varies with severity. *Allergy* 72:888-95
62. Gavin MA, Torgerson TR, Houston E, DeRoos P, Ho WY, et al. 2006. Single-cell analysis of normal and FOXP3-mutant human T cells: FOXP3 expression without regulatory T cell development. *Proc Natl Acad Sci U S A* 103:6659-64
63. Gerriets VA, Kishton RJ, Johnson MO, Cohen S, Siska PJ, et al. 2016. Foxp3 and Toll-like receptor signaling balance Treg cell anabolic metabolism for suppression. *Nat Immunol* 17:1459-66
64. Gong YN, Crawford JC, Heckmann BL, Green DR. 2019. To the edge of cell death and back. *FEBS J* 286:430-40
65. Gonzalez-Cabrero J, Wise CJ, Latchman Y, Freeman GJ, Sharpe AH, Reiser H. 1999. CD48-deficient mice have a pronounced defect in CD4(+) T cell activation. *Proc Natl Acad Sci U S A* 96:1019-23

66. Green DR, Droin N, Pinkoski M. 2003. Activation-induced cell death in T cells. *Immunological reviews* 193:70-81
67. Guo S, Liang Y, Murphy SF, Huang A, Shen H, et al. 2015. A rapid and high content assay that measures cyto-ID-stained autophagic compartments and estimates autophagy flux with potential clinical applications. *Autophagy* 11:560-72
68. Hadas Pahima PGP, Francesca Levi-Schaffer. 2018. 2B4 and CD48: A powerful couple of the immune system. *Clinical Immunology*
69. Hansen W, Westendorf AM, Buer J. 2008. Regulatory T cells as targets for immunotherapy of autoimmunity and inflammation. *Inflamm Allergy Drug Targets* 7:217-23
70. Hayatsu N, Miyao T, Tachibana M, Murakami R, Kimura A, et al. 2017. Analyses of a Mutant Foxp3 Allele Reveal BATF as a Critical Transcription Factor in the Differentiation and Accumulation of Tissue Regulatory T Cells. *Immunity* 47:268-83 e9
71. Herbel C, Patsoukis N, Bardhan K, Seth P, Weaver JD, Boussiotis VA. 2016. Clinical significance of T cell metabolic reprogramming in cancer. *Clin Transl Med* 5:29
72. Hernandez Del Pino RE, Pellegrini JM, Rovetta AI, Pena D, Alvarez GI, et al. 2017. Restimulation-induced T-cell death through NTB-A/SAP signaling pathway is impaired in tuberculosis patients with depressed immune responses. *Immunol Cell Biol* 95:716-28
73. Herranz D, Ambesi-Impiombato A, Sudderth J, Sanchez-Martin M, Belver L, et al. 2015. Metabolic reprogramming induces resistance to anti-NOTCH1 therapies in T cell acute lymphoblastic leukemia. *Nat Med* 21:1182-9
74. Himmel ME, MacDonald KG, Garcia RV, Steiner TS, Levings MK. 2013. Helios+ and Helios- cells coexist within the natural FOXP3+ T regulatory cell subset in humans. *J Immunol* 190:2001-8
75. Hislop AD, Palendira U, Leese AM, Arkwright PD, Rohrlich PS, et al. 2010. Impaired Epstein-Barr virus-specific CD8+ T-cell function in X-linked lymphoproliferative disease is restricted to SLAM family-positive B-cell targets. *Blood* 116:3249-57
76. Ho PC, Bihuniak JD, Macintyre AN, Staron M, Liu X, et al. 2015. Phosphoenolpyruvate Is a Metabolic Checkpoint of Anti-tumor T Cell Responses. *Cell* 162:1217-28
77. Ichimura Y, Kirisako T, Takao T, Satomi Y, Shimonishi Y, et al. 2000. A ubiquitin-like system mediates protein lipidation. *Nature* 408:488-92
78. Im SJ, Hashimoto M, Gerner MY, Lee J, Kissick HT, et al. 2016. Defining CD8+ T cells that provide the proliferative burst after PD-1 therapy. *Nature* 537:417-21
79. Ito T, Hanabuchi S, Wang YH, Park WR, Arima K, et al. 2008. Two functional subsets of FOXP3+ regulatory T cells in human thymus and periphery. *Immunity* 28:870-80
80. Jacobs SR, Herman CE, Maciver NJ, Wofford JA, Wieman HL, et al. 2008. Glucose uptake is limiting in T cell activation and requires CD28-mediated Akt-dependent and independent pathways. *J Immunol* 180:4476-86

81. Jiang Y, Li Y, Zhu B. 2015. T-cell exhaustion in the tumor microenvironment. *Cell Death Dis* 6:e1792
82. Johnson MO, Siska PJ, Contreras DC, Rathmell JC. 2016. Nutrients and the microenvironment to feed a T cell army. *Semin Immunol* 28:505-13
83. Kang CW, Dutta A, Chang LY, Mahalingam J, Lin YC, et al. 2015. Apoptosis of tumor infiltrating effector TIM-3+CD8+ T cells in colon cancer. *Sci Rep* 5:15659
84. Katz G, Krummey SM, Larsen SE, Stinson JR, Snow AL. 2014. SAP facilitates recruitment and activation of LCK at NTB-A receptors during restimulation-induced cell death. *J Immunol* 192:4202-9
85. Katz G, Snow AL. 2013. Fluorescence-activated cell sorting-based quantitation of T cell receptor restimulation-induced cell death in activated, primary human T cells. *Methods Mol Biol* 979:15-23
86. Katz G, Voss K, Yan TF, Kim YC, Kortum RL, et al. 2018. FOXP3 renders activated human regulatory T cells resistant to restimulation-induced cell death by suppressing SAP expression. *Cell Immunol*
87. Kim HJ, Barnitz RA, Kreslavsky T, Brown FD, Moffett H, et al. 2015. Stable inhibitory activity of regulatory T cells requires the transcription factor Helios. *Science* 350:334-9
88. Kim JM, Rudensky A. 2006. The role of the transcription factor Foxp3 in the development of regulatory T cells. *Immunol Rev* 212:86-98
89. Kim JY, Kim HJ, Hurt EM, Chen X, Howard OM, Farrar WL. 2007. Functional and genomic analyses of FOXP3-transduced Jurkat-T cells as regulatory T (Treg)-like cells. *Biochem Biophys Res Commun* 362:44-50
90. Kim YC, Bhairavabhotla R, Yoon J, Golding A, Thornton AM, et al. 2012. Oligodeoxynucleotides stabilize Helios-expressing Foxp3+ human T regulatory cells during in vitro expansion. *Blood* 119:2810-8
91. Klebanoff CA, Gattinoni L, Restifo NP. 2012. Sorting through subsets: which T-cell populations mediate highly effective adoptive immunotherapy? *J Immunother* 35:651-60
92. Klein L, Robey EA, Hsieh CS. 2019. Central CD4(+) T cell tolerance: deletion versus regulatory T cell differentiation. *Nat Rev Immunol* 19:7-18
93. Klysz D, Tai X, Robert PA, Craveiro M, Cretenet G, et al. 2015. Glutamine-dependent alpha-ketoglutarate production regulates the balance between T helper 1 cell and regulatory T cell generation. *Sci Signal* 8:ra97
94. Koyama J, Watanabe J, Yamada A, Koseki Y, Konno Y, et al. 2002. Evaluation of heart-rate turbulence as a new prognostic marker in patients with chronic heart failure. *Circ J* 66:902-7
95. Kunkele A, Johnson AJ, Rolczynski LS, Chang CA, Hoglund V, et al. 2015. Functional Tuning of CARs Reveals Signaling Threshold above Which CD8+ CTL Antitumor Potency Is Attenuated due to Cell Fas-FasL-Dependent AICD. *Cancer Immunol Res* 3:368-79
96. Kurachi M, Barnitz RA, Yosef N, Odorizzi PM, DiIorio MA, et al. 2014. The transcription factor BATF operates as an essential differentiation checkpoint in early effector CD8+ T cells. *Nat Immunol* 15:373-83

97. Kurts C, Kosaka H, Carbone FR, Miller JF, Heath WR. 1997. Class I-restricted cross-presentation of exogenous self-antigens leads to deletion of autoreactive CD8(+) T cells. *J Exp Med* 186:239-45
98. Kwon HK, Chen HM, Mathis D, Benoist C. 2017. Different molecular complexes that mediate transcriptional induction and repression by FoxP3. *Nat Immunol* 18:1238-48
99. Larsen SE, Bilenkin A, Tarasenko TN, Arjunaraja S, Stinson JR, et al. 2017. Sensitivity to Restimulation-Induced Cell Death Is Linked to Glycolytic Metabolism in Human T Cells. *J Immunol* 198:147-55
100. Larsen SE, Voss K, Laing ED, Snow AL. 2017. Differential cytokine withdrawal-induced death sensitivity of effector T cells derived from distinct human CD8+ memory subsets. *Cell Death Discov* 3:17031
101. Layman AAK, Deng G, O'Leary CE, Tadros S, Thomas RM, et al. 2017. Ndfip1 restricts mTORC1 signalling and glycolysis in regulatory T cells to prevent autoinflammatory disease. *Nat Commun* 8:15677
102. Lenardo MJ. 1991. Interleukin-2 programs mouse alpha beta T lymphocytes for apoptosis. *Nature* 353:858-61
103. Lenardo MJ. 1991. Interleukin-2 programs mouse alpha beta T lymphocytes for apoptosis. *Nature* 353:858-61
104. Li B, Samanta A, Song X, Iacono KT, Bembas K, et al. 2007. FOXP3 interactions with histone acetyltransferase and class II histone deacetylases are required for repression. *Proc Natl Acad Sci U S A* 104:4571-6
105. Li KP, Shanmuganad S, Carroll K, Katz JD, Jordan MB, Hildeman DA. 2017. Dying to protect: cell death and the control of T-cell homeostasis. *Immunol Rev* 277:21-43
106. Lindenstrom T, Knudsen NP, Agger EM, Andersen P. 2013. Control of chronic mycobacterium tuberculosis infection by CD4 KLRG1- IL-2-secreting central memory cells. *J Immunol* 190:6311-9
107. Lissina A, Ambrozak DR, Boswell KL, Yang W, Boritz E, et al. 2016. Fine-tuning of CD8(+) T-cell effector functions by targeting the 2B4-CD48 interaction. *Immunol Cell Biol* 94:583-92
108. Liu Z, Gerner MY, Van Panhuys N, Levine AG, Rudensky AY, Germain RN. 2015. Immune homeostasis enforced by co-localized effector and regulatory T cells. *Nature* 528:225-30
109. Lochner M, Berod L, Sparwasser T. 2015. Fatty acid metabolism in the regulation of T cell function. *Trends Immunol* 36:81-91
110. Logan MR, Jordan-Williams KL, Poston S, Liao J, Taparowsky EJ. 2012. Overexpression of Batf induces an apoptotic defect and an associated lymphoproliferative disorder in mice. *Cell Death Dis* 3:e310
111. Long SA, Buckner JH. 2011. CD4+FOXP3+ T regulatory cells in human autoimmunity: more than a numbers game. *J Immunol* 187:2061-6
112. Lu B, Finn OJ. 2008. T-cell death and cancer immune tolerance. *Cell Death Differ* 15:70-9
113. Lu L, Barbi J, Pan F. 2017. The regulation of immune tolerance by FOXP3. *Nat Rev Immunol* 17:703-17

114. Lum JJ, Bauer DE, Kong M, Harris MH, Li C, et al. 2005. Growth factor regulation of autophagy and cell survival in the absence of apoptosis. *Cell* 120:237-48
115. Maciver NJ, Jacobs SR, Wieman HL, Wofford JA, Coloff JL, Rathmell JC. 2008. Glucose metabolism in lymphocytes is a regulated process with significant effects on immune cell function and survival. *J Leukoc Biol* 84:949-57
116. MacIver NJ, Michalek RD, Rathmell JC. 2013. Metabolic regulation of T lymphocytes. *Annu Rev Immunol* 31:259-83
117. Maekawa Y, Ishifune C, Tsukumo S, Hozumi K, Yagita H, Yasutomo K. 2015. Notch controls the survival of memory CD4+ T cells by regulating glucose uptake. *Nat Med* 21:55-61
118. Magg T, Mannert J, Ellwart JW, Schmid I, Albert MH. 2012. Subcellular localization of FOXP3 in human regulatory and nonregulatory T cells. *Eur J Immunol* 42:1627-38
119. Maira SM, Stauffer F, Brueggen J, Furet P, Schnell C, et al. 2008. Identification and characterization of NVP-BEZ235, a new orally available dual phosphatidylinositol 3-kinase/mammalian target of rapamycin inhibitor with potent in vivo antitumor activity. *Mol Cancer Ther* 7:1851-63
120. Marino G, Niso-Santano M, Baehrecke EH, Kroemer G. 2014. Self-consumption: the interplay of autophagy and apoptosis. *Nat Rev Mol Cell Biol* 15:81-94
121. Marsden VS, Strasser A. 2003. Control of apoptosis in the immune system: Bcl-2, BH3-only proteins and more. *Annu Rev Immunol* 21:71-105
122. Masahiro Ono HY, Naganari Ohkura, Issay Kitabayashi, Yuko Nagamura, Takashi Nomura, Yoshiki Miyachi, Toshihiko Tsukada & Shimon Sakaguchi. 2007. Foxp3 controls regulatory T-cell function by interacting with AML1/Runx1. *Nature* 446
123. Mason EF, Rathmell JC. 2011. Cell metabolism: an essential link between cell growth and apoptosis. *Biochim Biophys Acta* 1813:645-54
124. McArdel SL, Brown DR, Sobel RA, Sharpe AH. 2016. Anti-CD48 Monoclonal Antibody Attenuates Experimental Autoimmune Encephalomyelitis by Limiting the Number of Pathogenic CD4+ T Cells. *J Immunol* 197:3038-48
125. McArdel SL, Terhorst C, Sharpe AH. 2016. Roles of CD48 in regulating immunity and tolerance. *Clin Immunol* 164:10-20
126. McLeod IX, Jia W, He YW. 2012. The contribution of autophagy to lymphocyte survival and homeostasis. *Immunol Rev* 249:195-204
127. McMurchy AN, Gillies J, Gizzi MC, Riba M, Garcia-Manteiga JM, et al. 2013. A novel function for FOXP3 in humans: intrinsic regulation of conventional T cells. *Blood* 121:1265-75
128. Mehrle S, Frank S, Schmidt J, Schmidt-Wolf IG, Marten A. 2005. SAP and SLAM expression in anti-CD3 activated lymphocytes correlates with cytotoxic activity. *Immunol Cell Biol* 83:33-9
129. Meyer Zu Horste G, Przybylski D, Schramm MA, Wang C, Schnell A, et al. 2018. Fas Promotes T Helper 17 Cell Differentiation and Inhibits T Helper 1 Cell Development by Binding and Sequestering Transcription Factor STAT1. *Immunity* 48:556-69 e7

130. Michalek RD, Gerriets VA, Jacobs SR, Macintyre AN, MacIver NJ, et al. 2011. Cutting edge: distinct glycolytic and lipid oxidative metabolic programs are essential for effector and regulatory CD4+ T cell subsets. *J Immunol* 186:3299-303
131. Miyao T, Floess S, Setoguchi R, Luche H, Fehling HJ, et al. 2012. Plasticity of Foxp3(+) T cells reflects promiscuous Foxp3 expression in conventional T cells but not reprogramming of regulatory T cells. *Immunity* 36:262-75
132. Monteiro M, Almeida CF, Caridade M, Ribot JC, Duarte J, et al. 2010. Identification of regulatory Foxp3+ invariant NKT cells induced by TGF-beta. *J Immunol* 185:2157-63
133. Moran AE, Holzapfel KL, Xing Y, Cunningham NR, Maltzman JS, et al. 2011. T cell receptor signal strength in Treg and iNKT cell development demonstrated by a novel fluorescent reporter mouse. *J Exp Med* 208:1279-89
134. Moreira-Teixeira L, Resende M, Devergne O, Herbeuval JP, Hermine O, et al. 2012. Rapamycin combined with TGF-beta converts human invariant NKT cells into suppressive Foxp3+ regulatory cells. *J Immunol* 188:624-31
135. Morel PA. 2018. Differential T-cell receptor signals for T helper cell programming. *Immunology* 155:63-71
136. Mosmann TR, Coffman RL. 1989. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol* 7:145-73
137. Mougiakakos D, Choudhury A, Lladser A, Kiessling R, Johansson CC. 2010. Regulatory T cells in cancer. *Adv Cancer Res* 107:57-117
138. Muhammad A, Schiller HB, Forster F, Eckerstorfer P, Geyeregger R, et al. 2009. Sequential cooperation of CD2 and CD48 in the buildup of the early TCR signalosome. *J Immunol* 182:7672-80
139. Muppidi JR, Siegel RM. 2004. Ligand-independent redistribution of Fas (CD95) into lipid rafts mediates clonotypic T cell death. *Nat Immunol* 5:182-9
140. Murera D, Arbogast F, Arnold J, Bouis D, Muller S, Gros F. 2018. CD4 T cell autophagy is integral to memory maintenance. *Sci Rep* 8:5951
141. Newton K, Strasser A. 2003. Caspases signal not only apoptosis but also antigen-induced activation in cells of the immune system. *Genes Dev* 17:819-25
142. Nicholls DG. 2009. Spare respiratory capacity, oxidative stress and excitotoxicity. *Biochem Soc Trans* 37:1385-8
143. Nishikawa H, Sakaguchi S. 2010. Regulatory T cells in tumor immunity. *Int J Cancer* 127:759-67
144. Okamoto S, Ji H, Howie D, Clarke K, Gullo C, et al. 2004. Expression of the SH2D1A gene is regulated by a combination of transcriptional and post-transcriptional mechanisms. *Eur J Immunol* 34:3176-86
145. Panchal N, Booth C, Cannons JL, Schwartzberg PL. 2018. X-Linked Lymphoproliferative Disease Type 1: A Clinical and Molecular Perspective. *Front Immunol* 9:666
146. Pandiyan P, Lenardo MJ. 2008. The control of CD4+CD25+Foxp3+ regulatory T cell survival. *Biol Direct* 3:6
147. Parmar S, Shpall EJ. 2016. Treg adoptive therapy: is more better? *Blood* 127:962-3

148. Passerini L, Bacchetta R. 2017. Forkhead-Box-P3 Gene Transfer in Human CD4+ T Conventional Cells for the Generation of Stable and Efficient Regulatory T Cells, Suitable for Immune Modulatory Therapy. *Front Immunol* 8:1282
149. Passerini L, Rossi Mel E, Sartirana C, Fousteri G, Bondanza A, et al. 2013. CD4(+) T cells from IPEX patients convert into functional and stable regulatory T cells by FOXP3 gene transfer. *Sci Transl Med* 5:215ra174
150. Patel CH, Powell JD. 2017. Targeting T cell metabolism to regulate T cell activation, differentiation and function in disease. *Curr Opin Immunol* 46:82-8
151. Patsoukis N, Bardhan K, Chatterjee P, Sari D, Liu B, et al. 2015. PD-1 alters T-cell metabolic reprogramming by inhibiting glycolysis and promoting lipolysis and fatty acid oxidation. *Nat Commun* 6:6692
152. Pauken KE, Wherry EJ. 2015. Overcoming T cell exhaustion in infection and cancer. *Trends Immunol* 36:265-76
153. Pearce EL, Poffenberger MC, Chang CH, Jones RG. 2013. Fueling immunity: insights into metabolism and lymphocyte function. *Science* 342:1242454
154. Pearce EL, Walsh MC, Cejas PJ, Harms GM, Shen H, et al. 2009. Enhancing CD8 T-cell memory by modulating fatty acid metabolism. *Nature* 460:103-7
155. Pereira LMS, Gomes STM, Ishak R, Vallinoto ACR. 2017. Regulatory T Cell and Forkhead Box Protein 3 as Modulators of Immune Homeostasis. *Front Immunol* 8:605
156. Pierson W, Cauwe B, Policheni A, Schlenner SM, Franckaert D, et al. 2013. Antiapoptotic Mcl-1 is critical for the survival and niche-filling capacity of Foxp3(+) regulatory T cells. *Nat Immunol* 14:959-65
157. Plitas G, Konopacki C, Wu K, Bos PD, Morrow M, et al. 2016. Regulatory T Cells Exhibit Distinct Features in Human Breast Cancer. *Immunity* 45:1122-34
158. Plitas G, Rudensky AY. 2016. Regulatory T Cells: Differentiation and Function. *Cancer Immunol Res* 4:721-5
159. Polansky JK, Kretschmer K, Freyer J, Floess S, Garbe A, et al. 2008. DNA methylation controls Foxp3 gene expression. *Eur J Immunol* 38:1654-63
160. Pollizzi KN, Patel CH, Sun IH, Oh MH, Waickman AT, et al. 2015. mTORC1 and mTORC2 selectively regulate CD8(+) T cell differentiation. *J Clin Invest* 125:2090-108
161. Pollizzi KN, Powell JD. 2014. Integrating canonical and metabolic signalling programmes in the regulation of T cell responses. *Nat Rev Immunol* 14:435-46
162. Pollizzi KN, Sun IH, Patel CH, Lo YC, Oh MH, et al. 2016. Asymmetric inheritance of mTORC1 kinase activity during division dictates CD8(+) T cell differentiation. *Nat Immunol* 17:704-11
163. Proskuryakov SY, Konoplyannikov AG, Gabai VL. 2003. Necrosis: a specific form of programmed cell death? *Exp Cell Res* 283:1-16
164. Puthalakath H, Strasser A. 2002. Keeping killers on a tight leash: transcriptional and post-translational control of the pro-apoptotic activity of BH3-only proteins. *Cell Death Differ* 9:505-12
165. Rabinovich GA, Toscano MA. 2009. Turning 'sweet' on immunity: galectin-glycan interactions in immune tolerance and inflammation. *Nat Rev Immunol* 9:338-52

166. Radoshevich L, Murrow L, Chen N, Fernandez E, Roy S, et al. 2010. ATG12 conjugation to ATG3 regulates mitochondrial homeostasis and cell death. *Cell* 142:590-600
167. Ramsdell F, Ziegler SF. 2014. FOXP3 and scurfy: how it all began. *Nat Rev Immunol* 14:343-9
168. Rathmell JC. 2012. Metabolism and autophagy in the immune system: immunometabolism comes of age. *Immunol Rev* 249:5-13
169. Rudra D dP, Chaudhry A, Niec RE, Arvey A, Samstein RM, Leslie C, Shaffer SA, Goodlett DR, Rudensky AY. 2012. Transcription factor Foxp3 and its protein partners form a complex regulatory network. *Nat Immunol* 13:1010-9
170. Ruffo E, Malacarne V, Larsen SE, Das R, Patrussi L, et al. 2016. Inhibition of diacylglycerol kinase alpha restores restimulation-induced cell death and reduces immunopathology in XLP-1. *Sci Transl Med* 8:321ra7
171. Russell JH, Rush B, Weaver C, Wang R. 1993. Mature T cells of autoimmune lpr/lpr mice have a defect in antigen-stimulated suicide. *Proc Natl Acad Sci U S A* 90:4409-13
172. Russell JH, White CL, Loh DY, Meleedy-Rey P. 1991. Receptor-stimulated death pathway is opened by antigen in mature T cells. *Proceedings of the National Academy of Sciences of the United States of America* 88:2151-5
173. Sadlon TJ, Wilkinson BG, Pederson S, Brown CY, Bresatz S, et al. 2010. Genome-wide identification of human FOXP3 target genes in natural regulatory T cells. *J Immunol* 185:1071-81
174. Schmidl C, Delacher M, Huehn J, Feuerer M. 2018. Epigenetic mechanisms regulating T-cell responses. *J Allergy Clin Immunol* 142:728-43
175. Schmidt A, Marabita F, Kiani NA, Gross CC, Johansson HJ, et al. 2018. Time-resolved transcriptome and proteome landscape of human regulatory T cell (Treg) differentiation reveals novel regulators of FOXP3. *BMC Biol* 16:47
176. Schmitz I, Krueger A, Baumann S, Schulze-Bergkamen H, Krammer PH, Kirchhoff S. 2003. An IL-2-dependent switch between CD95 signaling pathways sensitizes primary human T cells toward CD95-mediated activation-induced cell death. *J Immunol* 171:2930-6
177. Sebastian M, Lopez-Ocasio M, Metidji A, Rieder SA, Shevach EM, Thornton AM. 2016. Helios Controls a Limited Subset of Regulatory T Cell Functions. *J Immunol* 196:144-55
178. Seddiki N, Santner-Nanan B, Martinson J, Zaunders J, Sasson S, et al. 2006. Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *J Exp Med* 203:1693-700
179. She J, Matsui K, Terhorst C, Ju ST. 1998. Activation-induced apoptosis of mature T cells is dependent upon the level of surface TCR but not on the presence of the CD3 zeta ITAM. *Int Immunol* 10:1733-40
180. Shen CR, Yang WC, Chen HW. 2014. The fate of regulatory T cells: survival or apoptosis. *Cell Mol Immunol* 11:11-3
181. Shevach EM. 2009. Mechanisms of foxp3+ T regulatory cell-mediated suppression. *Immunity* 30:636-45
182. Shevach EM. 2018. Foxp3(+) T Regulatory Cells: Still Many Unanswered Questions-A Perspective After 20 Years of Study. *Front Immunol* 9:1048

183. Shi G, Ramaswamy M, Vistica BP, Cox CA, Tan C, et al. 2009. Unlike Th1, Th17 cells mediate sustained autoimmune inflammation and are highly resistant to restimulation-induced cell death. *J Immunol* 183:7547-56
184. Singh R, Cuervo AM. 2012. Lipophagy: connecting autophagy and lipid metabolism. *Int J Cell Biol* 2012:282041
185. Snow AL, Marsh RA, Krummey SM, Roehrs P, Young LR, et al. 2009. Restimulation-induced apoptosis of T cells is impaired in patients with X-linked lymphoproliferative disease caused by SAP deficiency. *J Clin Invest* 119:2976-89
186. Snow AL, Oliveira JB, Zheng L, Dale JK, Fleisher TA, Lenardo MJ. 2008. Critical role for BIM in T cell receptor restimulation-induced death. *Biol Direct* 3:34
187. Snow AL, Oliveira JB, Zheng L, Dale JK, Fleisher TA, Lenardo MJ. 2008. Critical role for BIM in T cell receptor restimulation-induced death. *Biol Direct* 3:34
188. Snow AL, Pandiyan P, Zheng L, Krummey SM, Lenardo MJ. 2010. The power and the promise of restimulation-induced cell death in human immune diseases. *Immunol Rev* 236:68-82
189. Solomon JC, Sharma K, Wei LX, Fujita T, Shi YF. 2003. A novel role for sphingolipid intermediates in activation-induced cell death in T cells. *Cell Death Differ* 10:193-202
190. Stadhouders R, Lubberts E, Hendriks RW. 2018. A cellular and molecular view of T helper 17 cell plasticity in autoimmunity. *J Autoimmun* 87:1-15
191. Stockinger B, Omenetti S. 2017. The dichotomous nature of T helper 17 cells. *Nat Rev Immunol* 17:535-44
192. Strasser A, Pellegrini M. 2004. T-lymphocyte death during shutdown of an immune response. *Trends Immunol* 25:610-5
193. Su HC, Lenardo MJ. 2008. Genetic defects of apoptosis and primary immunodeficiency. *Immunol Allergy Clin North Am* 28:329-51, ix
194. Sukumar M, Liu J, Ji Y, Subramanian M, Crompton JG, et al. 2013. Inhibiting glycolytic metabolism enhances CD8+ T cell memory and antitumor function. *J Clin Invest* 123:4479-88
195. Takahashi HK, Cambiaghi TD, Luchessi AD, Hirabara SM, Vinolo MA, et al. 2012. Activation of survival and apoptotic signaling pathways in lymphocytes exposed to palmitic acid. *J Cell Physiol* 227:339-50
196. Tamura A, Uemura S, Yamamoto N, Saito A, Kozaki A, et al. 2018. Hematopoietic cell transplantation for asymptomatic X-linked lymphoproliferative syndrome type 1. *Allergy Asthma Clin Immunol* 14:82
197. Tangye SG. 2014. XLP: clinical features and molecular etiology due to mutations in SH2D1A encoding SAP. *J Clin Immunol* 34:772-9
198. Tarasenko TN, Pacheco SE, Koenig MK, Gomez-Rodriguez J, Kapnick SM, et al. 2017. Cytochrome c Oxidase Activity Is a Metabolic Checkpoint that Regulates Cell Fate Decisions During T Cell Activation and Differentiation. *Cell Metab* 25:1254-68 e7
199. Tkachev V, Goodell S, Opipari AW, Hao LY, Franchi L, et al. 2015. Programmed death-1 controls T cell survival by regulating oxidative metabolism. *J Immunol* 194:5789-800

200. Torgerson TR, Genin A, Chen C, Zhang M, Zhou B, et al. 2009. FOXP3 inhibits activation-induced NFAT2 expression in T cells thereby limiting effector cytokine expression. *J Immunol* 183:907-15
201. Vaeth M, Schliesser U, Muller G, Reissig S, Satoh K, et al. 2012. Dependence on nuclear factor of activated T-cells (NFAT) levels discriminates conventional T cells from Foxp3+ regulatory T cells. *Proc Natl Acad Sci U S A* 109:16258-63
202. van der Windt GJ, Everts B, Chang CH, Curtis JD, Freitas TC, et al. 2012. Mitochondrial respiratory capacity is a critical regulator of CD8+ T cell memory development. *Immunity* 36:68-78
203. van der Windt GJ, O'Sullivan D, Everts B, Huang SC, Buck MD, et al. 2013. CD8 memory T cells have a bioenergetic advantage that underlies their rapid recall ability. *Proc Natl Acad Sci U S A* 110:14336-41
204. Varadhachary AS, Perdow SN, Hu C, Ramanarayanan M, Salgame P. 1997. Differential ability of T cell subsets to undergo activation-induced cell death. *Proc Natl Acad Sci U S A* 94:5778-83
205. Verbist KC, Guy CS, Milasta S, Liedmann S, Kaminski MM, et al. 2016. Metabolic maintenance of cell asymmetry following division in activated T lymphocytes. *Nature* 532:389-93
206. Vesely MD, Schreiber RD. 2013. Cancer immunoediting: antigens, mechanisms, and implications to cancer immunotherapy. *Ann N Y Acad Sci* 1284:1-5
207. Vignali DA, Collison LW, Workman CJ. 2008. How regulatory T cells work. *Nat Rev Immunol* 8:523-32
208. Voss K, Larsen SE, Snow AL. 2017. Metabolic reprogramming and apoptosis sensitivity: Defining the contours of a T cell response. *Cancer Lett* 408:190-6
209. Wang J, Ioan-Facsinay A, van der Voort EI, Huizinga TW, Toes RE. 2007. Transient expression of FOXP3 in human activated nonregulatory CD4+ T cells. *Eur J Immunol* 37:129-38
210. Wang L, Liu Y, Han R, Beier UH, Thomas RM, et al. 2013. Mbd2 promotes foxp3 demethylation and T-regulatory-cell function. *Mol Cell Biol* 33:4106-15
211. Wang RF. 2006. Functional control of regulatory T cells and cancer immunotherapy. *Semin Cancer Biol* 16:106-14
212. Weant AE, Michalek RD, Khan IU, Holbrook BC, Willingham MC, Grayson JM. 2008. Apoptosis regulators Bim and Fas function concurrently to control autoimmunity and CD8+ T cell contraction. *Immunity* 28:218-30
213. Wei J, Long L, Yang K, Guy C, Shrestha S, et al. 2016. Autophagy enforces functional integrity of regulatory T cells by coupling environmental cues and metabolic homeostasis. *Nat Immunol* 17:277-85
214. Weiss EM, Schmidt A, Vobis D, Garbi N, Lahl K, et al. 2011. Foxp3-mediated suppression of CD95L expression confers resistance to activation-induced cell death in regulatory T cells. *J Immunol* 187:1684-91
215. Wesselborg S, Kabelitz D. 1993. Activation-driven death of human T cell clones: time course kinetics of the induction of cell shrinkage, DNA fragmentation, and cell death. *Activation-driven death of human T cell clones: time course kinetics of the induction of cell shrinkage, DNA fragmentation, and cell death*
216. Wherry EJ, Kurachi M. 2015. Molecular and cellular insights into T cell exhaustion. *Nat Rev Immunol* 15:486-99

217. Wherry EJ, Teichgraber V, Becker TC, Masopust D, Kaech SM, et al. 2003. Lineage relationship and protective immunity of memory CD8 T cell subsets. *Nat Immunol* 4:225-34
218. Whiteside TL. 2002. Tumor-induced death of immune cells: its mechanisms and consequences. *Semin Cancer Biol* 12:43-50
219. Wofford JA, Wieman HL, Jacobs SR, Zhao Y, Rathmell JC. 2008. IL-7 promotes Glut1 trafficking and glucose uptake via STAT5-mediated activation of Akt to support T-cell survival. *Blood* 111:2101-11
220. Wu N, Veillette A. 2016. SLAM family receptors in normal immunity and immune pathologies. *Curr Opin Immunol* 38:45-51
221. Wu Y, Borde M, Heissmeyer V, Feuerer M, Lapan AD, et al. 2006. FOXP3 controls regulatory T cell function through cooperation with NFAT. *Cell* 126:375-87
222. Xu X, Araki K, Li S, Han JH, Ye L, et al. 2014. Autophagy is essential for effector CD8(+) T cell survival and memory formation. *Nat Immunol* 15:1152-61
223. Yamamoto TN, Lee PH, Vodnala SK, Gurusamy D, Kishton RJ, et al. 2019. T cells genetically engineered to overcome death signaling enhance adoptive cancer immunotherapy. *J Clin Invest* 130
224. Yang XF. 2008. Factors regulating apoptosis and homeostasis of CD4+ CD25(high) FOXP3+ regulatory T cells are new therapeutic targets. *Front Biosci* 13:1472-99
225. Yen B, Fortson KT, Rothman NJ, Arpaia N, Reiner SL. 2018. Clonal Bifurcation of Foxp3 Expression Visualized in Thymocytes and T Cells. *Immunohorizons* 2:119-28
226. Yolcu ES, Ash S, Kaminitz A, Sagiv Y, Askenasy N, Yarkoni S. 2008. Apoptosis as a mechanism of T-regulatory cell homeostasis and suppression. *Immunol Cell Biol* 86:650-8
227. Yuzefpolskiy Y, Baumann FM, Kalia V, Sarkar S. 2015. Early CD8 T-cell memory precursors and terminal effectors exhibit equipotent in vivo degranulation. *Cell Mol Immunol* 12:400-8
228. Zhang X, Brunner T, Carter L, Dutton RW, Rogers P, et al. 1997. Unequal death in T helper cell (Th)1 and Th2 effectors: Th1, but not Th2, effectors undergo rapid Fas/FasL-mediated apoptosis. *J Exp Med* 185:1837-49
229. Zhao F, Cannons JL, Dutta M, Griffiths GM, Schwartzberg PL. 2012. Positive and negative signaling through SLAM receptors regulate synapse organization and thresholds of cytolysis. *Immunity* 36:1003-16
230. Zheng L, Fisher G, Combadiere B, Hornung F, Martin D, et al. 1996. Mature T lymphocyte apoptosis in the healthy and diseased immune system. *Adv Exp Med Biol* 406:229-39
231. Zheng L, Li J, Lenardo M. 2017. Restimulation-induced cell death: new medical and research perspectives. *Immunol Rev* 277:44-60
232. Zheng Y. 2017. A Rogue Foxp3 Mutant Undermines Treg Cell Function. *Immunity* 47:211-4
233. Zhou X, Bailey-Bucktrout SL, Jeker LT, Penaranda C, Martinez-Llordella M, et al. 2009. Instability of the transcription factor Foxp3 leads to the generation of pathogenic memory T cells in vivo. *Nat Immunol* 10:1000-7

234. Zhu C, Anderson AC, Schubart A, Xiong H, Imitola J, et al. 2005. The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. *Nat Immunol* 6:1245-52
235. Zhu J, Paul WE. 2008. CD4 T cells: fates, functions, and faults. *Blood* 112:1557-69
236. Zhu J, Yamane H, Paul WE. 2010. Differentiation of effector CD4 T cell populations (*). *Annu Rev Immunol* 28:445-89
237. Ziegler SF. 2007. FOXP3: not just for regulatory T cells anymore. *Eur J Immunol* 37:21-3