Dissertations   **Theses and Dissertations**

2021

Investigating the Effects of TGF-β on T Cell Activation-Induced Cell Death

Christina Rose Cunha

Follow this and additional works at: https://ecommons.luc.edu/luc_diss

Part of the Immunology and Infectious Disease Commons

LOYOLA UNIVERSITY CHICAGO

INVESTIGATING THE EFFECTS OF

TGF-β ON T CELL ACTIVATION-

INDUCED CELL DEATH

A DISSERTATION SUBMITTED TO

THE FACULTY OF THE GRADUATE SCHOOL

IN CANDIDACY FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

PROGRAM IN MICROBIOLOGY AND IMMUNOLOGY

BY

CHRISTINA ROSE CUNHA

CHICAGO, ILLINOIS

DECEMBER 2021

ACKNOWLEDGEMENTS

First, I would like to acknowledge and extend my deepest appreciation to my mentor, Dr. Makio Iwashima. Throughout the past six years, he has pushed me to reach my true potential as a scientist and trained me to think critically. He has fostered development of my scientific mind and has been a joy to work with. We have had deep discussions about not only interesting data, but also about exciting topics in Immunology. His mentorship has allowed me to develop into an independent scientist, and I will cherish my time in his lab and the lessons I have learned from him throughout my career.

I am thankful for my dissertation committee, Dr. Mike Nishimura, Dr. Ed Campbell, Dr. Yee Ling Wu, and Dr. Katherine Knight for providing critical and valuable insight for developing and shaping my project. These are scientists that I look up to, and they pushed me to think deeply about my data and pursue interesting and relevant scientific questions. Both in and out of committee meetings, they have guided me not only in my science, but in my career. I am grateful for how much they have cared about my success and personal development and will forever remember their support.

I would like to thank the members of the Iwashima Lab for support, guidance, laughs, friendship, and much more throughout the years. This work would not have been possible without their advice, expertise, and help. Especially Saska and Wei, who have been by my side for all six years. I would also like to thank the Department of Microbiology and Immunology staff for their support. Additionally, I am very grateful for

Finally, I would like to acknowledge my friends and family who have been so incredibly thoughtful and supportive throughout the years. To my parents, Julia and Allyn. Thank you for constantly answering all my questions about the world as a child and supporting me in my goals and dreams to pursue science. You both have parented with such love and support, and I'm so grateful to be your daughter. To my brother, John, for years of laughs and friendship. Finally to my partner, Mihai, who always knows how to bring a smile to my face. Thank you for being my rock and for pushing me to be the best version of myself.

TABLE OF CONTENTS

LIST OF FIGURES

x

LIST OF ABBREVIATIONS

aa	amino acid
AICD	activation-induced cell death
AIRE	autoimmune regulator
ALPS	autoimmune lymphoproliferative syndrome
APC	antigen presenting cell
BCR	B cell receptor
CAR	chimeric antigen receptor
CD	cluster of differentiation
CNS	conserved noncoding region
CRS	cytokine release syndrome
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T lymphocyte associated protein-4
D	diversity
DAG	diacylglycerol
DC	dendritic cells
DISC	death-inducing signaling complex
DN	double negative
DP	double positive
ETP	early thymic progenitor
FcR	Fc receptor
FoxO	forkhead-box O

Foxp3	forkhead box protein P3
GAP	GTPase activating protein
GEF	guanine nucleotide exchange factor
GITR	glucocorticoid-Induced TNFR-related protein
HA	hyaluronic acid
HSC	hematopoietic stem cells
IDO	Indeolamine 2,3-dioxygenase
IEL	intraepithelial lymphocytes
IFN-γ	interferon gamma
iNOS	inducible nitric oxide synthase
IPEX	Immunodysregulation polyendocrinopathy enteropathy x-linked
ITAMS	immunoreceptor tyrosine-based activation motifs
J	joining
KO	knock out
LAP	latency-associated peptide
LCK	lymphocyte-specific kinase
LPL	lamina propria lymphocytes
LTBP-1	latent TGF-β binding protein-1
MHC	major histocompatibility complex
MLN	mesenteric lymph node
NK	natural killer
OVA	ovalbumin
PD-1	programmed cell death protein-1
PD-L1/2	programmed death ligand 1/2
PICA	p53 induced CD28 dependent apoptosis

PIP2	phosphatidylinositol-4,5- bis phosphate
PKC	protein kinase C
PLC-γ	phospholipase C-γ1
pTreg	peripheral Treg
RasGRP	Ras guanyl nucleotide releasing protein
S1P1	sphingosine-1-phosphate receptor 1
scFv	single-chain variable fragment
SH2	src homology 2
SLC	small latency complex
SLE	systemic lupus erythematosus
SOS	Son of Sevenless
SP	single positive
TAA	tumor associated antigen
T_{CM}	Central memory T cell
TCR	T cell receptor
T_{EM}	Effector memory T cell
Tfh	T follicular helper
TGF-β	Transforming growth factor β
Th	T helper
TIL	tumor infiltrating lymphocytes
TM	transmembrane
TME	tumor microenvironment
TNF	tumor necrosis factor
TRAIL-DR5	tumor necrosis factor related apoptosis inducing ligand death receptor 5

Treg	regulatory T cell
T_{RM}	Tissue-resident memory T cell
tTreg	thymic-derived Treg
V	variable
WT	wild type
ZAP-70	Zeta chain-associated protein
α	alpha
γ	gamma
δ	delta
ε	epsilon
ζ	zeta

ABSTRACT

Achieving immune homeostasis requires the functions and numbers of suppressive regulatory T cells (Tregs) and effector conventional T cells (conv T) to be balanced and precisely controlled. A decrease in Treg numbers is associated with autoimmune diseases such as type I diabetes and multiple sclerosis, while Treg numbers are increased in the tumor microenvironment. Conversely, an increase in conv inflammatory T cells in the tumor microenvironment is associated with better outcomes for cancer patients. Thus, maintaining the appropriate balance between Treg and conventional T cell populations is essential for controlling immune responses against non-self and self-antigens, as well as tumor antigens. Signaling mechanisms that promote the balance between regulatory and conv T cell populations remain largely unknown. One mechanism that could help control the balance between Treg and conv T cell populations is activation induced cell death (AICD). This type of apoptosis occurs when either Tregs or conv T cells undergo repeated stimulation in the presence of the T cell growth factor IL-2. If conv T cells undergo extensive AICD, then Tregs would dominate, and vice versa: if Treg cells undergo extensive AICD, then conv T cells would dominate. Our goal is to determine if conv T cells and Treg cells have different signaling mechanisms that lead to AICD.

We previously discovered a novel form of AICD that occurs *in vitro* with plate bound stimulation of T cells with anti-CD3 and anti-CD28 coated antibodies. Under these conditions, mouse Foxp3$^+$ Tregs expand while Foxp3$^-$ conv T cells undergo

massive apoptosis, indicating that these cells respond differently to anti-CD3/anti-CD28 stimulation. Unlike classical AICD, this form of apoptosis depends on p53, and thus we termed the process PICA (p53-induced CD28-dependent apoptosis). We wanted to understand why Tregs could expand and survive, while conv T cells died. We found that the expansion and survival of Tregs requires autocrine TGF-β signaling. Conversely, addition of TGF-β could promote conv T cell survival. Our work has identified that Tregs and conventional T cells have differences in the MAPK/ERK signaling pathways, which controls survival after stimulation. Tregs maintain low RasGRP1 expression by autocrine TGF-β signaling, and RasGRP1 expression in conventional T cells is suppressed by TGF-β. Furthermore, we found that RasGRP1 signaling is required for conv T cell apoptosis during PICA. For TGF-β to promote survival of conv CD4 T cells, we found that the transcription factor FoxO3 is critical. Finally, we studied AICD in a more physiological manner in CD19-specific chimeric antigen receptor (CAR) T cells. We found that CAR T cells undergo AICD *in vitro* when stimulated with large numbers of tumor antigens and the surviving CAR T cells are CD25$^+$Foxp3$^+$. Like PICA, addition of TGF-β can promote CAR T cell survival, but markers of CAR functionality are reduced.

Taken together, we have identified distinct signaling pathways that promote AICD in T cells and determined mechanisms by which TGF-β controls survival. We propose that the crosstalk between the RasGRP1 and FoxO3 pathways with TGF-β signaling are critical for T cell survival. Results from this study could inform how conventional and Treg populations survive and undergo apoptosis during AICD in memory formation and the tumor microenvironment, among other disease states.

CHAPTER ONE

LITERATURE REVIEW

Section 1: T Cell Differentiation and Function in the Immune System

T Cell Development

T cells originate from hematopoietic stem cells (HSCs) that are found in the bone

marrow. T cell progenitors then migrate via the blood and colonize the thymus. These

progenitors are known as thymocytes, and thymocytes must undergo a series of

maturation and selection steps during development before export to the periphery. The

thymus is critical for T cell development, as mice that are thymectomized immediately

after birth have a defect in T cell numbers and severe immunodeficiency (Miller 1961;

Marshall and White 1961). The earliest T cell lineage cell that seeds the thymus is

termed the early thymic progenitors (ETP). Early thymic progenitors undergo a series of

differentiation steps, which are classically defined by the cell surface expression of the

co-receptors CD4 and CD8, with thymocytes first starting as CD4-CD8- double negative

(DN), then progressing to CD4+CD8+ double positive (DP), and lastly single positive

(SP) CD4 or CD8 T cells (Figure 1). The DN subset can be further classified into

distinct subsets (DN 1-4) based on surface expression of CD25 (IL-2 receptor α) and

CD44 (adhesion molecule). During this early DN stage, Notch signaling has been

shown to be critical for induction of T cell lineage commitment, as mice with neonatally

induced inactivation of Notch1 have a severe reduction in thymocyte development

(Radtke et al. 1999). CD44+ DN1 cells that acquire CD25 expression are termed DN2

1

cells. Committed DN2 cells initiate T cell receptor (TCR)-β, TCR-γ and TCR-δ gene rearrangements. During this process, the T cells undergo one of two developmental programs, one generating αβ T cells and the other generating γδ T cells. DN3 cells successfully express the pre-TCR, which is composed of the rearranged TCR-β chain and invariant pre-Tα chain (For TCRαβ T cells) (Mombaerts, Iacomini, et al. 1992; Mombaerts, Clarke, et al. 1992) or the TCR-γ and δ chains for TCRγδ T cells (Burtrum et al. 1996; Taghon et al. 2006). Cells die if rearrangement is unsuccessful. For TCRγδ T cells, expression of the γδTCR receptor allows for functional maturation and proliferation, without the need for further developmental stages (Prinz et al. 2006). For TCR αβ T cell development, expression of the pre-TCR complex at the DN3 stage promotes survival and extensive proliferation, enforces *tcrb* allelic exclusion, initiates TCR-α germline transcription, and silences *tcrg* expression to promote the αβ lineage (Falk et al. 2001; Hager-Theodorides et al. 2007). With a rearranged αβ TCR on the surface, the T cells move onto the double positive stage (DP), where the T cells express both CD4 and CD8 co-receptors. For T cells to become single positive CD4 and CD8 αβ T cells, the DP thymocytes must undergo the processes of positive and negative selection.
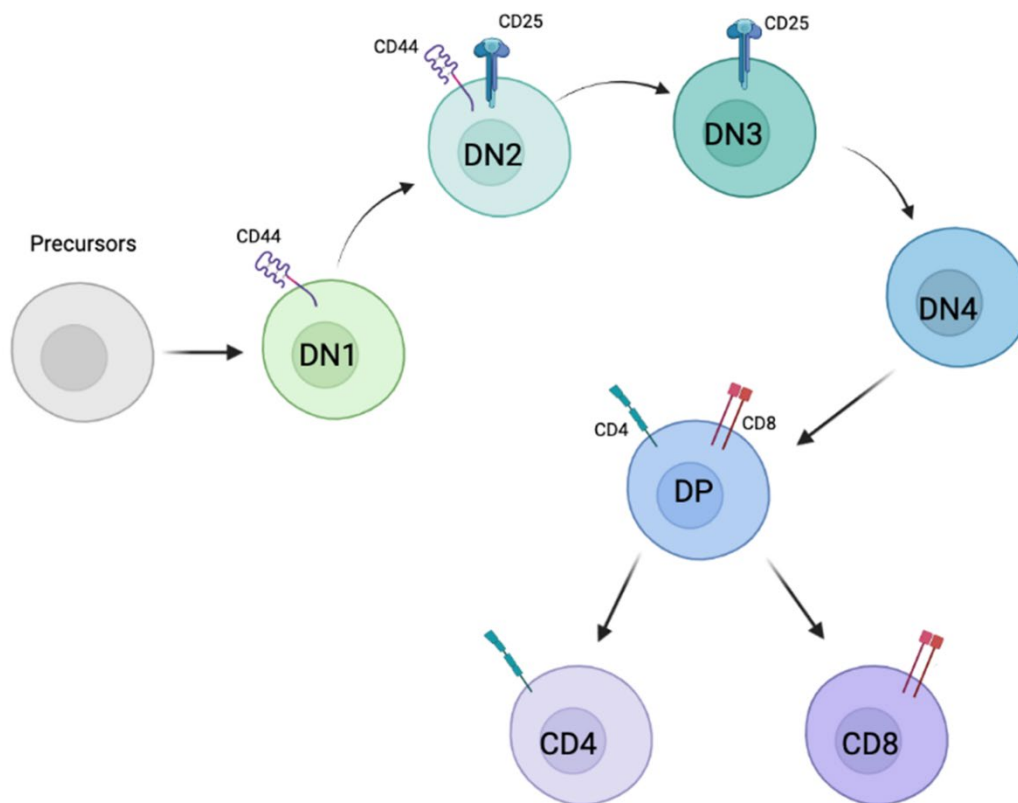
Figure 1. T Cell Development in the Thymus. Early thymic progenitors are termed double negative (DN) for CD4 and CD8 expression. DN1 cells express CD44 and DN2 cells acquire expression of CD25. Committed DN2 cells undergo TCR gene rearrangement, and cells die if this rearrangement in unsuccessful. For αβ T cells, with a rearranged αβ TCR on the surface, the T cells move to the double positive stage (DP), where the T cells express both CD4 and CD8 co-receptors. The DP thymocytes must undergo the processes of positive and negative selection to generate CD4 and CD8 single positive T cells. For γδ T cells, the successful rearrangement of γδ TCR on the surface at DN3 stage is sufficient for functional maturation.

Positive and negative selection. During positive and negative selection, T cells recognize antigen in the context of major histocompatibility complex (MHC) (Zinkernagel and Doherty 1974; Reinherz et al. 1982; Bjorkman et al. 1987). For T cells to be able to recognize the vast array of pathogenic and non-pathogenic antigens, there is a tremendous amount of diversity in the TCR. Variation is achieved through somatic rearrangement of gene segments that allows for recognition of approximately 10^{18}

epitopes (Market and Papavasiliou 2003). This occurs by recombining variable (V), diversity (D), and joining (J) gene segments in a process called V(D)J recombination, which requires RAG1 and RAG2 enzymes (Mombaerts, Iacomini, et al. 1992; Shinkai et al. 1993). These recombination events are required for the vast diversity achieved in TCRs, which can then recognize diverse antigens presented in the context of MHC. The fate of double positive thymocytes depends on the signaling events mediated by interaction of the TCR with self-peptide MHC ligands during selection.

Positive and negative selection of T cells occurs in the thymic cortex and medulla, respectively. During positive selection, thymocytes are selected that recognize peptide in the context of MHC Class I or Class II molecules expressed by dendritic cells (DCs) and cortical epithelial cells. These self-peptide-MHC interactions are generally weak, but are critical to activate the T cells and prevent apoptosis (Hogquist et al. 1994). If there is no signaling or weak signaling from the TCR-self-peptide-MHC interaction, the cells undergo death by neglect. Double positive (CD4+CD8+) thymocytes that recognize peptide presented by MHC Class I develop into single positive CD8 T cells, while those that recognize MHC Class II develop into CD4 T cells (Teh et al. 1988).

Negative selection removes thymocytes that bind too strongly to self-peptide-MHC and induces downstream signaling that promotes apoptosis (Kappler, Roehm, and Marrack 1987). This is critical to prevent autoimmunity and promote tolerance. If T cells recognize self-peptide too strongly in the periphery, this can promote inflammatory responses to self and adverse disease pathology. Therefore, TCRs that induce too weak or too strong of signaling processes lead to apoptosis, leaving mature T cells with

an "intermediate" affinity. This is critical to recognize foreign antigens in the context of MHC, and to eliminate conventional T cells that strongly recognize self-antigens. It is also critical that non-thymic antigens are presented in the thymus to prevent autoimmunity in other tissues. A transcription factor called autoimmune regulator (AIRE) is critical for the expression of these antigens from other tissues by thymic epithelial cells (Aaltonen et al. 1997; X. Zheng et al. 2004). Mutations in the AIRE gene have been linked with autoimmunity in humans (Aaltonen et al. 1997). Therefore, AIRE is critical for negative selection of thymocytes that recognize self-antigens from tissues outside the thymus.

After positive and negative selection is complete, single positive CD4 and CD8 thymocytes undergo final maturation in the thymus before export to the periphery. When developing thymocytes receive TCR stimulation, they will proliferate and upregulate the chemokine receptor CCR7 (Ueno et al. 2004). The ligands for CCR7 are expressed in the medullary region of the thymus, so this supports migration from the cortical region to the medulla. Additionally, T cells express the G protein coupled receptor sphingosine-1-phosphate receptor 1 (S1P1), that is critical for T cell egress from the thymus to the periphery (Matloubian et al. 2004). The ligand for S1P1, S1P, is present in the blood, which acts as a chemoattractant to promote T cell migration. Once in the bloodstream, these naïve T cells can circulate between the blood and peripheral lymphoid organs, until they encounter antigen presented by antigen presenting cells (APCs) and initiate adaptive immune responses.

Treg development. Regulatory T cells (Tregs) are critical for promoting tolerance and suppressing T cell responses to self and other harmless antigens such as food and commensal bacteria. The function and regulation of Tregs will be discussed later in detail, but first we will discuss the development of thymic-derived Tregs (tTregs). tTregs constitute ~3-5% of developing single-positive CD4 thymocytes, and ~10-15% of CD4 T cells in secondary lymphoid organs. Currently, the prevailing dogma is that tTregs develop in a two-step process (Lio and Hsieh 2008; Burchill et al. 2008). The first step is driven by strong TCR stimulation in developing single positive CD4 thymocytes. The second is cytokine-dependent conversion of Treg progenitors into mature tTregs via upregulation of the transcription factor forkhead box protein P3 (Foxp3).

As mentioned previously, the strength of TCR stimulation dictates the fate of developing thymocytes. Several studies have provided evidence that the tTreg TCR repertoire has higher self-reactivity than its conventional counterparts. Since tTregs require TCR stimulation to suppress autoreactive T cell responses, it was hypothesized that stronger and higher affinity antigen-peptide interactions would favor tTreg development over conventional CD4 T cell development (Takahashi et al. 1998; Thornton and Shevach 1998). It was demonstrated using TCR transgenic mice that autoreactive thymocytes that recognize a self-peptide can induce $CD4^+CD25^+$ tTreg development (Jordan et al. 2001). This was dependent on the affinity of the TCR for the self-peptide, as thymocytes with high-affinity TCRs developed into Tregs, while thymocytes with low-affinity TCRs did not. Sequencing variable TRAV14 (Vα2) TCRα chains paired with a fixed TCRβ chain uncovered that tTreg TCRs are largely distinct from $CD4^+CD25^-$ conventional CD4 T cells (less than 25% overlap) (Hsieh et al. 2004).

Additionally, tTregs have more efficient interactions and proliferation with self-peptides presented on MHC Class II compared to $CD4^+CD25^-$ conventional counterparts. Single-cell analysis also revealed that tTregs have distinct TCRs compared to $CD4^+Foxp3^-$ counterparts, and that the diversity of tTreg TCRs was greater than that of $Foxp3^-CD4^+$ naïve T cells (Pacholczyk et al. 2006). Later work using a range of TCRs with varying affinity for ovalbumin (OVA) demonstrated a positive linear relationship between affinity for OVA and Treg development (H. M. Lee et al. 2012). The threshold of TCR-reactivity required to elicit negative selection was ~100-fold higher than that required for Treg generation. Together, these studies support the current dogma that Treg generation occurs with peptide-MHC interactions that have a level of affinity greater than the signal strength that induces conventional CD4 T cells, but lower than the strength that induces cell death (negative selection) (Figure 2).
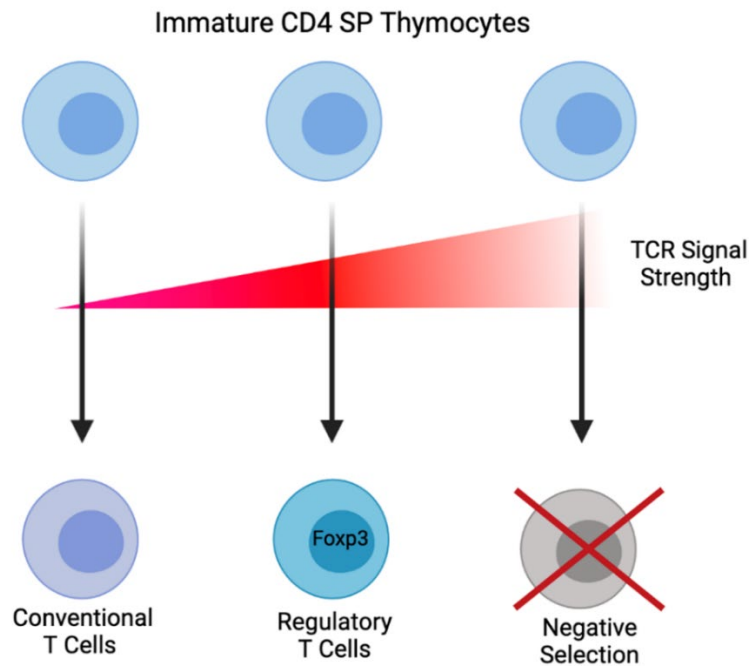
Figure 2. TCR Strength Determines Conventional and Treg Survival. Conventional T cells that express TCRs that react too strongly with self-antigens under negative selection, while those that express TCRs with a lower level of reactivity to self-antigens survive. Treg generation and survival occurs with TCRs that have higher affinity for self-antigens than conventional T cells.

The second step in tTreg development is the maturation of CD4$^+$CD25$^+$Foxp3$^+$ tTregs that requires a specific cytokine environment to induce Foxp3 expression and survival. IL-2 has been implicated as a critical cytokine for this maturation process. IL-2 deficient mice were shown to have increased numbers of activated T cells and developed colitis, which gave clues that IL-2 may be important in Treg development (Sadlack et al. 1993). Further analysis revealed that while IL-2 deficient mice have a large defect in Treg numbers, IL-2Rβ deficient mice have an even larger block in Treg development (Burchill et al. 2007; Soper, Kasprowicz, and Ziegler 2007). Further experiments revealed that IL-2 and IL-15, which both bind IL-2Rβ, were critical for Treg development and Foxp3 induction (Zorn et al. 2006; Burchill et al. 2007). Transforming

growth factor-β (TGF-β) is another cytokine that is critical for the formation and function of Tregs (discussed in detail later). Conditional deletion of TGF-βRI in T cells blocked the development of CD4^{+}CD25^{+}Foxp3^{+} tTregs days 3-5 after birth (Yongzhong Liu et al. 2008). TGF-β was found to protect nTregs from apoptosis/negative selection, as TGF-βRII deficient thymocytes had enhanced anti-CD3 induced-T cell apoptosis (Ouyang et al. 2010). Therefore, TGF-β signaling is critical for the formation of tTregs, where studies thus far point to a role in tTreg survival rather than a role during positive selection. Further investigation is needed to determine the source of these cytokines in the thymic milieu, as well as if there are specific APCs that are critical for Treg generation. Additionally, the mechanisms that render the *Foxp3* locus to be available for binding by transcription factors downstream of cytokine signaling remain to be elucidated.

T Cell Subsets and Functions

　　Naïve T cells. Naïve CD4 and CD8 T cells that migrate to periphery can persist for many months to even years, until they encounter and recognize antigen presented by APCs (Sprent and Basten 1973; Tough and Sprent 1994). Proliferation of naïve T cells is rare, where less than 20% of naïve T cells were shown to divide in a five-week time period. (Tough and Sprent 1994). This low level of homeostatic proliferation is thought to maintain naïve population numbers. Studies have shown that naive T cell homeostasis requires recognition of self-antigens in the context of MHC Class I (CD8) and Class II (CD4) to maintain their long-term survival (Takeda et al. 1996; Tanchot et al. 1997).

When TCR interactions with self-ligands presented on MHC are blocked, naïve CD8 T cells die rapidly, with a half-life on the order of days (Tanchot et al. 1997). CD4 T cells, on the other hand, die more slowly when these interactions are disrupted, with a half-life of months (Rooke et al. 1997). Naïve T cells were also shown to have sub-optimal basal TCR signaling *in vivo*, that is thought to help maintain survival. This was seen in thymocytes and mature T cells that were shown to have low levels of phosphorylated TCR ζ chain, which occurs after TCR ligation (Nakayama et al. 1989; van Oers, Killeen, and Welss 1994). This notion is supported by studies that showed when TCRs were inducibly deleted in mature T cells, both CD4 and CD8 T cells died with similar kinetics as in studies where MHC-self ligand interactions were lost (Polic et al. 2001; Labrecque et al. 2001). This suggests that naïve T cells require TCR expression for survival, and together with other studies suggests that this TCR signaling occurs from self-ligand MHC interactions.

Naïve T cells also require cytokine signaling to maintain their survival and homeostatic proliferation. IL-4 and IL-7 both promote naïve T cell survival *in vitro*, while IL-7 is the predominant cytokine required for survival *in vivo* (Vella et al. 1997; Schluns et al. 2000; Vivien, Benoist, and Mathis 2001). IL-7 promotes survival in part by Bcl-2 expression, and withdrawal of IL-7 can promote apoptosis through Bax-mediated signaling pathways (Von Freeden-Jeffry et al. 1997; Khaled et al. 1999). The mechanisms by which IL-7 promotes survival, as well as the contribution of other common γ chain cytokines such as IL-2 and IL-15 requires further investigation. So, in response to these various stimuli, how does a naïve T cell "decide" to proliferate or merely survive? Evidence suggests that the size of the naïve T cell compartment may

contribute to this "decision". In a T cell depleted environment, T cell proliferation can be successfully reduced by co-transfer of increasing numbers of competitor T cells (Dummer et al. 2001; Ernst et al. 1999). Two models have been proposed for how bystander T cells can limit proliferation of naïve T cells: direct contact inhibition by bystander T cells and/or competition for resources. Competition may could include access to IL-7 and APCs that are presenting suitable self-ligands for promoting naïve T cell survival. Overexpression of IL-7 was shown to increase the size of the naïve T cell pool and promote homeostatic proliferation, which suggests that IL-7 availability may limit naïve T cell expansion (Tan et al. 2001; Geiselhart et al. 2001).

Overall, naïve T cells actively require IL-7 and APC/self-ligand interactions to promote survival and homeostatic proliferation. Once naïve CD4 and CD8 T cells recognize foreign antigen in the context of MHC and the appropriate cytokine milieu, such as during infection, differentiation into distinct T cell subsets occurs. This includes CD4 T helper subsets, and multiple subsets of effector and memory T cells that can mediate pathogen clearance and provide long-term protection and immunological memory.

Kinetics of CD4 and CD8 responses. Upon infection with a pathogen, both CD4 and CD8 T cell responses can be broken down into three phases: (1) activation and expansion, (2) contraction, and (3) memory formation (Figure 3). During the initial phase that lasts about one week, naïve T cells recognize antigen presented by APCs, then differentiate into effector and helper subsets. In several viral models of infection, between 100- and 5000-fold expansion of antigen-specific CD4 and CD8 T cells occurs (Tripp et al. 1995; Moskophidis et al. 1987; McHeyzer-Williams and Davis 1995). A

period of death then ensues, which lasts between ~days 7-30. Most of the activated T

cells undergo apoptosis, a process termed activation-induced cell death (AICD). As the

amount of antigen declines, effector activity is diminished. AICD occurs through

Fas/FasL interactions and through TNF-cell death pathways (discussed in detail in a

subsequent section). About 5-10% of T cells survive as memory T cells, which

characterizes the third phase (Yang Liu and Janeway 1990; S. Webb, Morris, and

Sprent 1990; Kawabe and Ochi 1991). These memory T cells can be divided into

distinct groups that perform specialized functions when pathogens are encountered

again in the future.
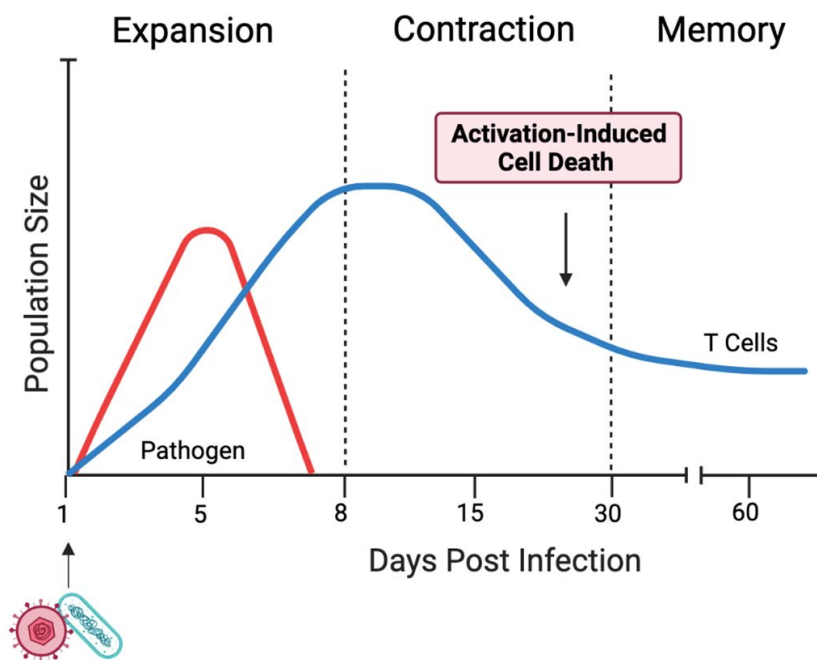
Figure 3. Kinetics of T Cell Responses after Infection. Upon pathogen encounter, T cells undergo three phases of expansion, contraction, and memory. Activation and expansion of T cells occurs when naïve T cells recognize antigen in the context of MHC presented by APCs (blue line). Once the pathogen has been cleared (red line), the T cells undergo a contraction phase to eliminate a large number of antigen-specific T cells. This occurs through activation-induced cell death (AICD). About 5-10% of antigen-specific memory T cells survive to rapidly respond if the pathogen is encountered again in the future.

Effector T cells. During the expansion phase, naïve T cells encounter antigen presented in the context of MHC in lymphoid tissues. This causes clonal expansion and differentiation into effector T cells, known as T helper (Th) for CD4s or cytotoxic T cells (CTLs) for CD8s. These CD4 and CD8 T cells have diverse functions, and the differentiation depends on the type of antigen encountered. We will first discuss CD4 Th subsets, which not only are important during viral infection, but also have roles in normal immune homeostasis and other states of disease such as allergy, autoimmunity, and cancer.

Conventional CD4 T helper cells. CD4$^+$ T lymphocytes are critical members of the adaptive immune response that are required for effective cell-mediated immunity. CD4$^+$ T cells have diverse functions, including promoting tolerance to commensals and self-antigens, directing inflammatory responses to pathogens, and orchestrating tumor immunity. To direct these various immune processes, naïve CD4$^+$ T cells differentiate into functionally distinct CD4$^+$ T helper (Th) subsets based on the antigen presented, co-stimulation, and the cytokine milieu. These subsets include: Th1, Th2, Th17, Th9, Th22, Treg, and T$_{FH}$ lineages. Th1 cells produce the proinflammatory cytokines TNF and IFN-ɣ and are critical for clearance of intracellular pathogens. Th2 cells produce IL-4, IL-5 and IL-13 and are critical for antibody production by B cells, clearance of extracellular microbes, and expulsion of helminths (Abbas, Murphy, and Sher 1996; Mosmann and Coffman 1989). Exaggerated Th2 responses can lead to allergic responses and asthma (Robinson et al. 1992; Finotto et al. 2002). Th17 cells produce IL-17A, IL-17F, IL-22 and IL-21, which play a critical role in recruitment of pro-inflammatory leukocytes and tissue inflammation, including autoimmunity (H. Park et al.

2005; Martinez et al. 2008). Th9 and Th22 cells have roles in tumor immunity, allergic inflammation and other inflammatory diseases and produce IL-9 and IL-22, respectively (Veldhoen et al. 2008; Eyerich et al. 2009). Tregs are critical for tolerance to self-antigens, preventing autoimmunity, and dampening inflammation via IL-10 and TGF-β (discussed in detail later).

The differentiation of naïve CD4 T cells into these distinct subsets requires activation by APCs. The first signal for differentiation is TCR recognizing antigen presented by APCs in the context of MHC class II (Eisenlohr and Hackett 1989; Weiss and Bogen 1989). The second signal is co-stimulation, whereby APCs provide ligands for co-stimulatory molecules on T cells. The major co-stimulatory molecule expressed on resting naïve CD4 T cells is CD28 (Burr et al. 2001; June et al. 1987). The third signal is the presence of cytokines in the environment of the naïve T cells, that are critical for polarization into distinct Th subsets (Curtsinger et al. 1999). These cytokines can enhance and suppress lineage decisions, and dictate signaling events that control expression of mater transcription factors for each lineage. IFN-γ and IL-12 are two critical cytokines for Th1 polarization, which are involved in expression of the master transcription factor T-bet (Hsieh et al. 1993; Smeltz et al. 2002). T-bet promotes IFN-γ synthesis and represses IL-4 and IL-5 production, blocking Th2 polarization (Szabo et al. 2000; Djuretic et al. 2007). During Th2 cell differentiation, IL-4 induces the expression of the master transcription factor GATA3 (W. P. Zheng and Flavell 1997). IL-2 also supports early Th2 differentiation by inducing expression of the IL-4 receptor α-chain and expression of GATA3 (W. Liao et al. 2008; Yamane, Zhu, and Paul 2005).

Together, these CD4 T cell subsets are required for mediating distinct inflammatory and

regulatory processes during infection and normal immune homeostasis (Figure 4).
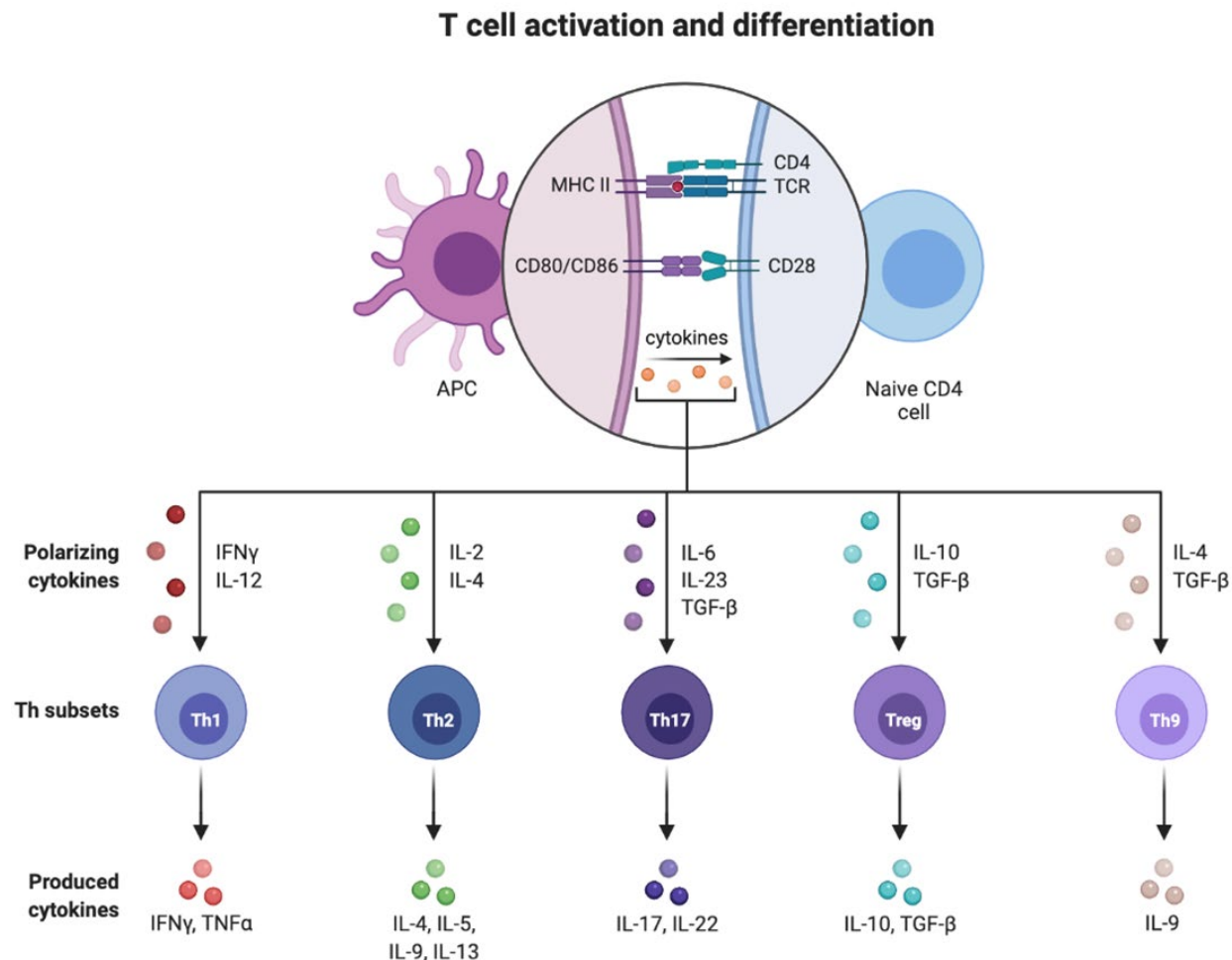
Figure 4. CD4 Th Differentiation. APCs present antigen to naïve CD4 T cells in the context of MHC Class II. Co-stimulation through CD28 and its ligands CD80/86 are required for activation. The polarizing cytokines in the environment dictate Th development fates. Th subsets secrete distinct cytokines and mediate a variety of effector and suppressive functions.

Effector CD8 cytotoxic T lymphocytes. Naïve CD8 T cells recognize antigenic

peptides (such as those from viruses) in the context of MHC Class I. This recognition in

the presence of IFN-α/β and IL-12 promotes differentiation into effector cytotoxic T

lymphocytes (CTLs). These CTLs specialize in the elimination of intracellular

pathogens such as viruses, and the elimination of tumor cells. Antigen-specific CTLs

expand in response to IL-2 and can enter the blood and migrate to the site of infection (Rollings et al. 2018). CD8 T cells can kill infected cells through the production of granzymes and perforin, and like Th1 cells also secrete inflammatory cytokines such as IFN-γ and TNF (Walsh et al. 1994). Like Th1 cells, CTLs are programmed through the transcription factor T-bet, but also rely on the transcription factor Eomesodermin (Pearce et al. 2003).

The contribution of costimulatory molecules to CD4 and CD8 effector differentiation are distinct. In the absence of costimulatory molecules, CD4 T cell responses in different infection models are severely impaired, while CD8 T cell responses are only moderately impaired or not affected at all (Shahinian et al. 1993; Whitmire et al. 1999; Andreasen et al. 2000). This suggests that different thresholds are required for CD4 and CD8 activation and differentiation. In order to become activated and proliferate, CD4 T cells required a minimum of 6 hours of stimulation with APCs, and more than 24 hours when co-stimulation is absent (Iezzi, Karjalainen, and Lanzavecchia 1998). In similar experiments, CD8 T cells could commit to activation and proliferation in a briefer amount of time (in as little as 2 hours) (Van Stipdonk, Lemmens, and Schoenberger 2001).

CD8 T cells also have higher rates of cell division compared to CD4 T cells after antigen encounter, both *in vitro* and *in vivo*. During the first 24 hours of stimulation, both CD4 and CD8 T cells become activated and increase in size, but no cell division is observed. CD8 division then commences at a rapid rate (6-8 hours per division), while CD4 T cell division is delayed for another 12 hours and then occurs at about 10 hours per division (Kaech and Ahmed 2001; Mercado et al. 2000). Therefore, after initial

antigen encounter with APCs, CD8 T cells can become activated more quickly than CD4 cells and proliferate more robustly.

Memory T cells. Following elimination of an infecting pathogen, T cells go through a contraction phase, where the majority of antigen-specific CD4 and CD8 T cells die by apoptosis. Typically, around 5-10% survive and mature into memory T cells. Memory T cells are maintained in an antigen-independent, cytokine dependent manner (IL-7 for CD4/CD8, IL-15 for CD8) (Surh and Sprent 2008; Seddon, Tomlinson, and Zamoyska 2003). A feature of memory CTLs is their ability to generate rapid effector responses after secondary exposure to infection.

Memory T cell subsets are diverse, which is thought to ensure optimal immune memory through cells with distinct localization and function. Three memory subsets have been described: central memory (T_{CM}), effector memory (T_{EM}), and tissue-resident memory (T_{RM}). These subsets are defined by expression of protein surface molecules, anatomical location, and function. Two markers distinguish T_{CM} and T_{EM}, CCR7 and CD62L. CCR7 is a chemokine that controls homing to secondary lymphoid organs, while CD62L is an adhesion molecule that is cleaved from antigen-activated cells in lymph nodes, allowing for T cells to reenter circulation and elicit effector functions (Förster et al. 1999; Galkina et al. 2003). T_{CM} mainly home to secondary lymphoid organs and the bone marrow, and are characterized by a CD62L^{high}CCR7^{high} phenotype. T_{EM} are characterized by low expression of these markers (CD62L^{low}CCR7^{low}), and are mostly found in non-lymphoid tissues (Sallusto et al. 1999). T_{CM} and T_{EM} are also functionally distinct. T_{CM} cells tend to mount a more robust recall response and produce IL-2, while CD4 T_{EM} cells produce cytokines such as IFN-γ and

TNF and CD8 T_{EM} make cytotoxic proteins such as perforin and granzyme (Masopust et al. 2014; Kaech and Wherry 2007).

T_{RM} cells reside long-term in mucosal and tissue sites, such as the brain, lungs, gut and skin (Teijaro et al. 2011; Masopust et al. 2010; X. Jiang et al. 2012). These memory T cells show very little migration, and at many sites they have been shown to be functionally and transcriptionally distinct from their circulating memory T cell counterparts. Since infection primarily occurs at tissue sites, these memory T cells are poised at these sites, ready for rapid detection and eliciting T cell immune responses. For example, in the gut, memory virus-specific intraepithelial lymphocytes (IELs) were found to be functionally and phenotypically distinct from antigen-specific T_{CM} and T_{EM} in the blood. The T_{RM} IELs had the highest expression of granzyme B compared to other subsets, contained very few actively dividing cells, and had very high expression of the antiapoptotic molecule Bcl2 (Masopust et al. 2006). Additionally, these cells show very little movement through circulation in parabiosis experiments (Klonowski et al. 2004). Similar findings have been shown for T_{RM} cells at other sites such as the lungs, skin, and brain. Overall, T_{RM} cells (a) express tissue-specific chemokine receptors and integrins (b) have low proliferative potential (c) low IL-2 production (d) little to no migration through circulation and (e) high levels of effector functions and cytotoxicity, especially viral antigen-specific T_{RM} cells.

Together, these memory subsets ensure optimal protective immunity with their distinct localization and functions. Following a secondary infection, T_{EM} and T_{RM} normally perform immediate effector functions and act as a first-line defense to

pathogens. T_{CM} cells generate a larger number of secondary effector cells more rapidly than during initial infection to control pathogens that breach initial tissue sites.

Section 2: Signaling Mechanisms of T Cell Activation, Proliferation and Survival

T Cell Signaling and Activation

T cell activation occurs when the TCR recognizes antigen peptide presented in the context of MHC. CD4 T cells use the CD4 coreceptor and CD8 T cells the CD8 coreceptor to bind MHC Class II and Class I, respectively. This promotes T cell to APC cell-cell adhesion and stabilizes the TCR-antigen-MHC protein complex (Doyle and Strominger 1987; Norment et al. 1988). This interaction initiates activation of T cells, leading to metabolic changes, proliferation, and cytokine production. Several signaling pathways coordinate to connect TCR recognition of antigen to these T cell outcomes.

Ligation of TCR with cognate antigen initiates TCR signaling by causing conformational changes in the CD3 signaling complex. The CD3 signaling complex consists of the following subunits: ε, γ, δ, and ζ. These subunits form a CD3εδ heterodimer, a CD3εγ heterodimer, and a CD3ζζ homodimer (Kjer-Nielsen et al. 2004). Upon antigen recognition, the Src-family kinase, Lck gets recruited to the CD3 complex and subsequently phosphorylates tyrosine residues within the immunoreceptor tyrosine-based activation motifs (ITAMs) of the CD3ζ chain (Gibson et al. 1996; Stirnweiss et al. 2013; Mustelin, Coggeshall, and Altman 1989). CD4 and CD8 coreceptors play a key role in the process, as they can directly bind and recruit Lck to the immunological synapse (Veillette et al. 1988; Kim et al. 2003; Shaw et al. 1990). The Src family kinase Fyn can also phosphorylate CD3 ITAMs (Samelson et al. 1990; Ley et al. 1994). The phosphorylated tyrosines within the ITAMs act as binding sites for the SH2 domains of

the Zeta chain-associated protein 70 (ZAP-70) kinase (Iwashima et al. 1994; Kane, Lin, and Weiss 2000; Straus et al. 1996). Lck binds ZAP-70 via SH2 domain interactions and ZAP-70 becomes activated by LCK phosphorylation of multiple tyrosine residues and autophosphorylation (Pelosi et al. 1999; Q. Yan et al. 2013; Neumeister et al. 1995; Chan et al. 1995; Watts et al. 1994). ZAP-70 phosphorylates multiple adaptor proteins including linker for activation of T cells (LAT) and SLP-76 (Wardenburg et al. 1996; W. Zhang et al. 1998). This promotes complex formation and activation of several proteins including Grb-2 and phospholipase C-γ1 (PLC-γ1), to initiate downstream signaling cascades (Williams et al. 1999; J. Lin and Weiss 2001; Yablonski, Kadlecek, and Weiss 2001). Son of Sevenless (SOS) is a substrate for Grb-2 and is recruited to the plasma membrane after Grb-2 is activated in the LAT complex. SOS is involved in ERK activation, as discussed more below (Ravichandran et al. 1995; Xavier et al. 1998). PLC-γ is also activated by phosphorylation in the LAT complex (Nishibe et al. 1990). Activated PLC-γ produces DAG and IP3 by cleaving phosphatidylinositol-4,5-bis phosphate (PIP2) (Nishibe et al. 1990). IP3 binding to IP3 receptors can lead to release of intracellular calcium from the ER, lysosomes and mitochondria, which promotes activation and nuclear localization of the transcription factor NFAT (Stankunas et al. 1999; Serafini et al. 1995). DAG activates another Ras activator, RasGRP1, leading to activation of the Erk MAPK pathway (discussed in more detail below) (Ebinu et al. 2000). All of these signaling pathways lead to T cell activation, proliferation, and cytokine production (Figure 5).

One of the most well-studied outputs of TCR signaling is IL-2 production. IL-2 was originally identified as a T cell growth factor that is essential for T cell activation and

proliferation (Morgan, Ruscetti, and Gallo 1976; Taniguchi et al. 1983). After TCR

activation, IL-2 transcription is initiated by the transcription factors NFAT and AP-1 (Jain

et al. 1993). NFAT is activated via the calcium signaling pathway and AP-1 is regulated

via the Ras-ERK MAP pathway. Therefore, both Ras-Erk and calcium signaling regulate

production of IL-2 in response to TCR stimulation.
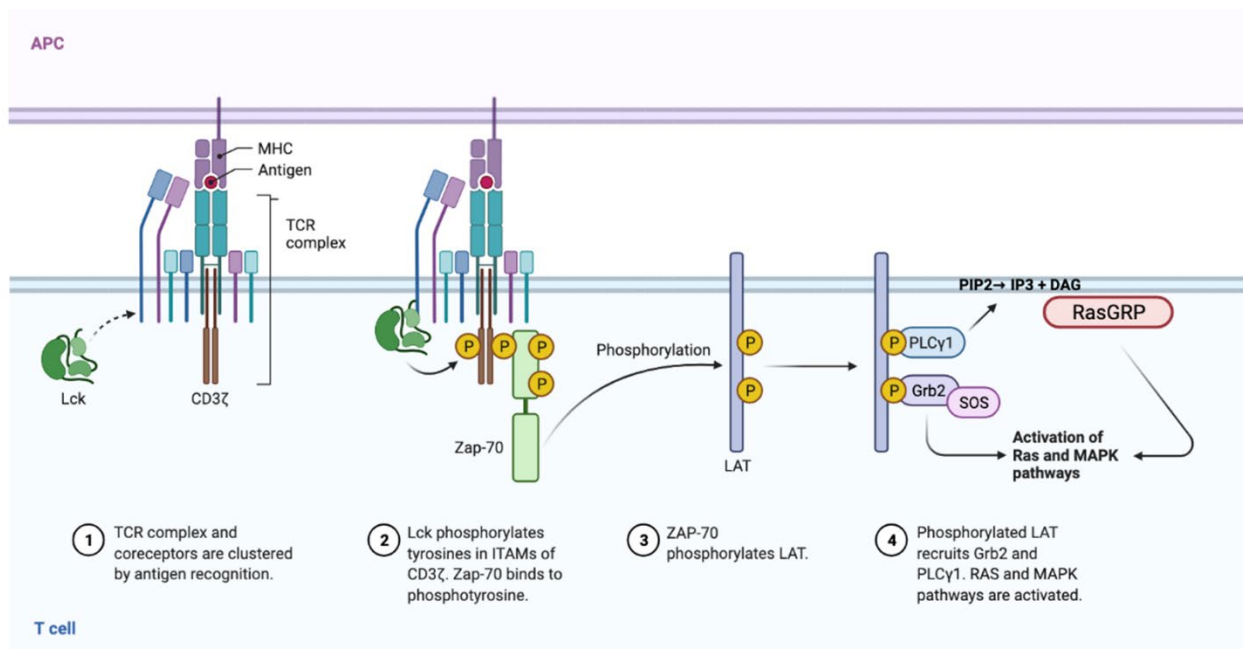
Figure 5. Initial TCR Signaling Events. Antigen recognition causes TCR clustering, and co-receptors recruit Lck to the immunological synapse. Lck phosphorylates CD3ζ and Zap-70 can bind to phosphorylated tyrosines. Activated ZAP-70 phosphorylates LAT, which allows for recruitment of Grb2 and SOS, as well as PLC-γ. PLC-γ generates IP3 + DAG, which recruits RasGRP. RasGRP and SOS activate the Ras and MAPK pathways. This together with other signaling pathways ultimately leads to IL-2 production, activation, and proliferation.

RasGRP and MAPK Pathway

It was known by the mid-90s that within minutes of TCR activation, PLC-γ1 is

recruited to phosphorylated adaptor proteins and activated by tyrosine phosphorylation

(Desai et al. 1990). Activated PLC-γ1 cleaves membrane phosphoinositides, which

generates the secondary messenger diacylglycerol (DAG). DAG subsequently activates

protein kinase C (PKC). The small GTPase Ras was known to be critical for activating a

kinase cascade that ultimately leads to transcription of the IL-2 gene and T cell proliferation (Gómez et al. 1996). Early protein kinase activity from proximal TCR signaling events was thought to activate Ras by somehow promoting the formation of Ras bound to GTP. Ras-GTP activates the Raf-Mek-Erk Map kinase (MAPK) cascade, which controls the level of a key transcription factor, AP1. AP1 transcription factors work in conjunction with NFAT to transcribe the IL-2 gene (Masuda et al. 1995; Jain et al. 1993).

In the late 1990s, the mechanism by which Ras was activated in T cells still was not fully understood. PKC was known to be critical for Ras activation, by inhibiting Ras GAPs (GTPase activating protein) to keep Ras in the active Ras-GTP bound state. However, it was also known that in most cell types, Ras is also positively regulated by Ras GEFs (guanine nucleotide exchange factors) that are recruited to the plasma membrane by tyrosine phosphorylated adaptor proteins. Due to a number of studies SOS was speculated to be the Ras GEF that activated Ras and MAPK signaling. In 1998 Ebinu et al described a novel Ras GEF, RasGRP (Ras guanyl nucleotide releasing protein) (Ebinu et al. 1998). RasGRP contained a catalytic domain responsible for catalyzing nucleotide release, a DAG-binding domain and a pair of calcium-binding EF hands. PMA treatment of fibroblasts caused an increased association of RasGRP with membranes and Ras activation (Ebinu et al. 1998). RasGRP RNA was expressed in numerous blood cells, including T cells (Tognon et al. 1998). These observations led to the hypothesis that upon TCR stimulation, activated PLC-γ1 generates DAG that along with possible calcium messengers binds and activates RasGRP to subsequently activate Ras-MAPK signaling in T cells.

Two studies in 2000 tested this hypothesis and found RasGRP to be the critical link between TCR signaling and Ras activation. Ebinu et al identified that RasGRP1 is expressed in primary mouse T cells and T cell tumor cell lines, but not in non-T cell lines such as human melanoma (Ebinu et al. 2000). Lorenzo et al showed that RasGRP1 possesses a single C1 domain that is homologous to the C1 domain in PKC (Lorenzo et al. 2000). This domain binds DAG as well as the DAG analog, PMA. Upon examining the localization of RasGRP1, it was shown that RasGRP1 translocated to the plasma membrane in response to PMA treatment, where it can be subsequently activated by DAG. The tail domain in RasGRP1 is required for localization to the plasma membrane (Fuller et al. 2012). Therefore, RasGRP1 is a high affinity receptor for DAG, which linked DAG generation to Ras-MAPK activation (Figure 6).
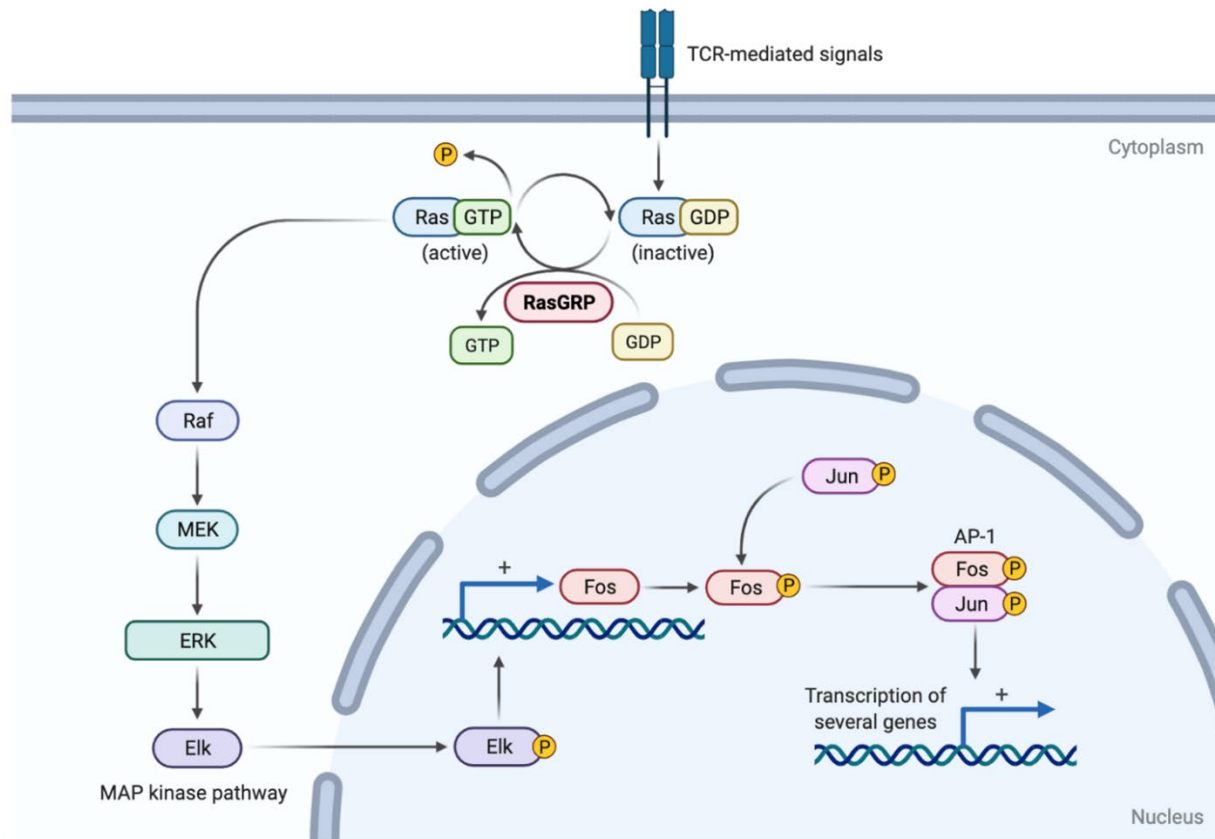
Figure 6. RasGRP Activation of Ras/MAPK Pathways. RasGRP is activated downstream of TCR signaling (detailed in Figure 5). RasGRP activates Ras by exchanging Ras-GDP to Ras-GTP. This active Ras initiates a phosphorylation cascade of the MAPK pathway, which leads to ERK phosphorylation of Elk. Elk positively controls transcription of Fos, which together with Jun can form the AP-1 complex. AP-1 targets multiple genes that control T cell activation and proliferation, including IL-2.

In 2005, Roose et al generated RasGRP1 deficient Jurkat T cell lines to confirm the role of RasGRP1 in Ras activation and the MAPK signaling pathway. RasGRP1 was required for Ras-Erk activation, and RasGRP1 deficient Jurkats had decreased expression of CD69, an early T cell activation marker (Roose et al. 2005). The phosphorylation of RasGRP by PKC was required for Erk signaling and T cell activation, which showed that RasGRP was essential for Erk activation. Although SOS is expressed in T cells, it could not compensate for RasGRP1.

Why T cells express both SOS and RasGRP1 after antigen stimulation was still puzzling, since they seem to have redundant functions. Although SOS is expressed in T cells, RasGRP1 was shown to have a dominant role in activating Ras and the MAPK pathway downstream of TCR stim. In 2007, Roose et al showed that the crystal structure of SOS has a Ras-GTP binding pocket (Roose et al. 2007). RasGRP was shown to orchestrate Ras downstream signaling by directly activating Ras, and by priming SOS with Ras-GTP that binds in an allosteric pocket. This Ras-GTP binding to SOS was shown to enhance its ability to activate Ras. This is thought to create a positive feedback loop that allows T cells to robustly respond to low levels of stimulation. In primary human T cells, RNA interference was used to demonstrate that TCR-mediated Erk activation requires RasGRP1, but not SOS (Warnecke et al. 2012). SOS, but not RasGRP1, was required for IL-2 mediated Erk activation. Thus, RasGRP1 and SOS lead to Ras activation induced by different stimuli.

RasGRP in T Cell Development and Function

RasGRP is critical for T cell development in the thymus. Ras signaling promotes thymopoiesis, as RasGRP deficient mice have a deficiency in single-positive (CD4 and CD8), mature thymocytes (Dower et al. 2000; Priatel et al. 2006). This deficiency causes an increased frequency of double positive T cells in the thymus. Conversely, RasGRP1 transgenic mice that overexpress RasGRP1 had a 4-fold increase in CD8 single-positive thymocytes (Norment et al. 2003). It was later found that thymocytes that express low affinity TCRs rely on Ras activation via RasGRP1, which promotes positive selection (Priatel et al. 2002). On the other hand, negative selection for thymocytes expressing high-affinity TCRs activates Ras via the SOS pathway. In proliferation

assays, RasGRP1 deficient thymocytes showed a complete lack of response to TCR stimulation or stimulation with DAG analogues, demonstrating that RasGRP1 is required for lymphocyte activation during the T cell differentiation processes in the thymus. This further confirmed that RasGRP is the link between TCR activation/generation of DAG and Ras signaling.

Although RasGRP signaling is important for T cell activation, proliferation, and cytokine production, sustained RasGRP/Erk signaling can promote apoptosis of lymphocytes. Overexpression of RasGRP1 in a B cell line was shown to increase apoptosis following B cell receptor ligation (Guilbault and Kay 2004). This enhanced apoptosis was due to the catalytic activity of RasGRP, as a point mutation that created a dominant negative mutant in RasGRP suppressed apoptosis. One of the direct targets of Erk is the proapoptotic protein Bim, which contributes to receptor-induced apoptosis (Stang et al. 2009). Erk was found to phosphorylate sites on Bim that are known to activate Bim and induce apoptotic signaling. In accordance with this role of RasGRP in T cell apoptosis, RasGRP deficient mice spontaneously develop autoimmunity, characterized by splenomegaly and autoantibodies (Dower et al. 2000). This autoimmunity is characterized by an activated and proliferating pool of CD4 T cells that have an exhausted phenotype, including expression of the checkpoint molecule PD-1 and high expression of CD44 and Helios (Daley et al. 2013; Priatel et al. 2007).

This autoimmunity manifests similar phenotypes as patients with systemic lupus erythematosus (SLE). Several studies have correlated defective RasGRP1 expression with autoimmunity in humans. One study examined RasGRP1 splice variants in SLE patients compared to healthy controls (Yasuda et al. 2007). Thirteen new splice variants

were identified. SLE patients had increased levels and types of defective RasGRP1

transcripts compared to healthy controls. These alternative transcripts in SLE patients

correlated with lower levels of RasGRP1 in the SLE patients' T cells. Furthermore, two

SLE patients had T cells that contained little, if any functional RasGRP1 protein.

RasGRP1 has differential roles in the thymus and in the periphery not only for

conventional T cells, but also for other T cell subsets. Tregs had impaired development

in the thymus of RasGRP1$^{-/-}$ mice, but there were increased frequencies of CD4$^+$Foxp3$^+$

Tregs in the periphery with a more activated cell surface phenotype (X. Chen et al.

2008). RasGRP1 deficient Tregs in the periphery were more suppressive than WT

Tregs. CD8$^+$CD44^{high} Tregs were also increased in the spleen of RasGRP1 deficient

mice, but production of IL-10 was reduced compared to WT counterparts. For γδ T cells,

RasGRP1 is not critical for thymic development, but RasGRP1 deficient mice have

increased γδ T cell numbers in the periphery. Furthermore, RasGRP1 was found to be

critical for γδ T cell proliferation and IL-17 production. RasGRP1 deficient mice also

have a deficiency in iNKT cells, which are a CD1d restricted T cell population that

responds to lipid stimulation (Shen et al. 2011). RasGRP1 was found to be critical for

iNKT development, maintenance, and TCR-induced proliferation *in vitro*. Therefore,

RasGRP1 plays differential roles for γδ and αβ T cell development but is critical for γδT

cell proliferation and production of IL-17, as well as iNKT development.

RasGRP1 deficient mice also display a weakened immune system, with a defect

in generating pathogen-specific T cells and delayed pathogen clearance after bacterial

or viral infection (Priatel et al. 2007). Several studies have linked mutations in RasGRP1

with autoimmunity in humans (Winter et al. 2018; Baars et al. 2021). A patient with

recurrent viral and bacterial infections was found to have a biallelic stop-gain variant in *RASGRP1,* which caused a defect in ERK activation in the patient's T cells (Salzer et al. 2016). Another study found two siblings with Autoimmune lymphoproliferative syndrome (ALPS), recurrent infections, and autoantibodies among other autoimmune problems (Mao et al. 2018). The brothers were found to have a mutation in *RasGRP1* that caused impaired T cell signaling, activation and proliferation of T cells, and defects in activation-induced cell death of their T cells. Upon examining numerous patients with autoimmunity, active inflammation in patients correlated with reduced RasGRP1 expression in CD4 T cells (Baars et al. 2021). In this study, A *RASGRP1* enhancer was discovered that has SNPs associated with autoimmunity. Disrupting this enhancer in healthy T cells with CRISPR-Cas9 decreased RasGRP1 expression, and decreased binding of two transcription factors: RUNX1 and CBFB. Reduced RUNX1 expression was also found in patients with the *RASGRP1* enhancer that harbors autoimmunity-associated SNPs. These studies suggest that RasGRP expression in T cells is critical for T cell homeostasis and preventing autoimmune phenotypes.

Activation-induced Cell Death (AICD) of T Cells

Activation-induced cell death (AICD) was first described in 1989 in thymocytes after engaging the CD3/TCR complex with anti-CD3 antibodies (Smith et al. 1989). This gave a possible explanation for a mechanism of deleting autoreactive clones in the thymus to promote self-tolerance. Accumulating evidence shows that mature T cells can also undergo AICD. AICD is recognized as an important mechanism to terminate immune responses, by eliminating T cells that have been repeatedly stimulated. After pathogen clearance, this allows for only a small number of memory T cells to remain,

poised for encountering the same pathogen again. In mature, conventional T cells, AICD is modeled *in vitro* by restimulating T cells that have been previously activated (Radvanyi, Mills, and Miller 1993). This can be done by stimulating T cells with anti-CD3 and anti-CD28 antibodies to mimic TCR and co-stimulation in the presence of IL-2. Then after several days (4-7 days), T cells are restimulated with anti-CD3 or PMA/Ionomycin. PMA is an analog for diacylglycerol and ionomycin is a calcium ionophore which can increase intracellular calcium levels. These two compounds can therefore strongly drive T cell activation.

Fas and FasL. Interaction of the Fas receptor with its ligand FasL is a well-known signaling pathway that promotes AICD of T cells. Fas is a death receptor that belongs to the tumor necrosis factor receptor family. In 1989 two groups identified monoclonal antibodies to Fas (also termed APO-1) that could induce apoptosis in activated lymphocytes and numerous lymphocytic tumor cell lines (Trauth et al. 1989; Yonehara, Ishii, and Yonehara 1989). Lymphoma cells that constitutively express Fas were established to further test the role of Fas in T cell apoptosis. When the T cells were treated with the anti-Fas antibody, they died within 5 hours, further confirming that Fas transduces apoptotic signals in T cells (Oehm et al. 1992). Mutational analysis of the cytoplasmic tail of Fas and TNF-RI showed a conserved region of about 70 aa that is sufficient for transducing apoptotic signals (N. Itoh and Nagata 1993). Fas was found to be highly expressed in activated mature lymphocytes, and IFN-γ can upregulate expression (Trauth et al. 1989; N. Itoh et al. 1991). In order to transduce apoptotic signals, Fas must bind to FasL either on the same cell or a neighboring cell (Dhein et al. 1995). FasL is only expressed after T cell activation (Anel et al. 1994; Vignaux et al.

1995). The precise mechanisms of FasL upregulation remain underexplored, but IL-2 signaling has been shown to be critical for transcription and subsequent cell surface expression of FasL (Refaeli et al. 1998). IL-2 knockout mice do not upregulate FasL and are more resistant to AICD. The ligation of Fas and FasL leads to activation of the Fas-associated death domain, followed by the formation of the death-inducing signaling complex (DISC) (Kischkel et al. 1995). The DISC complex can activate caspase 8 or 10 to induce caspase-dependent apoptotic signaling events. Caspases cleave a number of proteins such as cytoskeletal proteins and nuclear lamins, which triggers cell fragmentation, blebbing, and apoptotic bodies, leading to destruction of the cell. Mice with mutations in *fas* or *fasl* develop autoimmunity and accumulate autoreactive antibodies, demonstrating the essential role for Fas/FasL in maintaining immune homeostasis and killing autoreactive lymphocytes (Hewicker, Kromschröder, and Trautwein 1990; Cohen and Eisenberg 1991). In humans, patients with autoimmune lymphoproliferative syndrome have mutations in the *fas* gene (Aspinall et al. 1999). Therefore, Fas/FasL is critical for inducing AICD in T cells and preventing autoimmune phenotypes.

CD44 in T cell activation and AICD. CD44 is a type I transmembrane glycoprotein that has critical roles in T cell activation, migration, and survival. CD44 binds to its ligand hyaluronic acid (HA), which is a glycosaminoglycan component of the extracellular matrix (ECM) that helps maintain integrity of tissues (Banerji et al. 1998; Bajorath et al. 1998). CD44 can also bind to other parts of the ECM including fibronectin and collagen, but these interactions have not been verified to be physiologically relevant *in vivo* and have not been well characterized. CD44 is expressed at low levels on naïve

T cells, then is upregulated after activation (Budd et al. 1987). Memory T cells maintain CD44 expression, and therefore CD44 has been used as a marker for memory T cells in the field. The affinity of CD44 for HA is regulated by post-translational modifications such as sialylation and glycosylation (Skelton et al. 1998; Katoh et al. 1999). CD44/HA interactions have been observed after TCR activation or exposure to pro-inflammatory cytokines (DeGrendele, Estess, and Siegelman 1997; Ariel et al. 2000). The most well-characterized role for CD44 in T cells is as an adhesion receptor, critical for mediating entry and movement of T cells into and within target tissue sites during infection. CD44 interacts with HA on endothelial cells to mediate tethering and rolling required for extravasation into an inflammatory site (DeGrendele, Estess, and Siegelman 1997; Mrass et al. 2008).

The intracellular domain of CD44 has no intrinsic enzymatic activity on its own, but several reports have shown that it can interact with receptor tyrosine kinases such as LCK and Fyn (Taher et al. 1996; Llangumaran, Briol, and Hoessli 1998). Crosslinking of CD44 leads to activation of the ERK signaling pathway and T cell activation (Föger, Marhaba, and Zöller, n.d.). CD44 crosslinking has been proposed to enhance the strength of TCR signaling by delivering LCK to the immunological synapse, as CD44 co-localizes with the TCR/CD3 complex when propagating these signaling events (Hegde et al. 2008). Deficiency of CD44 in T cells causes a defect in Th1 and Th17 differentiation, as well as a defect in the survival of Th1 memory T cells (Baaten et al. 2010; Guan, Nagarkatti, and Nagarkatti 2011). Survival of Th2, Th17, and CD8 T cells does not depend on CD44, demonstrating a specific role for Th1 memory effector T cell survival (Baaten et al. 2010). It has been proposed that CD44 enhances TCR signaling

through recruiting LCK to active signaling sites, which could be required to generate Th1/Th17 cells, but not Th2 (Schumann et al. 2015). This notion of CD44 strengthening TCR signaling is further supported by the fact that DCs express the ligand HA, which has been shown to bind to CD44 on T cells and enhance DC-T cell clustering as well as stabilize DC-T cell interactions to enhance T cell activation and proliferation (Do, Nagarkatti, and Nagarkatti 2004; Hegde et al. 2008). Therefore, CD44 has an active role in T cell activation as well as survival and migration of memory T cells.

While CD44 is appreciated as having a role in T cell activation and memory, there is now accumulating evidence that CD44 has an active role in promoting T cell death. Crosslinking of CD44 on peripheral T cells enhances surface expression of FasL, which leads to AICD (Nakano et al. 2007). HA binding to CD44 can induce apoptosis, and CD44 deficiency confers resistance to apoptosis (Ruffell and Johnson 2008; McKallip et al. 2002). HA is not expressed on resting T cells but is expressed on activated T cells (Mummert et al. 2002). AICD of purified T cells was reduced in the presence of the hyaluronic acid cleaving enzyme, hyaluronidase, suggesting that HA binding to CD44 can promote AICD in T cells (Nakano et al. 2007). Together, these studies demonstrate that CD44 has distinct roles in all phases of the T cell lifecycle, including T cell activation, differentiation, survival, and death.

FoxO Signaling

The Forkhead-box O (FoxO) family of transcription factors control a wide array of cellular processes, including metabolism, cellular differentiation, survival, cell-cycle arrest, and tumor suppression. A FoxO ortholog (Daf-16) was first identified in the roundworm *Caenorhabditis elegans* (Daf-16) (Riddle, Swanson, and Albert 1981). Daf-

16 was found to be a critical regulator for a Dauer or a "non-aging" larval stage. In the 1990s, a mutation in Daf-2 was found to double the lifespan of *C. elegans,* and this effect required Daf-16 (Kenyon et al. 1993). Daf-16 was found to mediate insulin-like metabolic signaling and confers resistance to stress as well as prolonging the *C. elegans* lifespan (Ogg et al. 1997; K. Lin et al. 1997). Shortly after in 2003, a homolog of *daf-16* was found in *Drosophila melanogaster*, termed dFOXO (Kramer et al. 2003). Overexpression of *dFOXO* decreased cell size and cell number, while flies lacking *dFOXO* appeared physically normal but had heightened sensitivity to oxidative stress (Jünger et al. 2003). Overexpression of *dFOXO* in adult *Drosophila* increased lifespan significantly (Giannakou et al. 2004; Hwangbo et al. 2004). The studies in *C. elegans* and *Drosophila* suggested that FoxO was a "longevity gene" and prompted numerous studies aiming at identifying how FoxO could be a key regulator of the aging process.

In mammals, the FoxO subclass contains four members: FoxO1, FoxO3, FoxO4 and FoxO6. These FoxO proteins share a winged helix forkhead DNA-binding domain (Furuyama et al. 2000). FoxO transcription factors can act as transcriptional activators or repressors, often depending on their binding partners (Dijkers et al. 2000; Paik et al. 2007). FoxO can form complexes with β-catenin, STAT3, Runx3, Smad3 or Smad4 to mediate different downstream transcriptional processes. FoxO1, 3 and 4 are all regulated via the PI3-kinase Akt pathway, which is one of the most well-studied mechanisms of FoxO regulation (A. Brunet et al. 1999). Phosphorylation and activation of Akt by PI3K results in phosphorylation of FoxO1, 3 and 4 at three conserved sites, diminishing their DNA-binding ability (Guertin et al. 2006; Rena et al. 2001). This causes nuclear exclusion and degradation, resulting in lack of FoxO binding and

transcription of target genes. In immune cells, the PI3K pathway can be activated by a variety of stimuli via specific receptors, including the B cell receptor (BCR), TCR, CD28 signaling, as well as cytokine and chemokine receptors (Stahl et al. 2002; Wood, Schneider, and Rudd 2006). Cytokine withdrawal can elicit dephosphorylation of FoxO proteins and activation (You et al. 2006).

FoxO1 and FoxO3 in the immune system. FoxO1 and FoxO3 are the main isoforms expressed in the immune system. FoxO1 is expressed abundantly in lymphoid cells, and can regulate many processes including differentiation, homing and survival (Dejean et al. 2009). Mice with a T cell-specific deletion in FoxO1 have an expanded population of activated and/or memory CD4$^+$CD44^{high} T cells, and develop autoimmunity, characterized by B cell activation, autoantibodies and hypergammaglobulinemia (Kerdiles et al. 2010). Young mice with a T cell-specific FoxO1 deletion had a decrease in the proportion and number of Foxp3$^+$ Tregs in the thymus, while Foxp3$^+$ Treg numbers were normal in the periphery. However, these Foxp3$^+$ Tregs in the periphery were not functionally suppressive, suggesting that autoimmunity in these mice could be due to impaired function of Treg cells. FoxO1 deficient Tregs also had decreased CTLA-4 and CD25 expression, two proteins critical for Treg function. FoxO1 was found to directly control CTLA-4 expression. Similar results were found in mice that had a T cell specific deletion of both FoxO1 and FoxO3. These mice developed systemic inflammatory disease that was fatal, impart due to impaired functionality of Foxp3$^+$ Tregs (Ouyang et al. 2010). Both FoxO1 and FoxO3 bind to the Foxp3 promoter region, and promote transcription of Foxp3. Together, these

data show that FoxO1 and FoxO3 are critical for Treg function and dampening autoimmunity to maintain immune homeostasis.

FoxO1 and FoxO3 appear to have some functional redundancy in T cell homeostasis as well as unique signaling targets. FoxO3 deficient mice show no significant immunological abnormalities such as spontaneous autoimmunity (Dejean et al. 2009; Hosaka et al. 2004). FoxO3 deficiency also does not alter the number or proportion of activated memory T cells in lymph nodes and spleen. Although there was not a T cell-intrinsic phenotype with the loss of FoxO3 alone, mice with a T cell specific deletion for both FoxO1 and FoxO3 developed accelerated autoimmunity compared to FoxO1 deficient mice alone (Kerdiles et al. 2010). In CD8 T cells, FoxO1 and FoxO3 have been found to have unique functions. FoxO1 deficient T cells have decreased IL-7R and naïve CD8 T cells, while FoxO3 does not affect IL-7R expression (Kerdiles et al. 2009; Ouyang et al. 2009). In CD8 memory and effector T cells, FoxO1 inhibits T-bet expression, IFN-γ, granzyme B, and promotes memory formation, while FoxO3 promotes apoptosis of memory T cells, as FoxO3 deficient mice have increased memory CD8 T cells after viral infection (J. A. Sullivan et al. 2012; Rao et al. 2012). Therefore, FoxO1 and FoxO3 have some redundant and some non-redundant functions, depending on the T cell subset and context.

Role of FoxO3 in CD4 T cell differentiation. In CD4 T cells, FoxO3 expression is low in resting T cells and increases after T cell receptor engagement. Activated CD4 cells have a 3-fold increase in FoxO3 expression compared to naïve CD4 T cells (Stienne et al. 2016). FoxO3 expression is positively correlated with the strength of TCR engagement. After TCR stimulation, FoxO3 expression steadily increases overtime,

peaking at 72 hours post stimulation (Stienne et al. 2016). Thus, FoxO3 expression is low in resting naïve T cells, and after TCR stimulation FoxO3 expression increases and is higher in memory T cells.

FoxO3 has an important role in Th1 differentiation. FoxO3 deficient CD4 T cells have a defect in T-bet and Eomes, two critical transcription factors for Th1 polarization. Mechanistically, FoxO3 was found to directly bind to Eomes locus and upregulate transcription of Eomes. Additionally, FoxO3 deficient CD4 T cells have a defect in IFN-γ and GM-CSF, cytokines that Th1 cells produce as part of mediating their critical functions (Stienne et al. 2016). Eomes transcriptionally controls GM-CSF and IFN-γ to promote T-bet expression and a Th1 phenotype. Overexpression of Eomes in FoxO3 deficient mice is sufficient to restore cytokine production. T cell-specific FoxO3 deficient mice have a defect in pathogenic Th1 responses, as the mice are less susceptible to EAE. Therefore, FoxO3 is critical for transcription factors and cytokines that promote Th1 responses. More research is needed to understand the role of FoxO3 in the differentiation of other CD4 Th subsets, as well as in CD8 T cell differentiation.

Role of FoxO3 in T cell survival, function, and memory. FoxO3 has a dynamic role in T cell survival and apoptosis. When T cells are initially stimulated, TCR and CD28 signaling as well as IL-2 production promote the phosphorylation of Foxo by Akt, which promotes nuclear exclusion of FoxO3 (Stahl et al. 2002). This promotes T cell survival and prevents FoxO3 from transcribing target genes that promote apoptosis (Bim and Puma). With cytokine withdrawal, FoxO3 is dephosphorylated, and can bind and induce transcription of Bim and Puma to promote apoptosis (You et al. 2006). Overexpression of FoxO3 is sufficient to trigger T cell apoptosis (Stahl et al. 2002).

Therefore, expression of FoxO3 in the nucleus is tightly controlled, to dictate T cell survival or apoptosis.

FoxO3 is a critical regulator of CD8 T cell memory and survival during infection. Studies using global and T cell-specific FoxO3-deficient mice have investigated CD8 T cell memory formation and survival in various viral and bacterial infections. During LCMV infection, FoxO3 deficiency causes an increase in CD8 T cell memory, due to a reduction in apoptosis of the T cells (J. A. Sullivan et al. 2012). Similarly, during *Listeria monocytogenes* (LM) infection, a higher number of memory CD8 T cells were detected in FoxO3 deficient mice (Tzelepis et al. 2013). During infection with vaccinia virus, FoxO3 limits the primary expansion of CD8 T cells (Togher et al. 2015). Although FoxO3 is critical for T cell memory and survival, FoxO3 is not required for CD8 T cell functionality. FoxO3 deficient CD8 T cells have similar activation, antigen presentation, cytokine production, and granzyme B production as FoxO3 sufficient T cells (Tzelepis et al. 2013; J. A. Sullivan et al. 2012). Therefore, FoxO3 is critical in CD8 T cells for limiting the expansion and survival after infection. A role for FoxO3 has also been found in the survival of CD4 central memory T cells, where TCR and cytokine signaling promoted FoxO3 phosphorylation which was shown to be critical for survival (Riou et al. 2007). Further research is needed to understand the dynamics of FoxO3 signaling in initial T cell signaling and differentiation compared to signaling mechanisms that promote apoptosis.

FoxO-TGF-β signaling crosstalk. TGF-β signaling (which will be discussed at length in the following section) can crosstalk with T cell signaling pathways, such as FoxO transcription factor signaling. As described previously, FoxO3 and FoxO1 work

cooperatively in T cells to control Treg differentiation (Kerdiles et al. 2010). FoxO

proteins directly controlled transcription of Foxp3, which suggests a crosstalk between

FoxO3 and TGF-β signaling. This is significant not only for its implications in Treg cell

biology, but because there is very little known about how pathways downstream of TCR

signaling (such as FoxO3) can interact with TGF-β signaling. TGF-β also suppresses

Tbet and Th1 differentiation, which required FoxO1 (Kerdiles et al. 2010). In non-

lymphoid cells such as keratinocytes, FoxO3 has been shown to directly interact with

smad transcription factors to mediate TGF-β signaling (Seoane et al. 2004; Gomis et al.

2006). Based on these data, FoxO transcription factors may also be critical for

mediating TGF-β signaling outcomes in conventional T cells, but this remains to be

elucidated.

Section 3: Transforming Growth Factor-β (TGF-β)

TGF-β Introduction

TGF-β is a pleiotropic cytokine, that can mediate a variety of cellular processes

and responses in numerous cell types. This includes cell development, fibrosis, wound

healing, carcinogenesis, metastasis, and immune responses. TGF-β is highly

evolutionarily conserved across species and has three known family members in

mammals: TGF-β1, 2 and 3. In the immune system, TGF-β1 is the predominant form

that is expressed and has been the most extensively studied (M. O. Li, Sanjabi, and

Flavell 2006; Shull et al. 1992). TGF-β1 will be the focus of our studies.

Processing and Activation of Latent TGF-β

Before TGF-β can mediate its diverse functions, it must be synthesized,

processed, and activated. TGF-β is initially synthesized as a precursor molecule. The

N-terminal region contains a pre-region that possesses a signal peptide, followed by a pro-region, also known as latency-associated peptide (LAP). The carboxy-terminal end contains the mature peptide region (Gray and Mason 1990; Wakefield et al. 1988). The pre-region is cleaved by endopeptidases, which allows for dimerization. In the Golgi, the endopeptidase furin cleaves LAP, which can then non-covalently associate with mature TGF-β and hold it in an inactive form (Claire M. Dubois et al. 2001; C. M. Dubois et al. 1995). This formation of LAP with mature TGF-β is termed the small latency complex (SLC) (Kohei Miyazono, Ichijo, and Heldin 1993; K. Miyazono et al. 1991). The SLC can be directly secreted from cells, or the SLC can first associate with latent TGF-β binding protein-1 (LTBP-1) to form a large complex that gets deposited onto the extracellular matrix (Irene Nunes et al. 1997; Olofsson et al. 1995). TGF-β remains inactive when bound to LAP and LTBP-1 and must be activated before binding receptors and mediating downstream signaling events.

Several mechanisms can trigger activation of latent TGF-β and its release from LAP. This process appears to be cell-type and TGF-β isoform specific. Proteases and other components of the extracellular matrix can release active TGF-β, as well as changes in pH, thrombospondin in serum, and ROS (I Nunes, Shapiro, and Rifkin 1995; Taylor 2008). Integrins have been shown to be important activators of TGF-β under physiological conditions. Integrins are type I transmembrane receptors that play critical roles in cell adhesion and signaling (Xiong et al. 2001). Integrins bind a RGD integrin binding motif on LAP to liberate active TGF-β (Munger et al. 1999). Mice that possess a single point mutation in the RGD integrin binding motif of LAP express only latent TGF-β and develop lethal systemic inflammation (Yang et al. 2007). Integrins that are capable

of activating TGF-β are expressed on a variety of cell types, including CD4 T cells, DCs and epithelial cells (Travis et al. 2007). In Tregs, the integrin αVβ8 has been shown to activate latent TGF-β to suppress immune cells within close proximity (Liénart et al. 2018). The precise mechanical mechanisms of integrin-mediated activation and liberation of TGF-β are still unknown and are actively being investigated and debated.

TGF-β Signaling

Once TGF-β is liberated from LAP, TGF-β can mediate downstream signaling events. TGF-β signals through two related type I and type II transmembrane serine/threonine kinase receptors that form heteromeric complexes (Gilboa et al. 1998). TGF-β first binds to the constitutively active TGF-β type II receptor (TGF-βRII) (Wrana et al. 1992). This binding brings the TGF-βRI in close proximity to TGF-βRII, and TGF-βRII then transphosphorylates serine and threonine resides on the glycine/serine rich region of the TGF-βRI that can now serve as a binding site for SMAD family of proteins (Luo and Lodish 1996; R. H. Chen et al. 1995; Wrana et al. 1994). Activation of TGF-βRI can lead to canonical and non-canonical signaling mechanisms.

Canonical signaling. During canonical TGF-β signaling, Smad proteins link receptor activation at the cell membrane to transcriptional regulation in the nucleus. Smads contain an amino-terminal MH1 domain that regulates nuclear import and DNA binding, and a MH2 domain that interacts with the TGF-βRI as well as other cytoplasmic adaptors (Abdollah et al. 1997; Lo et al. 1998). TGF-βRI activates Smads2/3 by phosphorylation within the MH2 domains (Qin et al. 2002). Smad2/3 can now associate with Smad4 and translocate into the nucleus (Y. Zhang et al. 1996; Tsukazaki et al. 1998; Correia et al. 2001). Once in the nucleus, Smads bind to smad binding elements

and can mediate activation or repression of target genes as well as interact with various DNA binding proteins. An inhibitory Smad, Smad7, acts as a key regulator of the TGF-β signaling pathway. Smad7 has been proposed to act as a negative feedback loop to control TGF-β signaling responses (Nakao et al. 1997). Smad7 acts at numerous stages in TGF-β signaling, where it can mediate degradation of TGF-βRI, inhibit phosphorylation of Smad2/3, and inhibit formation of Smad2/3 with co-Smad4, preventing translocation into the nucleus (Hayashi et al. 1997; S.-J. Zheng and Chen 2003; Ebisawa et al. 2001).

Non-canonical signaling. Non-canonical TGF-β signaling is Smad independent, where the TGF-βRI transmits signals and interacts with a variety of different cellular pathways. These signaling processes have been shown to be context and cell-type specific. These pathways include: TNF receptor associated factor 4 and 6 (TRAF4 and 6), TGF-β activated kinase 1 (Tak1), phophoinositide 3-kinase (PI3K), extracellular signal-regulated kinase (ERK), nuclear factor-κB (NF-κB), JUN N-terminal kinase (JNK), among others (Hartsough and Mulder 1995; Bakin et al. 2002). These non-canonical signaling pathways have been investigated in tumor cells, epithelial cells, and fibroblasts among others, but little is known about non-canonical TGF-β signaling in T cells.

TGF-β in T Cells

The role of TGF-β in T cell differentiation, function and survival is highly context dependent. TGF-β is critical for aspects of T cell differentiation, modulation of activation and proliferation, as well as survival. TGF-β can promote the differentiation of distinct Th subsets, as well as inhibit differentiation of subsets that do not require TGF-β. TGF-β

can also mediate the suppression of T cell activation and proliferation, but also promote survival of T cells during continuous stimulation. These different functions of TGF-β will be discussed in detail in the sections below.

T cell development and differentiation. As discussed above, the cytokine milieu that T cells encounter during T cell activation dictates the subsequent subset and function of the T cell. Naïve T cells that are activated in the presence of TGF-β and IL-2 can differentiate into peripheral Tregs (pTregs). pTregs are critical to maintain tolerance and suppress autoreactive T cell responses. These suppressive functions require the transcription factor Foxp3 (discussed in detail in Treg section). Smad3 binds at the conserved noncoding region (CNS1) in the *Foxp3* locus and regulates the transcription of *Foxp3* along with other transcription factors and suppresses IL-2 signaling (Tone et al. 2008; McKarns, Schwartz, and Kaminski 2004). Th17 cells also require TGF-β for differentiation. Th17 cells promote inflammation at sites of infection and contribute to autoimmune disease. Mice with defective TGF-β signaling have fewer Th17 (IL-17 producing) cells and overexpression of *Tgfb1* increases the number of Th17 cells (Veldhoen et al. 2006; Manel, Unutmaz, and Littman 2008). TGF-β upregulates expression of the IL-23 receptor, which signals to induce the development of Th17 cells (Mangan et al. 2006). While TGF-β is critical for Treg and Th17 development, TGF-β also actively inhibits Th1 and Th2 development. TGF-β signaling inhibits T-bet (master transcription factor for Th1 differentiation) and IFN-γ signaling (H. Park et al. 2005; I. K. Park, Letterio, and Gorham 2007). TGF-β1^{-/-} mice spontaneously develop lethal necroinflammatory hepatitis, with a large number of CD4 Th1 cells that produce IFN-γ (Gorham et al. 2001). Therefore, TGF-β is critical for preventing Th1 inflammatory

responses. Additionally, TGF-β inhibits GATA3 expression, which is critical for Th2

differentiation (Gorelik, Fields, and Flavell 2000). The precise signaling mechanisms by

which TGF-β inhibits these critical Th1 and Th2 transcription factors and cytokines

remains unknown.

Suppression of T cell proliferation. TGF-β signaling can directly suppress the

proliferation and activation of T cells. Without TGF-β signaling *in vivo*, lethal

autoimmunity can develop. In TGF-β deficient mice, about 20 days post-birth, mice

exhibit acute wasting disease, followed by death (Shull et al. 1992). This is

characterized by excessive immune cell infiltration and tissue necrosis. Gorelik and

Flavell expressed a dominant-negative TGF-βRII under a T cell specific promoter, to

determine if TGF-β signaling in T cells is required to prevent this lethal autoimmunity

(Gorelik and Flavell 2000). These mice have circulating autoantibodies, immune

infiltration in multiple organs, and hyperactivation of T cells. Older mice especially have

increased frequencies of activated memory T cells and loss of naïve T cells compared

to WT controls. These data suggested that TGF-β signaling in T cells is required to

prevent hyperactivation and suppress autoimmunity.

Further investigation uncovered that TGF-β signaling acts primarily on naïve T

cells to inhibit their proliferation and growth. Naïve T cells are inherently more

susceptible to TGF-β, as they have TGF-βRI and RII on their cell surface, while

activated T cells lose expression of the receptors (Cottrez and Groux 2001; Sanjabi,

Mosaheb, and Flavell 2009; Tu et al. 2018). Patients with autoimmunity have been

found to have decreased TGF-βRI on their naïve T cells, which is believed to contribute

to spontaneous T cell activation (Tu et al. 2018). TGF-β/Smad3 signaling has been

shown to dampen the effect of CD28 co-stimulation on T cell activation and growth.

Smad3 specifically modulates the expression of >400 transcripts to suppress co-

stimulation dependent mobilization of T cell proliferation (Delisle et al. 2013). TGF-β

also actively promotes survival of naïve T cells by contributing to expression of IL-7Rα,

which enables naïve T cells to conduct IL-7 signaling that is critical for survival (Ouyang

et al. 2013). After naïve T cells encounter antigen presented by APCs and co-

stimulation, CD28 signaling has been found to inhibit the anti-proliferative effects of

TGF-β, which can subsequently drive activation and proliferation (Sung, Lin, and

Gorham 2003). In activated T cells, IL-10 has been shown to upregulate expression of

TGF-βR expression, which can make activated/memory T cell susceptible to TGF-β

mediated suppression (Cottrez and Groux 2001).

Another mechanism by which TGF-β blocks T cell proliferation is through

inhibition of the T cell growth factor IL-2 (Kehrl et al. 1986; Brabletz et al. 1993). IL-2 is

critical for driving mitosis and expansion of T cells after activation. TGF-β blocks IL-2

transcriptionally through Smad signaling (Tzachanis et al. 2001). Smads can bind to the

smad binding element in the IL-2 promoter and interact with recruited histone methyl

transferases to directly inhibit IL-2 transcription (Wakabayashi et al. 2011). Smad

signaling also upregulates Foxp3, which can compete with transcription factors for

binding to the IL-2 promoter. NFAT and AP1 are two transcription factors that

cooperatively form a complex to mediate IL-2 transcription. Foxp3 binds to NFAT to

reduce NFAT/AP1 complex formation (Wu et al. 2006; C. Chen et al. 2006).

Role of TGF-β in T cell survival. The effects of TGF-β on T cells depends on

their differentiation status and history of activation. TGF-β can potently suppress the

activation and proliferation of naïve T cells, but in memory T cells TGF-β promotes their survival and function (Filippi et al. 2008). TGF-β has been shown to rescue antigen-specific effector T cells from AICD. Antigen-specific effector T cells were found to robustly proliferate and then undergo AICD *in* vitro after stimulation, and IL-2 and TGF-β could rescue cell death and promote survival (X. Zhang et al. 1995). Similarly, TGF-β protects conventional mouse CD4 T cells from AICD caused by continuous stimulation with plate-bound anti-CD3 and CD28 antibodies (Singh et al. 2010; Takami, Love, and Iwashima 2012). TGF-β-treated T cells have reduced Fas and FasL, which promotes survival and prevents apoptosis (Genestier et al. 1999; Cerwenka et al. 1996). Therefore, TGF-β is thought to have a critical role in promoting survival of memory T cells.

In one study, Ma et al used conditional deletion of *Tgfbr2* in antigen-specific T cells to study the effect of TGF-β signaling in T cells during the effector and memory phases (Ma and Zhang 2015). After bacterial infection, the number of antigen-specific CD8 T cells was significantly decreased in the absence of TGF-β signaling. Continuous TGF-β signaling in memory T cells was found to be critical to maintain their memory-specific transcriptional programming. Similar findings were shown in CD8 memory T cells after *Listeria* infection. The concentration of TGF-β in serum was positively correlated with CD8 memory formation (Sanjabi, Mosaheb, and Flavell 2009). Blocking TGF-β signaling did not affect the effector functions of these antigen specific T cells. However, it did reduce the number of memory T cells by reducing pro-survival factor Bcl-2 and promoting apoptosis. In autoimmune disease, TGF-β has also been shown to promote T cell survival. In a mouse model for diabetes, the survival and function of

autoreactive memory T cells that promote islet destruction were dramatically reduced in the absence of TGF-β signaling (Filippi et al. 2008).

Therefore, TGF-β signaling has pleiotropic effects on T cell differentiation and function. TGF-β can potently suppress the activation and proliferation of T cells, especially naïve T cells. Additionally, TGF-β can promote the differentiation of distinct Th subsets such as Th17 and Treg cells. During memory formation, TGF-β signaling is critical for T cells to survive repeated stimulation and prevents AICD, while also maintaining memory T cell numbers and functions (Figure 7).
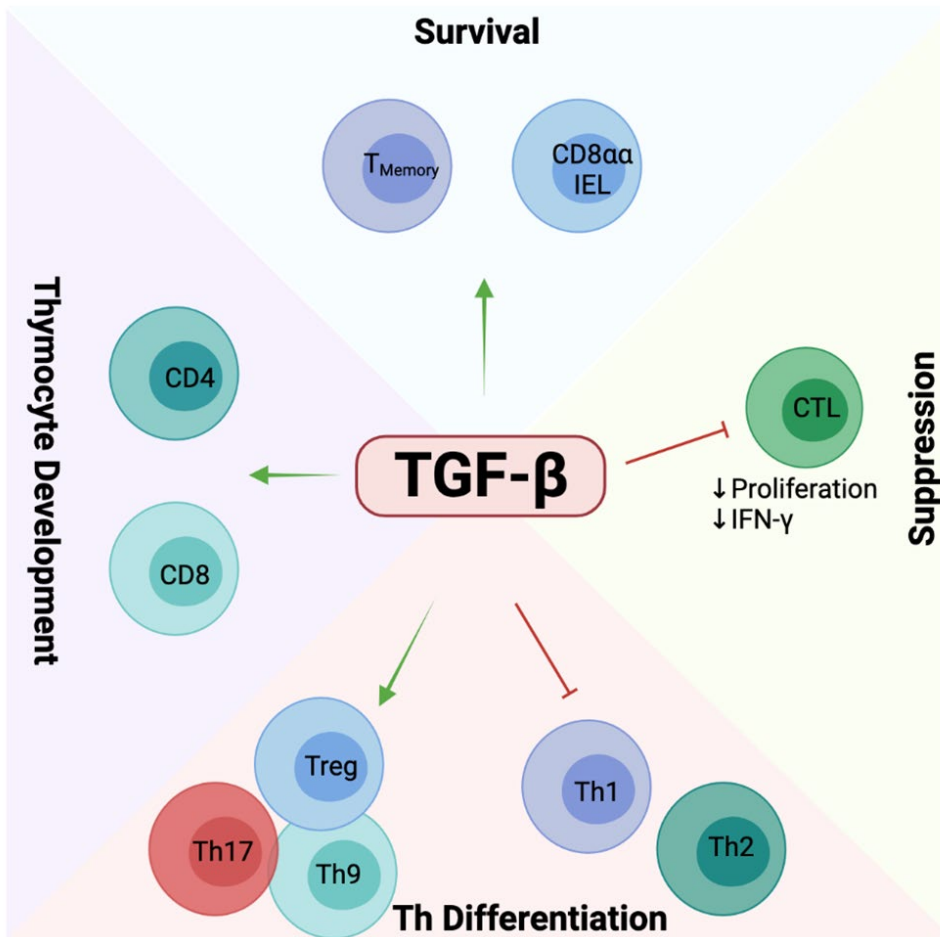
Figure 7. Pleiotropic Roles of TGF-β Signaling in T Cells. TGF-β signaling can affect Th cell differentiation by suppressing Th1 and Th2 differentiation, and promoting Treg, Th17, and Th9 differentiation. In the thymus, TGF-β signaling supports the differentiation and survival of CD4 and CD8 T cells. In the periphery, TGF-β supports the survival of memory T cells and protects from AICD and has a role in survival of gut IEL CD8αα T cells. TGF-β suppresses CTL responses by blocking proliferation and inflammatory cytokine production, especially in the context of the tumor microenvironment.

Section 4: Treg Differentiation, Function, and Homeostasis

Regulatory T cells (Tregs) are a critical CD4 Th subset that maintains self-tolerance and suppresses inflammatory immune responses. After birth, our bodies are constantly exposed to harmless and beneficial non-self-antigens from commensal bacteria, food, and our environment. Tregs are essential for suppressing T cells that recognize these foreign antigens and self-antigens to prevent autoimmune disease. A

decrease in Treg numbers is associated with autoimmune diseases such as type I diabetes, multiple sclerosis, and inflammatory bowel disease, while Treg numbers are increased in the tumor microenvironment. Thus, maintaining the appropriate balance between Treg and conventional T cell populations is essential for controlling immune responses against non-self and self-antigens, as well as tumor antigens. Tregs can be generated in the thymus (tTregs) during negative selection, as well as the periphery (pTregs). pTregs are induced when naïve CD4 T cells are stimulated in the presence of the cytokine TGF-β and IL-2 (W. J. Chen et al. 2003).

Discovery and Function of Tregs

The idea of suppressive T cells dates back to 1969 (Nishizuka and Sakakura 1969), but it took several years to identify a definitive marker for Tregs. Because of this, skepticism grew in the 1980s and without a definitive marker, it was difficult to show that Tregs were indeed responsible for suppressing autoimmunity. In a ground-breaking discovery in 1995, Sakaguchi et al identified that Tregs express high levels of CD25 (IL-2 receptor α chain) (Matoba et al. 2019). When T cells depleted of CD25$^+$ cells were transferred into athymic nude mice, this caused autoimmune disease, including colitis and the development of autoantibodies. Co-transfer of a small number of CD4$^+$25$^+$ T cells could prevent autoimmunity.

Like other T cell subsets, Tregs develop in the thymus. It was discovered in 1999 that TCR transgenic mice develop suppressive CD4+CD25+ T cells that primarily express TCRs that utilize endogenous alpha-chains, while RAG-2-deficient TCR transgenic mice do not develop Tregs (M. Itoh et al. 1999). This suggested that Tregs

require TCR for thymic selection and development. Tregs make up approximately 10% of peripheral T cells in mice and humans.

TGF-β Signaling and Foxp3

TGF-β signaling is required for the differentiation, maintenance, and suppressive function of Tregs. TGF-β signaling induces a variety of signaling processes critical for Tregs, including induction of the master transcription factor Foxp3 (Hori, Nomura, and Sakaguchi 2003). Foxp3 programs the development and function of regulatory T cells, and stabilizes lineage commitment (Gavin et al. 2007; Fontenot, Gavin, and Rudensky 2003). In 2003, Hori et al mechanistically demonstrated that Foxp3 controls regulatory T cell development (Hori, Nomura, and Sakaguchi 2003). Foxp3 was shown to be expressed in naturally arising CD4 Tregs. Additionally, retroviral gene transfer of Foxp3 could convert naïve T cells to a regulatory phenotype, similar to Tregs. This demonstrated that Foxp3 is sufficient for regulatory T cell phenotypes. While the precise mechanisms of Foxp3 in controlling Treg functions remain poorly understood, Foxp3 has been found to act as a transcriptional activator or repressor. Foxp3 can act with other transcription factors to control expression of thousands of genes (Y. Zheng et al. 2007; Sadlon et al. 2010). This is why Foxp3 is often deemed the "master" transcription factor in Tregs. Foxp3 can form a complex with NFAT, which has been shown to upregulate CTLA-4, CD25 and repress expression of IL-2 (Marson et al. 2007; Wu et al. 2006). Foxp3 can also interact with AML1 or Runx1 to suppress IL-2 and IFN-γ production, while enhancing suppressive activity and upregulating glucocorticoid-induced TNF-receptor family-related protein (GITR) (Ono et al. 2007). GITR

engagement has been shown to enhance Treg proliferation and is a marker of functional Tregs (Ronchetti et al. 2015; G. Liao et al. 2010).

Because Foxp3 is so critical for Treg differentiation and functions, mutations in Foxp3 cause fatal multi-organ autoimmune disease. In humans, mutations in Foxp3 cause immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome and a similar lymphoproliferative disease in Scurfy mice (Bennett et al. 2001; Brunkow et al. 2001). In Scurfy mice, transfer of Tregs from healthy mice can prevent systemic inflammation (Fontenot, Gavin, and Rudensky 2003). This demonstrated that Foxp3 expression in Tregs is critical to prevent systemic autoimmunity. A critical distinction between mice and humans is that Foxp3 expression in mice is limited to Tregs, while human non-Tregs can transiently express Foxp3 after activation. However, only Tregs have stable expression of Foxp3 in humans (Gavin et al. 2006).

Mechanisms of Treg Generation and Survival

TGF-β. TGF-β signaling is critical not only for Treg differentiation, but also for survival, maintenance, and expansion in the periphery. Tregs were examined in transgenic mice with a dominant negative form of TGFBRII under control of the CD2 promoter (marker for T cells and NK cells). $CD4^+CD25^+$ Tregs were decreased in these mice, and upon transferring TGF-βRII deficient Tregs into mice with DSS-induced colitis, the Tregs proliferated less than WT counterparts (Huber et al. 2004). This suggested that TGF-β signaling was critical for the expansion and maintenance of Tregs *in vivo.* Mice that harbor a T cell-specific deletion of TGF-βRII develop lethal inflammation and autoimmunity, with severely diminished survival of Tregs as well as other CD4 T cell subsets (M. O. Li, Sanjabi, and Flavell 2006). In 8-10 day old TGF-β1

deficient mice, peripheral, but not thymic Tregs were reduced, suggesting a critical function of TGF-β in the maintenance of peripheral Tregs (Marie et al. 2005). When *Tgfbr1* is specifically deleted in Tregs, this causes a dramatic defect in the recruitment and retention of Tregs in the GI tract, resulting in excessive inflammation in the intestines (Konkel et al. 2017). These studies point to a critical role for TGF-β signaling in the maintenance and survival of Tregs, although the source of TGF-β that is required for *in vivo* maintenance of Tregs still remains controversial. Conventional T cells, Tregs, and numerous other cell types including other leukocytes and stromal cells can produce TGF-β. TGF-β1 produced by T cells is dispensable for Treg generation and maintenance. However, TGF-β1 deficient Tregs were shown to have reduced suppressive function in an *in vivo* transfer model (M. O. Li, Wan, and Flavell 2007). Therefore, TGF-β produced by Tregs is critical for suppressive functions. Other cell types that provide TGF-β for Tregs remain to be elucidated.

  IL-2. CD25 (IL-2 receptor α chain) is not only a marker for Tregs, but is required for IL-2 signaling that is critical for the development, survival, and suppressive functions of Tregs (D'Cruz and Klein 2005; Fontenot et al. 2005). IL-2 signaling controls numerous signaling pathways that promote cell growth, division, survival and metabolic activity. IL-2 deficient mice have a dramatic decrease in the number of Tregs and develop autoimmunity (Schorle et al. 1991). Additionally, antibody-mediated blockade of IL-2 in normal mice can cause an onset of autoimmunity and a decrease in Treg numbers, suggesting IL-2 is critical for peripheral maintenance of Tregs (Setoguchi et al. 2005). IL-2 is required for the expression of Foxp3 as IL-2 induced Stat5 can bind to the Foxp3 promoter and stabilize expression (Setoguchi et al. 2005; Sakaguchi et al.

1995). Tregs cannot make their own IL-2, and thus rely on other cell types such as activated T cells to produce IL-2 (Yamamoto-Taguchi et al. 2013; Cousens, Orange, and Biron 1995). Intravital imaging has beautifully shown that Tregs and conventional T cells colocalize with DCs, and in these clusters conventional T cells respond to antigen and produce IL-2, while Tregs are recipients of IL-2 (Z. Liu et al. 2015).

      Other survival factors. Although IL-2 is critical for survival and expansion of Tregs, other survival factors have been identified. Tregs are not completely absent in IL-2 deficient mice, suggesting other survival factors in the absence of IL-2 (Schorle et al. 1991). Additionally, IL-2 is not readily available in all tissue sites, and is produced primarily by activated T cells and in the lamina propria (Yamamoto et al. 2013). One factor Tregs rely on for survival is CD28. Inducible CD28 deletion demonstrated a selective loss of Treg cells that showed reduced homeostatic proliferation (Gogishvili et al. 2013). The authors postulated that this reduction in proliferation was probably due to a lack of co-stimulation during self-antigen recognition. Another survival factor is TNF (tumor necrosis factor). Tregs express membrane-bound TNF, which is a potent activator of the type 2 TNF receptor (Nelson et al. 2018). The type 2 TNF receptor promotes cell survival and growth. If autocrine TNF is blocked in human Tregs *in vitro* in the absence of IL-2, Tregs stop proliferating. This showed that TNF can act as an autocrine Treg-derived growth factor. Although several mechanisms of Treg survival and homeostasis have been studied, there is still much to be discovered regarding how Tregs survive *in vivo* in distinct immunological niches.

Mechanisms of Treg-mediated Suppression

Tregs suppress inflammatory responses and proliferation of conventional T cells by a variety of mechanisms. To date, many of the proposed mechanisms fit into two broad categories: contact-dependent and contact-independent suppression. Treg-mediated suppression can directly target the function of effector T cells (through suppressor cytokines, cytolysis or consumption of IL-2) or target the APCs that are required for activating effector T cell responses. While Treg suppression requires TCR activation, after activation Tregs can mediate suppression in an antigen-independent manner (Hori, Nomura, and Sakaguchi 2003).

CTLA-4. One vital Treg suppressive mechanism is delivered through surface expression of cytotoxic T lymphocyte associated protein (CTLA-4). While activated T cells can transiently express CTLA-4, constitutive expression of CTLA-4 is restricted to Tregs (Read, Malmström, and Powrie 2000). Blocking CTLA-4 *in vivo* results in the development of autoimmunity and colitis in mice and prevents Tregs from mediating suppression of conventional T cells *in vitro* (Takahashi et al. 2000; Tang et al. 2004). Specific deletion of CTLA-4 in Tregs results in spontaneous lymphoproliferation, fatal T cell autoimmunity, and potent anti-tumor immunity (Wing et al. 2008). Mechanistically, CTLA-4 binds B7 ligands on APCs, sequestering these ligands from effector T cells that require them for CD28 co-stimulation and T cell activation (Oderup et al. 2006; Wing et al. 2008). Sequestration of these B7 ligands by CTLA-4 binding can prevent availability of B7 ligands for T cell co-stimulation and lead to effector T cell anergy. Additionally, binding of CTLA-4 to CD80/86 B7 ligands causes an increase in IDO (Indeolamine 2,3-dioxygenase) production by DCs (Grohmann et al. 2002; Munn et al. 1999). IDO breaks

down tryptophan, which can limit the availability of this proinflammatory resource to T cells. IDO expressing DCs possess potent tolerogenic properties that can limit T cell responses to antigen stimulation and promote the conversion of naïve T cells to regulatory T cells (Baban et al. 2009; Y. Yan et al. 2010). Thus, CTLA-4 is critical for Treg function, and can block effector T cell activation by sequestering B7 ligands and modulating APCs to indirectly block T cell responses.

Direct killing of APcs and effector T cells. Tregs can directly kill target cells through cell contact-dependent cytolysis. Tregs produce granzyme A and B, which enters target T cells and APCs through pores produced in the membrane by perforin (Grossman et al. 2004; Cao et al. 2007). Granzymes then activate caspase-mediated apoptosis in these target cells. Tregs can directly kill effector T cells or kill APCs, which is thought to indirectly diminish effector T cell responses. Another mechanism of direct T cell killing is through the TRAIL-DR5 (Tumor-necrosis-factor-related apoptosis-inducing ligand–death receptor 5) pathway. Tregs upregulate and express TRAIL on the surface after activation (Ren et al. 2007). TRAIL can bind the DR5 receptor on T cells, leading to effector T cell apoptosis. Blocking this TRAIL-DR5 axis reduces the suppressive activity and cytotoxic effects of Tregs *in vivo*.

Cytokine-mediated suppression. Although contact-dependent suppression is critical for Treg functional responses, several contact-independent mechanisms of suppression have been discovered. Production of IL-10 and TGF-β by Tregs has been shown to suppress inflammatory T cell responses. IL-10 production by Tregs inhibits intestinal inflammation and colitis pathologies, and promotes tolerance to alloantigens (Asseman et al. 1999; Hara et al. 2001). Tregs can express soluble and membrane-

bound TGF-β, which can act as an immunosuppressive cytokine to block T cell activation and proliferation. Blocking TGF-β in an *in vitro* suppression assay prevents Treg mediated suppression of T cell proliferation (K. Nakamura et al. 2004). Production of TGF-β is enhanced by co-stimulation via CTLA-4.

Another cytokine that is critical for suppression is IL-2. Tregs express high levels of the IL-2 receptor alpha chain, CD25, on their cell surface. In addition to promoting survival and growth, the consumption of IL-2 by Tregs can limit IL-2 availability for effector T cells. This deprivation of IL-2 can cause Treg-induced apoptosis of effector T cells, and exogenous IL-2 can rescue effector T cells from apoptosis (Pandiyan et al. 2007). Additionally, Tregs that are activated in the presence of anti-IL-2 antibodies cannot mediate suppression of responder T cells (de la Rosa et al. 2004). IL-2 also primes Tregs to produce IL-10 upon secondary stimulation. Therefore, IL-2 can act as a survival factor for Tregs and promote suppressive functions.

Section 5: T Cells in the Tumor Microenvironment

T Cell Recognition of Tumor Antigens

Conventional T cells recognize foreign antigens in the context of MHC, as T cells that recognize self-antigens with high affinity are eliminated during negative selection in the thymus. This allows for T cells to respond to pathogens and prevents autoimmunity. So, how do T cells recognize tumor cells, which are derived from our own cells? It was discovered that T cells can recognize tumor associated antigens (TAA), which are antigens that are highly overexpressed in tumor cells compared to normal cells. Because of these antigens, it was demonstrated in the 1980s that tumor infiltrating lymphocytes (TIL) can be expanded with IL-2 and infused back into mice to target and

kill tumor cells (Rosenberg et al. 1988; Rosenberg, Spiess, and Lafreniere 1986). This was used early on to treat melanoma in mice, and TAAs such as MART-1, tyrosinase and MAGE-1 were among the first identified to be presented in the context of HLA-A2 (Brichard et al. 1993; Van Der Bruggen et al. 1991; Coulie et al. 1994; Kawakami et al. 1994). Since the discovery that T cells can target and eliminate tumor cells, there has been great interest in understanding T cells in the tumor microenvironment. Much emphasis has been put on understanding how to enhance T cell mediated killing, survival, and on the development of novel immune-based therapies.

Over the past couple of decades, it has been well documented that the tumor microenvironment (TME) can be hostile and prevent successful immune-mediated anti-tumor responses. This includes: (i) the induction of negative regulators on immune cells to shut down effector T cell responses (ii) the recruitment and induction of suppressive innate and adaptive immune cells and (iii) the presence of tumor metabolic pathways that causes a depletion of nutrients that are critical for the function and survival of T cells.

Negative Regulators of T Cell Function

As discussed previously, T cells become activated when recognizing antigen presented by APCs in the context of MHC. This orchestrates activation, proliferation, cytokine production and killing of target cells during infection. In the tumor microenvironment, T cells must recognize TAAs to become activated and eliminate tumor cells. It has been documented that even if tumors express antigens that can be recognized by T cells, one reason T cells cannot elicit effective immunity against tumors is a lack of costimulatory ligands that are needed for activation (L. Chen et al. 1992;

Townsend and Allison 1993). Expression of B7 ligands that can bind CD28 and provide T cell co-stimulation has been shown to enhance T cell anti-tumor responses (Baskar et al. 1993; Allison, Hurwitz, and Leach 1995). After T cells become activated and perform their effector functions, T cells upregulate negative feedback responses to shut down T cell responses. This negative feedback process is critical during homeostasis to prevent aberrant T cell responses and autoimmunity. However, in the TME, these negative feedback responses can prevent successful elimination of tumors and anti-tumor immunity.

　　　　Immune checkpoint molecules. Immune checkpoint molecules are the most well-studied molecules that promote negative feedback responses, and have been found to block inflammatory T cell responses in the TME. The first identified inhibitory receptor was CTLA-4. CTLA-4 is a homolog of CD28 that is upregulated after T cell activation and binds B7 family ligands with much higher affinity than CD28 (J. F. Brunet et al. 1988; Linsley et al. 1994; Krummel and Allison 1995). This allows CTLA-4 to compete with B7 ligands to block CD28 binding and thus T cell co-stimulation. Additionally, binding of CTLA-4 to B7 ligands negatively regulates multiple aspects of TCR signaling to dampen T cell responses. CTLA-4 can recruit phosphatases that can decrease phosphorylation of several key proteins in the TCR signaling cascade, such as CD3ζ and LAT (Marengère et al. 1996; K. M. Lee et al. 1998). CTLA-4 can also recruit PI3-K to promote signaling that prevents death in anergic T cells (Schneider et al. 2008). Crosslinking of CTLA-4 *in vitro* inhibits T cell proliferation and IL-2 production induced by anti-CD3 activation and blocking CTLA-4 with Fab fragments enhances T cell proliferation (Walunas et al. 2011; Krummel and Allison 1995). This is required to

dampen overactivation of T cells and reduce inflammatory processes after pathogen clearance. CLTA-4 is required for immune homeostasis in T cells, as mice deficient in CTLA-4 develop lethal autoimmunity from massive lymphoproliferation and tissue destruction (Tivol et al. 1995; Waterhouse et al. 1995).

Because CTLA-4 blocks T cell activation, it was postulated that CTLA-4 may function to inhibit T cell responses in the TME. Indeed, blocking CTLA-4 via injection of antibodies that prevent CTLA-4 ligand binding was shown to successfully eliminate B7-1 positive colon carcinoma tumors in mice, as well as B7 negative tumors (Leach, Krummel, and Allison 1996). Mice treated with blocking antibodies to CTLA-4 were also protected from secondary challenge of tumor cells. Because of numerous promising studies in mice, CTLA-4 blockade was one of the first negative regulators to be targeted clinically. In 2011, the Food and Drug Administration approved the use of a monoclonal antibody that blocks CTLA-4, ipilimumab, in metastatic melanoma. A second monoclonal antibody, tremelimumab, was approved in 2015 for mesothelioma. Both antibodies now have been used for clinical trials targeting other tumor types (Szostak et al. 2019). CTLA-4 is also highly expressed on Tregs and contributes to their suppressive functions, as discussed previously. Tregs with high expression of CTLA-4 have been found in multiple tumor types, and these cells were shown to be $Foxp3^+CD25^+$ and have suppressive functions (Matoba et al. 2019). Additionally, CTLA-4^+ Tregs have been found to suppress NK cell cytotoxicity and correlate with poor prognosis (Jie et al. 2015). Therefore, there has been increasing interest in blocking CTLA-4 not only to remove effector T cell exhaustion, but also to block Treg responses in the tumor.

Another very well-known and studied checkpoint inhibitor is programmed cell death protein-1 (PD-1). Like CTLA-4, PD-1 is a critical regulator of normal T cell homeostasis. PD-1 is expressed by T cells after activation, and acts as a break to inhibit overactivation of T cell responses. Mice deficient in Pdcd1 (which encodes PD1) develop lupus-like autoimmune disease (Nishimura et al. 1999). PD-1 counters T cell activation signals by binding to its ligand PD-L1 and/or PD-L2, that is present on APCs as well as tumor cells (Freeman et al. 2000; Latchman et al. 2001). High PD-1 expression in T cells is associated with exhaustion in many biological scenarios, including chronic viral infection and in the TME. Blocking PD-1 or its ligands has been shown in these settings to partially reverse T cell exhaustion and promote T cell activation and killing (Barber et al. 2006; Iwai et al. 2002; Hirano et al. 2005). Similar to CTLA-4, because of success in anti-tumor responses in mice, several antibodies that target PD-1 and its ligands were approved for use in a variety of human cancers. Although results were promising, most patients do not show long-lasting remission. Therefore, there has been great interest in combining CTLA-4 and PD-1 therapy, which in certain cancers such as melanoma can promote enhanced efficacy of tumor immune responses (Wolchok et al. 2013). As we learn more, the treatment of patients with cancer becomes more complex and personalized, with the need to often do combination therapies. This includes combining PD-1/CTLA-4 blockade with other inhibitors such as LAG3, TIM3 or TIGIT; blocking suppressive cytokines such as IL-10, administering cytokines that promote T cell survival such as IL-2, IL-7 and IL-15, and delivering engineered T cells (discussed later on). Greater understanding of the biology of these

receptors and cytokines, as well as the unique tumor environments is needed to facilitate better treatment.

Suppressive Immune Cells and Hostile Metabolic Environment

When tumors start to develop, a variety of cell types are recruited to the TME that can antagonize effective T cell responses. The TME is highly complex, containing fibroblasts, myeloid cells, suppressive B cells, endothelial cells, Tregs and γδ T cells. These cells can inhibit T cell responses through contact-dependent mechanisms and the production of soluble molecules. Although Tregs are classically thought of as CD4+, CD8+ Tregs are also found in the TME and can suppress inflammatory T cell responses (Kiniwa et al. 2007; J. Li et al. 2011). Tolerogenic APCs can directly mediate the induction and/or expansion of these regulatory T cells (Sisirak et al. 2012; Faget et al. 2012). Many of the APCs in the TME express low levels of co-stimulatory molecules and upregulate enzymes like inducible nitric oxide synthase (iNOS) and indoleamine 2,3 dioxygenase (IDO) that create metabolic challenges for T cells. IDO converts tryptophan into metabolic byproducts called kynurenines (Higuchi and Hayaishi 1967; Shimizu et al. 1978). High IDO is associated with reduced T cell infiltration into tumors and increased numbers of Tregs (Pelak et al. 2015; T. Nakamura et al. 2007). The diminished tryptophan levels in the TME prevent T cell proliferation and can induce T cell death (Munn et al. 2004). Another metabolic barrier is hypoxia, where areas of low oxygen in tumors can cause T cell dysfunction. Growing evidence shows that T cell growth and survival is impaired in hypoxic conditions (Atkuri, Herzenberg, and Herzenberg 2005; Caldwell et al. 2001). Hypoxia can inhibit CTL differentiation from precursors and can shift cells from IL-2, IFN-γ producing Th1 cells to a Th2 phenotype (Zuckerberg,

Goldberg, and Lederman 1994; Caldwell et al. 2001). In addition to hypoxia, the TME

has low glucose availability, which can dampen T effector functions. Tregs mainly rely

on oxidative phosphorylation, and thus can survive better than CTLs, which rely

primarily on glycolysis and glucose to mount their inflammatory functions (Michalek et

al. 2011; Chang et al. 2013). Foxp3 was found to be necessary and sufficient to

program Tregs to utilize fatty acids and perform oxidative phosphorylation (Howie et al.

2017). Therefore, there are several molecules and metabolic conditions in the TME that

promote Treg survival and effector T cell disfunction.

Role of TGF-β in Tumor-specific T Cells

TGF-β is present in a vast variety of tumor types and has been shown to promote

tumor growth. TGF-β can be produced by tumor cells themselves, or it can be

produced by other cells in the TME. Specifically, T cells, natural killer cells,

macrophages and epithelial cells have all been shown to produce TGF-β in the TME

(Tamada et al. 1997; Donkor et al. 2011). Regardless of its source, TGF-β in the TME

can have a critical role in suppressing anti-tumor immune responses. T cells, which are

necessary for killing tumors cells and promoting inflammation, can be negatively

affected by TGF-β in the TME.

TGF-β can suppress the proliferation, inflammatory cytokine production, and

cytotoxic functions of T cells. Consistent with this notion, neutralization of TGF-β in the

TME using TGF-β specific antibodies enhanced IL-2 receptor expression (CD25) and

Th1 cytokine production by T cells (Maeda and Shiraishi 1996). This demonstrated that

TGF-β can suppress inflammatory activities of T cells. To test if there was a specific role

for TGF-β signaling in suppression of T cell responses, Gorelik and Flavell generated

transgenic mice with T cells expressing a dominant negative form of TGF-βRII, which prevents T cells from propagating TGF-β signaling (Gorelik and Flavell 2000). When mice were challenged with tumor cells, transgenic mice were able to eradicate tumors and survive, while WT littermates had rapid tumor progression and death. This showed that blocking TGF-β signaling in T cells is sufficient for T cell-mediated anti-tumor responses. Transgenic mice also had a greater expansion of tumor specific CD8 T cells, demonstrating that TGF-β actively inhibits T cell proliferation in the TME.

TGF-β was shown to inhibit expression of several gene products that are critical for cytotoxic functions in tumors. These include granzyme A, granzyme B, perforin, Fas Ligand, and IFN-γ. Mechanistically, TGF-β activated smads cooperated with ATF1 transcription factors to bind and block transcription of granzyme B and IFN-γ (Thomas and Massagué 2005). To suppress these cytotoxic T cell responses, TGF-β was also found to control the transcription factors Tbet and Eomes that direct CTL differentiation and activation. Tbet and Eomes upregulate cytotoxic molecules such as granzyme B, IFN-γ and perforin (Pearce et al. 2003; B. M. Sullivan et al. 2003). In a B16 melanoma model, a TGF-βRI pharmacological inhibitor blocked tumor progression and enhanced CTL responses through restoration of Eomes and cytotoxic molecules in CTLs (Yoon et al. 2013). TGF-β was shown to inhibit Tbet expression in Th1 cells, which resulted in decreased CD8 CTL responses in human melanoma-reactive memory T cells (Ahmadzadeh and Rosenberg 2005). TGF-β not only inhibits Th1, but also can promote PD-1 expression, which can block effective T cell responses (B. V. Park et al. 2016). Therefore, TGF-β signaling has a role in suppressing T cell inflammatory responses through multiple mechanisms, which can dampen anti-tumor immunity.

Although TGF-β is classically thought of as inhibitory to T cell responses in the TME, more evidence is mounting that the effect of TGF-β on T cells is not just immunosuppressive. TGF-β is critical for Th9, Th17 and resident memory T cell subsets, which can all initiate pro-inflammatory responses in the tumor and improved control of tumors. Th9 cells are CD4 T cells that are characterized by IL-9 production (Schmitt et al. 1994). IL-4 and TGF-β are required for induction of Th9 cells from naïve T cells (Veldhoen et al. 2008). Th9 cells have several properties that promote anti-tumor immunity. IL-9 can promote apoptosis of melanoma cells and can promote survival of tumor infiltrating T cells (Fang et al. 2015; Parrot et al. 2016). Th9 cells also recruited DCs to tumor sites, which were able to deliver antigen to tumor draining lymph nodes and activate CD8 T cells (Lu et al. 2012). Therefore, TGF-β promoting Th9 cell development could be beneficial for cytotoxic T cell responses.

TGF-β also has a role in promoting resident memory T cells (T_{RM}). These long-lived memory T cells reside in tissues to mediate robust responses against pathogens, and have also been found in the TME of several cancers. A marker for these cells is CD103, which is an integrin that promotes T cell contact with the epithelium (MacKay et al. 2013). TGF-β upregulates CD103 in T_{RM} cells (Robertson 2001). TGF-β also downregulates sphingosine-1-phosphate receptor 1 (S1P1) expression, which promotes retention of T_{RM} cells in tissues (Skon et al. 2013). In numerous cancers, the presence of CD103+ T_{RM} cells in the TME correlates with better prognosis and improved survival (J. R. Webb et al. 2014; Paul 2010; Djenidi et al. 2015). This raises the possibility that TGF-β promotes survival of T_{RM} cells in the TME, and could enhance their retention. Overall, the role of TGF-β on T cells the TME is complex, and more research is needed

to understand how to prevent the negative effects of TGF-β on T cell function while still allowing for the beneficial/homeostatic effects on certain T cell subsets.

CAR T Cells

Chimeric antigen receptor (CAR) T cells have revolutionized cancer immunotherapy over the last few decades. As described previously, some major challenges in the TME is the lack of antigen presentation of tumor antigens and the lack of co-stimulatory molecules present to activate T cells. Additionally, there can be a lack of tumor-specific T cells present in patients with a variety of tumors. CAR T cells were developed to attempt to overcome many of these barriers. CAR T cells combine the effector functional responses of T cells with the diversity of antibody recognition.

CAR T cell design. The first CAR T cell developed contained an extracellular single-chain variable fragment (scFv) linked to an intracellular signaling domain (Gross, Waks, and Eshhar 1989). This CAR contained a Fc receptor (FcR) γ chain signaling domain. Subsequent CARs contained the CD3ζ chain, which has three ITAMs compared to a single ITAM in the FcR γ chain, which can enhance signaling. Tumor antigen specificity from the extracellular domain combined with the CD3ζ intracellular signaling domain constitutes a first-generation CAR (Figure 8). Second generation CARs were developed that add a costimulatory domain, such as CD28 or 4-1BB. This allows the CAR T cells to receive signal 1 from antigen binding, and signal 2, through co-stimulation. The addition of a costimulatory domain showed enhanced cytokine secretion, tumor killing, and reduced apoptosis (Savoldo et al. 2011; D. W. Lee et al. 2015).
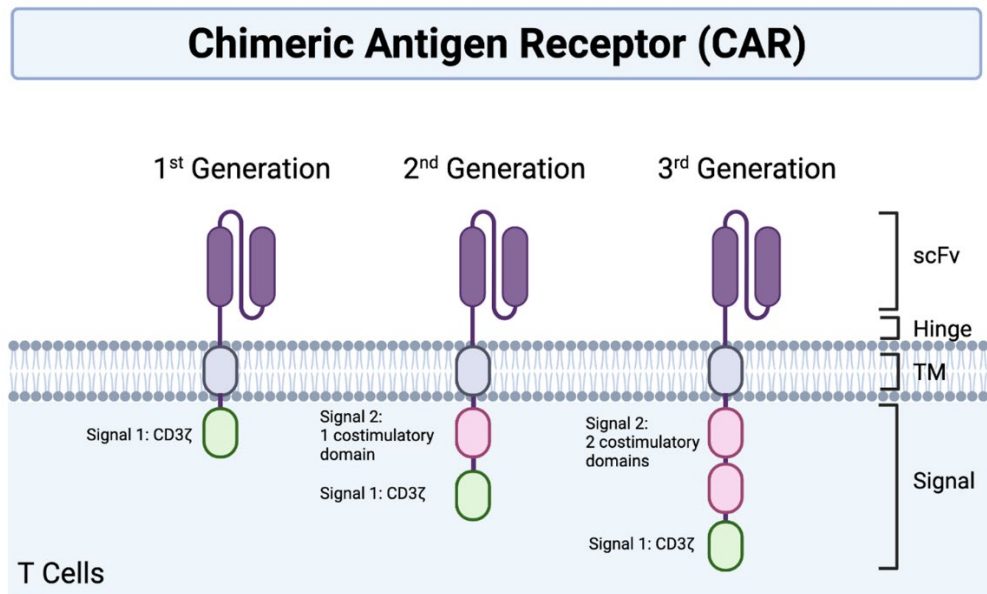
Figure 8. CAR T Cell Design. CARs contain a scFv that recognize tumor antigens, followed by a hinge and transmembrane (TM) domain. The 1st generation contains an intracellular CD3ζ signaling domain. The 2nd generation adds one costimulatory domain such as CD28. The 3rd generation has two costimulatory domains, such as CD28 and 4-1BB.

There has been much interest in enhancing CAR T cell effector functions, survival, and persistence, including the creation of third generation CARs that have two co-stimulatory domains, and armored CARs that can produce cytokines such as IL-12 and IL-15. These CARs have also shown enhanced tumor killing and functionality compared to 1st and 2nd generation CARs (Carpenito et al. 2009; Zhong et al. 2010; Brentjens et al. 2003). Many clinical trials and research are ongoing to determine the optimal construction of the CARs, which will also depend on the specific tumor type.

CAR T cell therapy. For treatment with CAR T cells, the patient's own T cells are activated and expanded *ex vivo*, and commonly transduced with retroviral vectors containing the CAR genes and a marker for transduction. These transduced T cells can be purified by sorting for the marker that is only expressed in transduced cells, then

infused back into the patient. These CAR T cells now have redirected specificity for the

tumor antigen of interest (Figure 9).
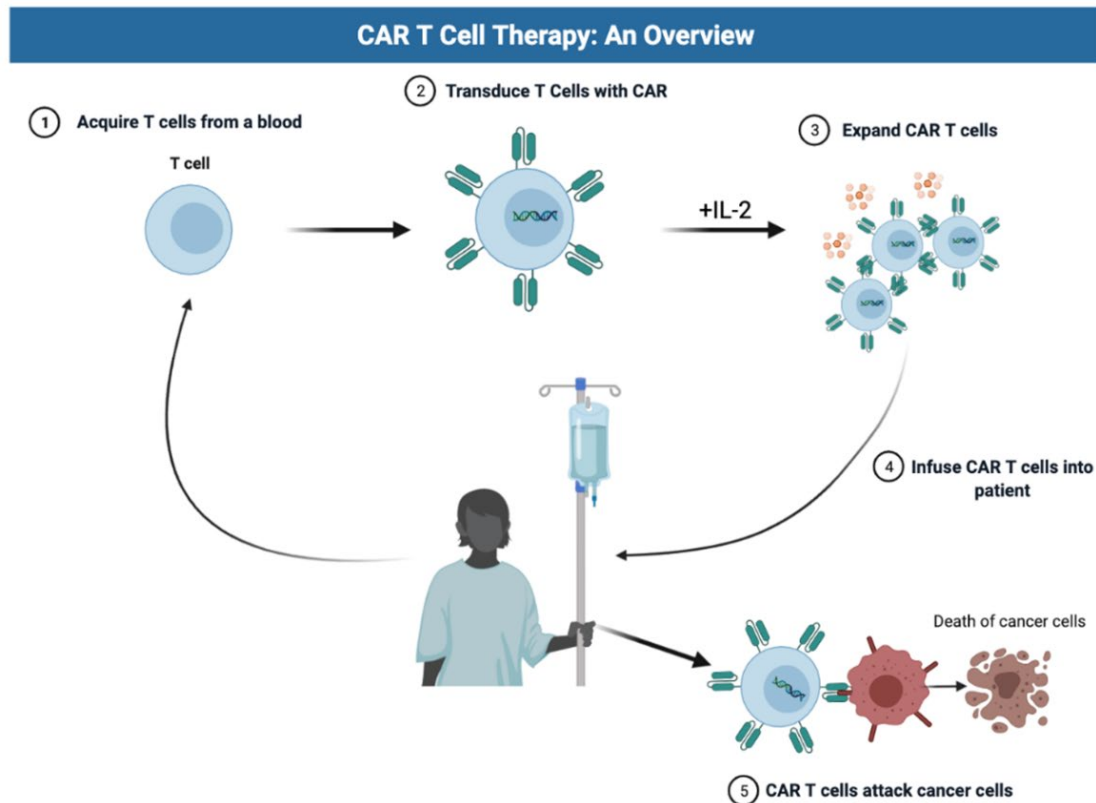
Figure 9. CAR T Cell Therapy. PBMCs are first removed from the patient, and T cells are activated *in vitro*. Activated and expanded T cells are transduced with CAR T cell vectors, then selected for by a marker gene (such as CD34). T cells are further expanded with IL-2 and infused back into the patient. These CAR T cells now have redirected specificity for the tumor antigen of interest.

CD19 CAR T cells are one of the most widely studied and have shown the most

promise clinically. One of the first trials at Memorial Sloan Kettering Cancer

Center in New York used 2nd generation CAR T cells (CD3ζ/CD28) to treat B-cell acute

lymphoblastic leukemia. An overall complete response rate of 91% was found for 32

patients (J. H. Park et al. 2015). Another clinical trial at UPenn treated pediatric and

adult patients with 2nd generation CD3ζ/4-1BB CD19 CARs, where a 90% response rate

was observed (Maude et al. 2014). Several other trials also saw promising results with the CD19 CARs. Common toxicities that were observed were cytokine release syndrome (CRS) and some neurological toxicities. Because of its success, the FDA approved CARs as a treatment for large B-cell lymphoma in 2017. Overall, the use CAR T cells, especially for treatment of B-cell malignancies, has been a significant breakthrough in the field of immunotherapy.

Challenges of CAR T cell therapy. While CAR T cell therapy has shown great promise, especially for B cell malignancies, treatment for solid tumors remains a challenge. One example is in glioblastoma, where only a minority of patients showed any objective clinical responses after CAR therapy (Brown et al. 2016; Louis et al. 2011). This lack of clinical efficacy in solid tumors has been attributed to a number of factors, including: (i) the immunosuppressive environment in the tumor, including regulatory T cells, that can shut down the killing activity of the CAR T cells, (ii) T cell exhaustion due to excessive activation and upregulation of checkpoint molecules, (iii) survival in the tumor microenvironment and activation induced cell death of CAR T cells.

Because of these barriers, different strategies have been implemented to engineer CARs that overcome resistance, as well as combination therapies that enhance CAR fitness. Like other tumor-specific T cells, CAR T cells can express checkpoint molecules such as PD-1 and CTLA-4 that can bind ligands on tumor cells and suppress their functions (Oh et al. 2020). Combination therapies with checkpoint inhibitors and CAR T cell treatment are currently under investigation (Chong et al. 2017; Heczey et al. 2017). Another strategy has been to engineer CAR T cells themselves to secrete molecules that block the interactions of inhibitory receptors and their ligands

such as PD-1 blocking antibodies or anti-PDL-1 antibodies (Suarez et al. 2016; Zhou et al. 2020). Overall, CAR T cells can be powerful tools for redirecting T cells to tumor targets, but more research will need to uncover the optimal constructs and treatments for different types of tumors.

Activation-induced cell death in CAR T cells. As described above, adoptive transfer of CAR T cells has shown great success for hematologic cancers such as B cell lymphomas. For those patients that respond, successful tumor elimination is correlated with the survival of CAR T cells post-transfusion (Kershaw et al. 2006; Jensen et al. 2010). Although CAR T cells have been successful clinically, one major drawback for many types of cancers is the lack of CAR T cell persistence *in vivo.* This is especially true for solid tumors (D'Aloia et al. 2018). After infusion, CAR T cells must traffic to the tumor and survive in a highly immunosuppressive environment. Both *in vitro* and *in vivo,* CAR T cells have been shown to undergo activation-induced cell death (AICD), where repeated stimulation of the CARs results in apoptosis (Gargett et al. 2016; Wang et al. 2021). Similar to AICD in conventional T cells, Fas/FasL causes AICD in CAR T cells (Künkele et al. 2015). CD28-based CAR T cells have been specifically shown to undergo apoptosis when repeatedly activated by antigen (Gargett et al. 2016; Guedan et al. 2018; Künkele et al. 2015). Changing the cytoplasmic region of CD28 to other co-stimulatory molecules (such as 4-1BB) made the CAR T cells more resistant to AICD (Guedan et al. 2018; Long et al. 2015). Philipson et al found that 4-1BB co-stimulation enhanced NF-κB signaling, which reduced expression of the pro-apoptotic protein Bim (Philipson et al. 2020). The data suggest that CD28-mediated co-stimulation in CAR T cells may promote apoptosis more than other co-stimulators.

Cytokine treatment of CARs also affects their survival and resistance to AICD *in vivo*. Because of their role in T cell survival, proliferation and function, IL-2, IL-15 and IL-7 are common cytokines used to expand CAR T cells *in vitro.* IL-7 promotes homeostasis and survival of naïve and memory T cells through the upregulation of Bcl-2 and repression of pro-apoptotic proteins (Q. Jiang et al. 2004). IL-15 signaling is critical for the development and homeostasis of CD8 T cells, and inhibits AICD through the upregulation of Bcl-2 and Mcl1, two anti-apoptotic proteins (Sandau et al. 2010). Culturing CAR T cells with IL-7 and IL-15 was shown to improve *in vivo* persistence of CARs (Ghassemi et al. 2016). IL-15 and IL-7 reduces the differentiation of central memory into effector memory, which preserves T cells in early differentiation states. Another study showed that culturing CAR T cells with IL-15 and IL-7 increases CD19 CAR T cells with a memory stem cell phenotype (Xu et al. 2014). These memory stem cell CAR T cells are able to resist AICD after repeated encounters with antigen and migrate to secondary lymphoid organs. These data suggest that IL-7 and IL-15 together can enhance CAR survival and resistance to AICD *in vivo.* Altogether, the design and cytokine signaling in CARs can greatly influences survival and functionality.

Concluding Remarks

T cells are critical members of the adaptive immune system, with diverse subsets and functions. T cells control tolerance to self-antigens and commensals, mount inflammatory responses to pathogens, immunological memory, and are critical for anti-tumor immunity. To perform these diverse functions, T cells have countless signals that control their differentiation, function, survival, and death. Although there is a great deal of literature on T cell functionality, much is left to uncover on the intricacies of the T cell

lifecycle, especially signaling mechanisms that activation-induced cell death of T cells. This is critical for tumor-specific T cells and CAR T cells, as their survival can be especially difficult in the tumor microenvironment. Continued research into the signaling mechanisms that control the survival and death of T cells and the complex role of TGF-β signaling will undoubtedly uncover novel pathways that can be therapeutically targeted in many disease states, including cancer, autoimmunity, and improving immunological memory.

CHAPTER TWO

MATERIALS AND EXPERIMENTAL METHODS

Mice

C57BL/6, CD4-CRE, and FoxO3$^{fl/fl}$ mice were purchased from Jackson

Laboratory. *Rasgrp1*$^{-/-}$ mice were kindly gifted by Dr. JC Stone. FoxO3$^{fl/fl}$ mice contain

loxP sites flanking exon 2 (the first coding exon) of *FoxO3* gene. FoxO3$^{fl/fl}$ mice from

Jackson are FVB/NJ strain. Because our *in vitro* activation-induced cell death assays

(PICA) were performed in C57BL/6 mice, we generated CD4-CRE FoxO3$^{fl/fl}$ mice in the

C57BL/6 background. FoxO3$^{fl/fl}$ mice were first backcrossed to C57BL/6 mice for 8

generations. Then, FoxO3$^{fl/+}$ mice (C57BL/6 background) were crossed with CD4-CRE

mice (C57BL/6 background) to generate CD4-CRE FoxO3$^{fl/+}$ mice. Subsequent

breeding was performed to generate CD4-CRE FoxO3$^{fl/fl}$ and littermate WT controls. All

mice were maintained under specific pathogen-free conditions. All procedures were

approved and monitored by the Institutional Animal Care and Use Committee of Loyola

University Chicago.

Culture Conditions

Primary mouse and human cells were maintained in RPMI 1640 (GE Healthcare)

supplemented with 10% fetal calf serum (FCS, Gemini Bioproducts), L-glutamine

(Hyclone), a 10% solution of penicillin/streptomycin (Hyclone), 1mM sodium pyruvate

(Corning), 0.1 M hepes (Corning), non-essential amino acids (Gibco), essential amino

acids (Corning), and 50 µM 2-ME (Fisher Scientific). Cells were maintained at 37°C with 5% CO_2.

Constructs

SAMEN retroviral vector and CAR construct: The SAMEN retroviral vector (including the CAR construct) was a kind gift from the Nishimura lab, and is modified from a MMLV-based splicing vector constructed in the Surgery Branch, NCI. The EMCV/IRES cassette was removed and replaced with a modified CD34 cassette which lacks it cytoplasmic domain, so it is incapable of signaling. The anti-CD19 CAR was cloned into the SAMEN retroviral vector and has been sequenced on multiple occasions.

Cell Lines

PG13 retroviral packaging cell line: The PG13 retroviral packaging line was purchased from ATCC. This cell line has been used repeatedly to make retrovirus for our transduction experiments. Cells were maintained in Iscove's media with 10% FCS and a 10% solution of penicillin/streptomycin (Hyclone).

Raji cell line: The Raji cell line is a human B cell line that was originally derived from a patient with Burkitt's lymphoma and was purchased from ATCC. This cell line has been used extensively in B cell lymphoma research and CD19 CAR research in the field. Cells were maintained in RPMI 1640 medium with 5% FCS and a 10% solution of penicillin/streptomycin (Hyclone).

Jurkat cell line: Jurkat T cells were a gift from Dr. Arthur Weiss (UCSF, San Francisco, CA). Cells were maintained in RPMI 1640 medium with 5% FCS and a 10% solution of penicillin/streptomycin.

Human PBMCs

All PBMCs used were de-identified apheresis products purchased from Key

Biologics (Memphis, TN) or Zen Bio (Research Triangle Park, NC). PBMCs were from

healthy donors. Mononuclear cells were enriched by density centrifugation using

Lymphopure density gradient medium (Biolegend, San Diego, CA) and red blood cells

were lysed with RBC lysis buffer (Biolegend, San Diego, CA).

Cell Culture and T Cell Activation

For CD4 and Treg isolation, splenocytes were labeled with fluorochrome

conjugated anti-CD4 and anti-CD25 antibodies, followed by sorting of

CD4$^+$CD25$^+$ tTregs and CD4$^+$ CD25$^-$ conventional T cells using a FACSAria (BD

Biosciences). For CD8 T cell isolation, total CD8 splenic T cells were isolated using

negative selection with magnetic sorting (Mojosort from Biolegend). For PICA

stimulation, tTregs and conventional T cells (1×10^5-3×10^5) were stimulated with anti-

CD3 and anti-CD28 (5 µg/mL each, leaf-grade, Biolegend) coated 60 mm plates in the

presence IL-2 (10 ng/mL, Peprotech). For TGF-β treated conditions, TGF-β was added

at 2.5 ng/mL on day 0 (Peprotech).

For the culture of conventional T cells for 7 days (Figure 11B), CD4$^+$25$^-$ T cells

were stimulated with plate-bound anti-CD3 (5 µg/mL) and soluble anti-CD28 antibodies

(1 µg/mL) +/- TGF-β (2.5 ng/mL,) and IL-2. After 3 days of stimulation, cells were

harvested and removed from stimulation and were further cultured in the presence of

TGF-β and IL-2.

For the tTreg culture with the TGF-β signaling inhibitor (Figure 13), tTregs were

expanded with anti-CD3/anti-CD28 antibody coated beads (4.5 µM polystyrene beads,

Polysciences, Inc) for 7 days in the presence of IL-2. After 7 days, tTregs were restimulated with immobilized anti-CD3/anti-CD28 antibodies (5 µg/mL each) coated on culture dishes with DMSO control or 10 µM SB-431542 (Sigma) in the presence of IL-2. For re-stimulation to assess TCR signaling events, expanded cells were stimulated with biotin-conjugated anti-CD3 Ab (5 µg/mL cross-linked by streptavidin (5 µg/mL) for indicated time points.

For re-stimulation to assess TCR signaling events (Figure 10), expanded conventional and Treg cells were stimulated with biotin- conjugated anti-CD3 Ab (5 µg/mL, eBiosciences, San Diego, CA USA) cross-linked by streptavidin (5 µg/mL, EMD Chemicals, Rockland, MA, USA) for time points indicated.

Isolation of Small Intestine IEL and LPL

Intraepithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL) were isolated from the mouse small intestine. Briefly, the small intestine was removed, and fecal matter and fat were removed. The intestinal tissues were opened longitudinally and cut into 1cm pieces. The tissues were incubated in calcium- and magnesium-free 10% HBSS supplemented with fetal calf serum, 10mM HEPES and 1mM Dithiothreitol (DTT) rocking for 30 minutes in 37˚C. The tissues were then vortexed vigorously and intestinal IEL were obtained by filtration of the supernatant through a 100µm filter. To isolate LPL, the remaining tissues were further cut into smaller pieces, and incubated in 10% HBSS supplemented with 0.1mg/ml DNAseI, 2mg/ml Collagenase D and 0.17U/ml Dispase, rocking for 30 minutes in 37˚C. To stop the digest, 10% HBSS supplemented with 5mM EDTA was added. LPL cells were then obtained by filtration of the supernatant through 100µm filter. Purified IEL were obtained by harvesting the cell

interphase of 40/70% Percoll centrifugation and LPL were obtained from 40/80% Percoll centrifugation.

Generating High Titer CAR PG13 Cell Line

Using a HEK293GP packaging cell line, retroviral supernatants were prepared and used to make a stable retroviral producer PG13 cell line expressing the CD19-specific CAR in the SAMEN vector. On day 0, 3 million HEK293GPs were plated in 10 cm plates and incubated overnight. HEK293GP cells were transiently co-transfected with 20 µg retroviral SAMEN vector DNA and 5 µg of a plasmid containing the VSV envelope gene using 50 µL Lipofectamine 2000 (Invitrogen) on day 1. Transfection medium was replaced 6 hours later with 10 mL fresh complete medium and incubated for 48 hours. On day 2, PG13 cells were seeded at 2 million in a 10 cm tissue culture plate. On day 3, fresh HEK293GP viral supernatant was collected and filtered using a 0.45 µm filter. PG13 media was replaced with 9 mLs of filtered HEK293GP viral supernatant and 3 mL complete medium. On day 6, PG13 cells were collected and stained using anti-CD34 mAb (BioLegend) and analyzed for CD34 expression by flow cytometry. CD34 high cells were sorted using a BD FACSAria cell sorter (BD BioSciences, San Jose, CA) and the final PG13 cells were maintained in complete medium.

Generating CAR T Cells

T cells from healthy donors were activated with 5 ug/mL plate-bound anti-CD3 and anti-CD28 (Biolegend, leaf-grade) in the presence of IL-2 (10 ng/mL, Peprotech). To make high titer retroviral supernatant, PG13 cell lines were seeded overnight at 8 million cells/T-175 cell culture flask and incubated. On day 2, 25 mLs of complete

Iscove's DMEM supplemented with 0.5 mLs (1 mM) sodium butyrate (Sigma-Aldrich) and 1 mL (10 mM) HEPES (Sigma-Aldrich) was added to flasks for 8-10 hours to stimulate virus production. Media was then replaced with fresh complete media and incubated overnight. Viral supernatants were collected on day 3 and filtered to remove any cellular debris using 0.45 μm filters.

Activated T cells were transduced by spinoculation on day 3. Cells were mixed with virus supernatant (0.5 million cells/mL) with 8 ug/ml Polybrene and placed into 24 well plates. The plates were spun for 2 hours at 2,000xg at 32°C and then incubated for 6 hours. Viral media was replaced with fresh media containing IL-2, and T cells were cultured in flasks for 2 days. 2 days after transduction, transduction efficiency was determined by FACS analysis using anti-CD34 mAb (Biolegend). TCR transduced T cells were purified by positive selection using CD34 immunomagnetic beads (Miltenyi Biotec, San Diego, CA) and maintained in complete media with IL-2.

CAR T Cell Culture Transwell Assay

CAR T cells were washed and placed in complete RPMI media (10% FCS) with no cytokines added. Raji or Jurkat cells were irradiated (50 grays) and placed in complete media. CAR T cells and Rajis were placed together in the top of a transwell (Corning). The transwell contained 200 ul of CARs and Rajis in the 0.33 cm^2 culture area. The pore size was 0.4 μM, so that cells could not travel to the bottom of the transwell. The media at the bottom of the transwell was replaced everyday with complete media, leaving the CAR T cells and Rajis undisturbed in the top of the transwell. Where indicated, TGF-β (10ng/uL, Peprotech) or Pan Caspase Inhibitor Z-VAD-FMK 5μM (R&D Systems) was added on Days 1 and 3, during the media change.

After 5 days, CAR T cells were counted via tryphan blue exclusion, then cells were stained for flow cytometry analysis. For restimulation, dead Rajis were removed via ficoll gradient and live CAR T cells were stimulated with Rajis at a 1:1 ratio in u-bottom 96 well plates overnight. Cytokines were collected in the supernatants prior to staining.

Flow Cytometry

All fluorochrome-conjugated antibodies used were from Biolegend, unless otherwise indicated. For the surface staining, cells were first treated with anti-CD16/32 Fc block followed by staining with surface antibodies on ice for 30 minutes in the dark. To assess psmad2/3 (BD Biosciences), cells were fixed using fix/perm reagents from BD Biosciences, according to the manufacturer's instructions. For transcription factor staining, the true nuclear kit from Biolegend was used, according to the recommended protocol. Cells were stained for Foxp3 and Helios using Biolegend's recommended protocol. Cells were analyzed on a BD FACSCanto II or LSRFortessa flow cytometer. Cell sorting was performed on a FACSAria cell sorter (BD Biosciences). Analyses were performed with FlowJo software (Tree Star, Ashland, OR). Cells were gated on live cells (exclusion of Zombie Aqua dye, Biolegend) followed by gating on single cells. Subsequent gating was performed as described in each figure.

Apoptosis Analysis

For analyzing apoptosis, mouse T cells were collected from PICA plates, washed in PBS, then subjected to staining. Surface markers were stained first, followed by Annexin V and 7AAD staining (1 ul per 0.5 million cells) in Annexin Binding Buffer at room temperature for 15 minutes (10X solution contains 0.1M Hepes (pH 7.4), 1.4M NaCl, and 25 mM CaCl2, which was diluted to 1X prior to use). Cells were immediately

analyzed after incubation by flow cytometry, without washing. In addition to 7AAD/Annexin V analysis by flow cytometry, cells were counted prior to staining using tryphan blue exclusion.

Western Blot Analysis

At indicated time points, cells were collected and whole cell lysates were prepared at a concentration of 1 x 10^6 cells/50uL in sodium dodecyl sulfate (SDS) lysis buffer (2% SDS, 125 mM DTT, 10% glycerol, 62.5 mM Tris-HCL (pH 6.8). Equivalent amounts of proteins adjusted by cell number were loaded in each lane and separated on SDS- PAGE gels. Proteins transferred on PVDF membranes were probed with the following primary antibodies: antibodies specific for phospho-Erk (Thr 202/Tyr204), phospho-Mek1/2 (Ser 217/221), phosphor-cRaf (Ser 259), phospho-smad 2/3 (Ser 423/425) and Mek were from Cell Signaling Technology. Antibodies specific for RasGRP1 and Sos1 were from Santa Cruz Biotechnology (Dallas, TX, USA). The anti-Ras antibody was from BD Biosciences, the anti-Erk Ab was from Millipore (Billerica, MA, USA), the anti-Foxp3 Ab was from eBiosciences, and the anti-β actin antibody was from Sigma (St. Louis, MO, USA). Enhanced Chemiluminescence (ECL) substrates were used to detect proteins of interest (GE Healthcare). The relative intensity of each band (number indicated under each lane) was normalized to β-actin as the loading control, as determined by ImageJ software (National Institutes of Health).

Statistical Analysis

Statistical significance was determined using a Student's t test (unpaired or paired), as indicated. These analyses were performed using the GraphPad Prism software (GraphPad Software; La Jolla, CA). A p value <0.05 was considered

statistically significant with p<0.001 denoted as ***, p = 0.001 to 0.01 denoted as **, and

p = 0.01 to 0.05 denoted as*. Not significant denoted as ns (p > 0.05).

CHAPTER THREE

EXPERIMENTAL RESULTS

Section 1: Identifying Signaling Differences Between Conventional and

Regulatory T Cells and the Role of TGF-β

Tregs and Conventional T Cells Differ in MAPK Pathway Signaling and RasGRP1

Expression

Activation-induced cell death (AICD) occurs after expansion of antigen-stimulated

T cells to reduce the number of these activated T cells (Smith et al. 1989). Previously,

we have established an *in vitro* system to study AICD by stimulating T cells with anti-

CD3 and CD28 antibodies coated on flat tissue culture plates. Under these conditions, a

majority of conventional T cells undergo apoptosis in a p53- dependent manner, while

Foxp3$^+$ tTregs are resistant to apoptosis and expand over 7000-fold within 10 days

(Singh et al. 2010). Since classical AICD is p53 independent (Boehme and Lenardo

1996), we concluded that plate-bound anti-CD3/anti-CD28 antibodies induce a distinct

mechanism of AICD and named the process p53-induced CD28-dependent T-cell

apoptosis (PICA). Further analysis revealed that Foxp3$^+$ Tregs require autocrine TGF-β

to resist PICA (Takami, Love, and Iwashima 2012). Conversely, addition of exogenous

TGF-β renders conventional T cells resistant to PICA. These data suggest that PICA

may play a role in immune regulation by controlling the balance between tTregs and

conventional T cells.

CD4$^+$CD25$^+$ Tregs expand and grow robustly with plate-bound antibody stimulation (PICA conditions), whereas conventional T cells CD4$^+$25$^-$ undergo apoptosis. Therefore, we hypothesized that there are TCR-associated signaling differences between Tregs and conventional T cells that allow for Tregs to resist PICA. To test this hypothesis, we analyzed known signaling molecules that function downstream of TCR stimulation and compared them between mouse splenic CD4$^+$CD25$^+$ Tregs and conventional T cells (Figure 10). We found differences in the Ras/ERK signaling pathway when comparing stimulated Tregs and conventional T cells. Conventional T cells showed upregulation of phosphorylated ERK (indicative of ERK activation) 15–30 min after TCR stimulation. Tregs showed little, if any, upregulation of ERK phosphorylation (Figure 10A). Compared to conventional T cells, Tregs also showed a substantial decrease in phosphorylation of two molecules upstream of ERK, MEK and c-Raf. These data suggest that the ERK/Ras signaling pathway is closely coupled to TCR stimulation in conventional T cells, but not in Tregs. In T cells, activation of the MAPK/ERK pathway is dependent on two Ras activators, RasGRP1 and SOS-1/SOS-2. Since ERK activation was significantly lower in Tregs compared to conventional T cells, we expected lower levels of RasGRP1 and SOS-1 expression in Tregs compared to conventional T cells (Figures 10B and C). We found that Tregs expressed lower levels of RasGRP1 protein and mRNA compared to conventional T cells. Expression levels of Ras were comparable between conventional T cells and Tregs. SOS-1 expression by Tregs was slightly lower than conventional T cells. Together, the data suggest that Tregs exhibit reduced ERK signaling, in part due to lower levels of RasGRP1 expression.
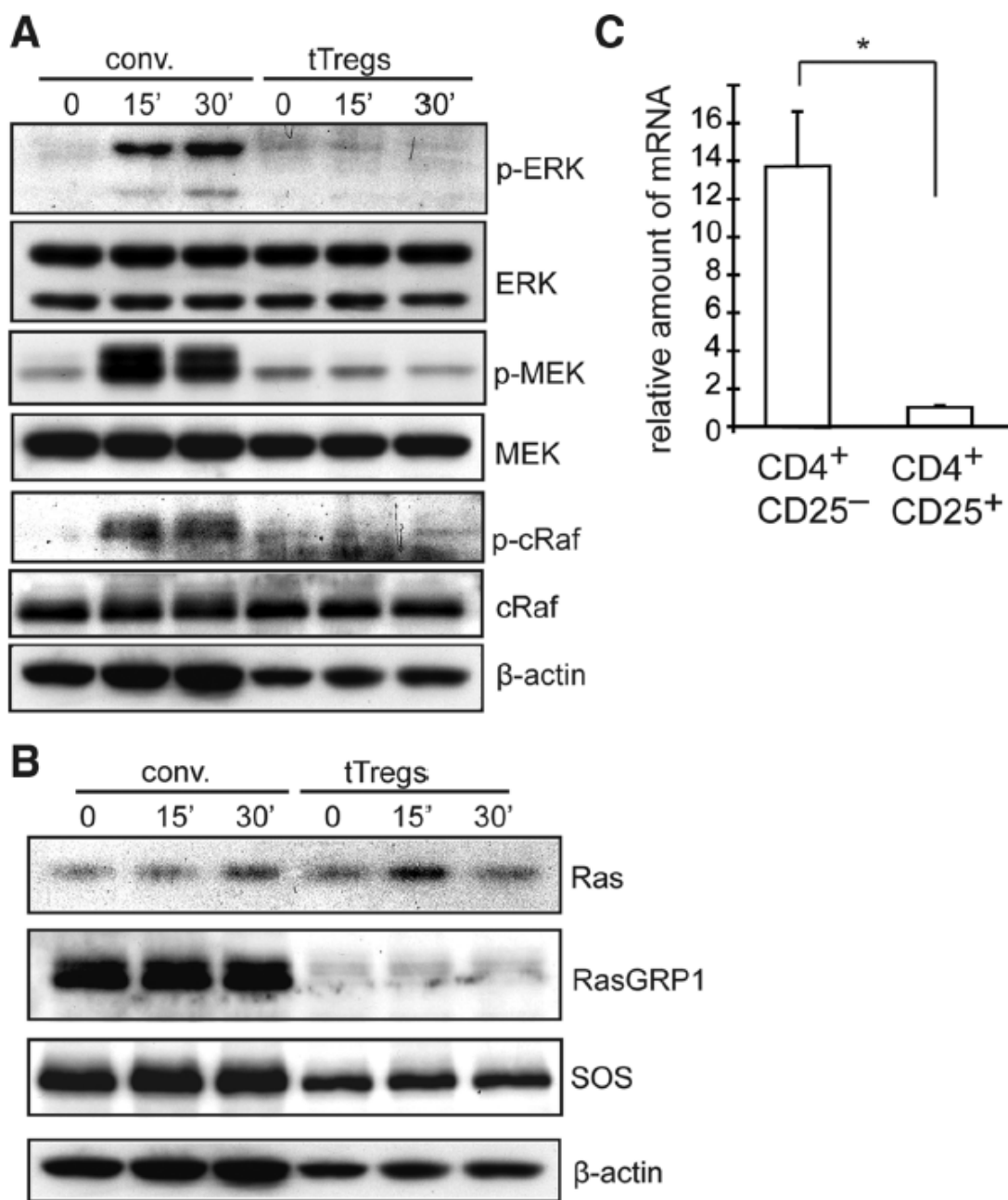
Figure 10. Expression and Activation of ERK Signaling Molecules in Conventional T Cells and tTregs. *Ex vivo* expanded CD4$^+$CD25$^+$ tTregs and conventional (conv) CD4$^+$ T cells (CD4$^+$CD25$^-$ cells) isolated from C57BL/6 spleens were stimulated with biotin-conjugated anti-CD3 crosslinked with avidin for 0, 15, or 30 min. **(A, B)** tTregs and conv cells were analyzed by western blot analysis. Blots are representative of three independent experiments. **(C)** Real-time PCR assays for expression of *Rasgrp1* mRNA by conventional CD4$^+$ T cells and tTregs. The relative amount of mRNA was determined using *gapdh* gene expression as a reference, *p<0.005 Student's *t*-test. The data are representative of three independent experiments. Experiments performed in conjunction with Dr. Mariko Takami (Takami and Cunha et al. 2018).

We sought to determine the mechanism by which tTregs express lower levels of RasGRP1. We first determined the kinetics of RasGRP1 expression in conventional T cells and Tregs in unstimulated cells and after 7 days of *in vitro* stimulation (Figure 11A). The expression of RasGRP1 did not significantly differ between freshly isolated conventional and regulatory T cells. However, after *in vitro* stimulation for 7 days, expression of RasGRP1 substantially increased in conventional T cells, but not dramatically in Tregs (Figure 11A). This demonstrates that Tregs and conventional T cells have mechanisms downstream of TCR activation that differentially controls the expression of RasGRP. The data suggest that Tregs produce an inhibitory molecule(s) for RasGRP1 expression and/or conventional T cells produce RasGRP1 promoting factors during expansion.

TGF-β Suppresses RasGRP1 in Conventional T Cells

Our previous work showed that Tregs require TGF-β signaling to resist PICA, and that exogenous TGF-β confers PICA resistance to conventional T cells (Takami, Love, and Iwashima 2012). Tregs express active TGF-β and its receptors (Liu et al. 2008; Chen et al. 2003). Conventional T cells also express TGF-βRI and TGF-βRII, but their expression of active TGF-β is limited due to a lack of TGF-β activation machinery (Tu et al. 2018). Based on these differences between conventional and regulatory T cells, we hypothesized that TGF-β controls RasGRP1 expression. If so, then conventional T cells upregulate RasGRP after activation due to a lack of TGF-β production, while production of TGF-β by Tregs suppresses RasGRP1 expression. Our hypothesis predicted that addition of TGF-β to activated conventional T cells would reduce expression of RasGRP1. When we stimulated conventional T cells, RasGRP1

expression substantially increased after 3 days, and this level of expression was maintained over 7 days (Figure 11B). In contrast, addition of exogenous TGF-β to conventional T cells resulted in little, if any, increase in RasGRP1 expression. These data show that TGF-β inhibits RasGRP1 expression in activated conventional T cells. TGF-β also increased Foxp3 in conventional T cells, which is a canonical transcription factor downstream of TGF-β signaling.
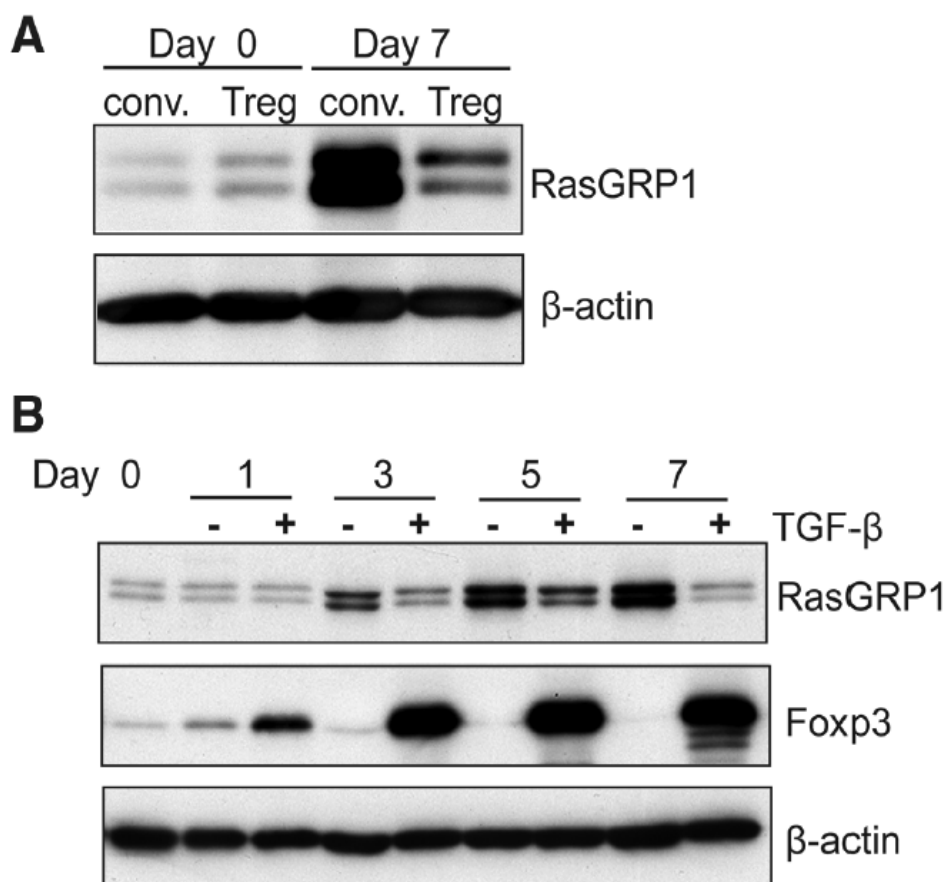
Figure 11. Regulation of RasGRP1 Expression by TGF-β. (A) Tregs (4^+25^+) and conventional T cells (4^+25^-) were isolated from spleens of C57BL/6 mice and stimulated with anti-CD3/28 coated beads for 7 days in the presence of IL-2. Cells harvested at day 0 and day 7 were lysed in SDS sample buffer for western blot analysis **(B)** Conventional T cells were stimulated with plate-bound anti-CD3 and soluble anti-CD28 antibodies in the presence or absence of TGF-β supplemented with IL-2. After 3 days of stimulation, cells were harvested to remove stimulation and were further cultured in the presence of TGF-β and IL-2 and analyzed by western blot. Data are representative of three independent experiments. Experiments performed in conjunction with Dr. Mariko Takami (Takami and Cunha et al. 2018).

Canonical TGF-β signaling induces phosphorylation of SMAD2/3, which regulates many transcriptional targets, including Foxp3. We sought to determine if TGF-β induces canonical phospho-smad signaling in conventional T cells to a similar extent as in regulatory T cells. To assess this, we sorted Tregs and Conventional T cells (Figure 12A), then stimulated for 30 min in the presence or absence of TGF-β. We found that Tregs and conventional T cells have a basal low level of pSMAD2/3 expression without stimulation, and upon stimulation with the addition of exogenous TGF-β, both Tregs and conventional T cells upregulated pSMAD2/3 expression (Figure 12B). The data suggest that signaling processes downstream of SMAD phosphorylation and/or non-canonical TGF-β signaling are involved in the regulation of RasGRP1 expression in both conventional and regulatory T cells.
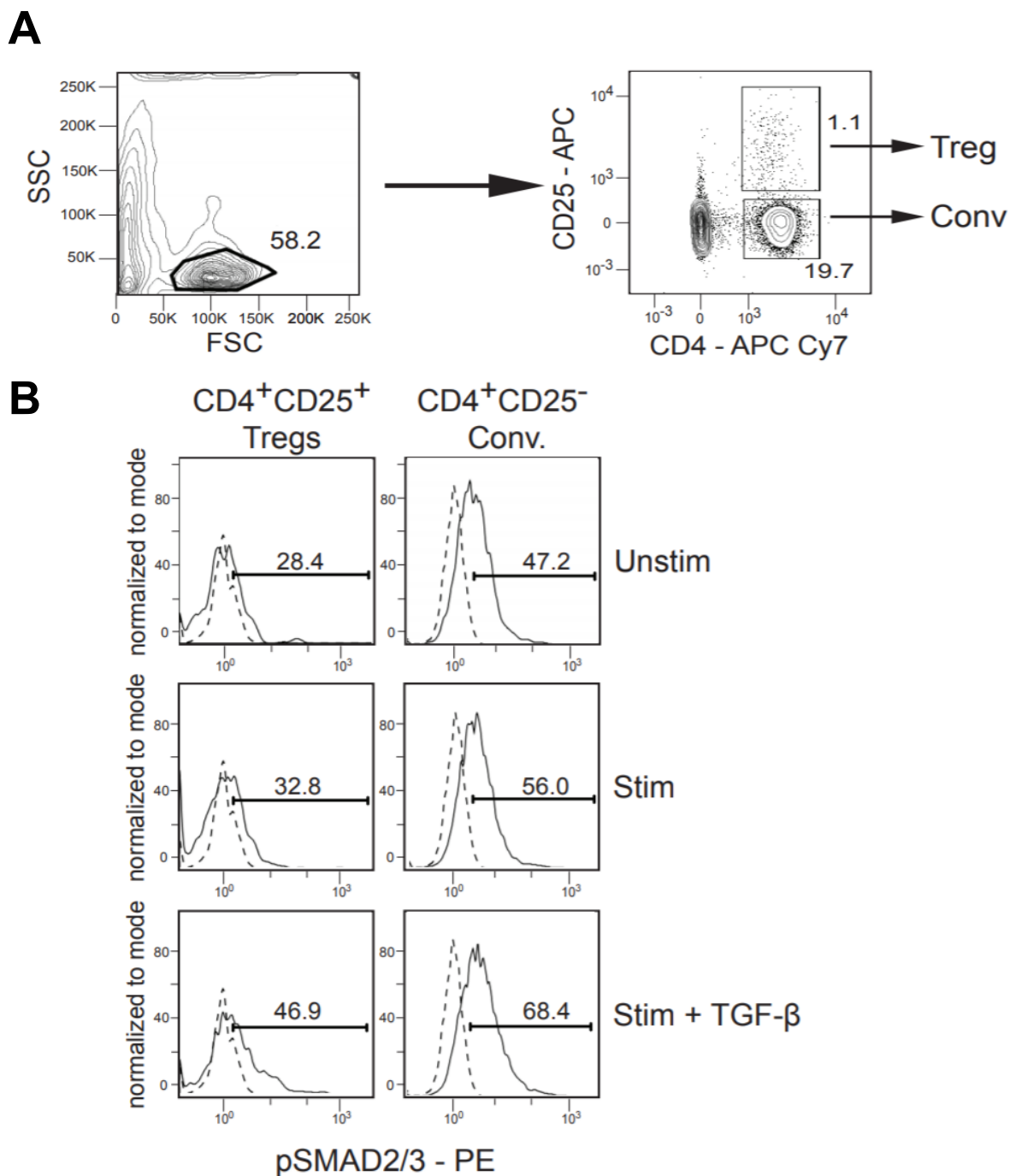
Figure 12. Expression of pSMAD2/3 in TGF-β Treated Tregs and Conventional T cells.
Splenocytes from C57BL/6 mice were left unstimulated or stimulated with anti-CD3 and anti-CD28 coated plates in the presence or absence of TGF-β for 30 minutes **(A)** Gating strategy for Tregs and conventional T cells. **(B)** pSMAD2/3 expression (PE) in Tregs and conventional T cells (Conv.) Dashed line indicates isotype control. Representative data from 3 samples. (Takami and Cunha et al. 2018).

Tregs Utilize Autocrine TGF-β Signaling to Suppress RasGRP1

Because we saw that TGF-β reduces RasGRP1 expression in conventional T cells, we next tested if TGF-β signaling is required for low levels of RasGRP1 expression in Tregs. If autocrine TGF-β is required, then inhibition of TGF-β signaling would increase RasGRP1 expression. To test this, we re-stimulated *ex vivo* expanded CD4$^+$CD25$^+$ Tregs in the presence or absence of a TGF-β type I receptor inhibitor (SB-431542). Cells were harvested 5 days after stimulation and the level of RasGRP1 expression was determined by western blot (Figure 13A). Tregs stimulated with the TGF-β receptor signaling inhibitor showed a significant increase in RasGRP1 expression compared to cells stimulated with a DMSO control (Figure 13A and 4B), suggesting that TGF-β signaling in Tregs is required for maintaining low RasGRP1 expression after activation. Inhibition of TGF-β signaling did not significantly reduce expression of Foxp3 by tTregs (Figure 13B). The data suggest that TGF-β inhibits RasGRP1 expression in a manner independent of Foxp3 expression.
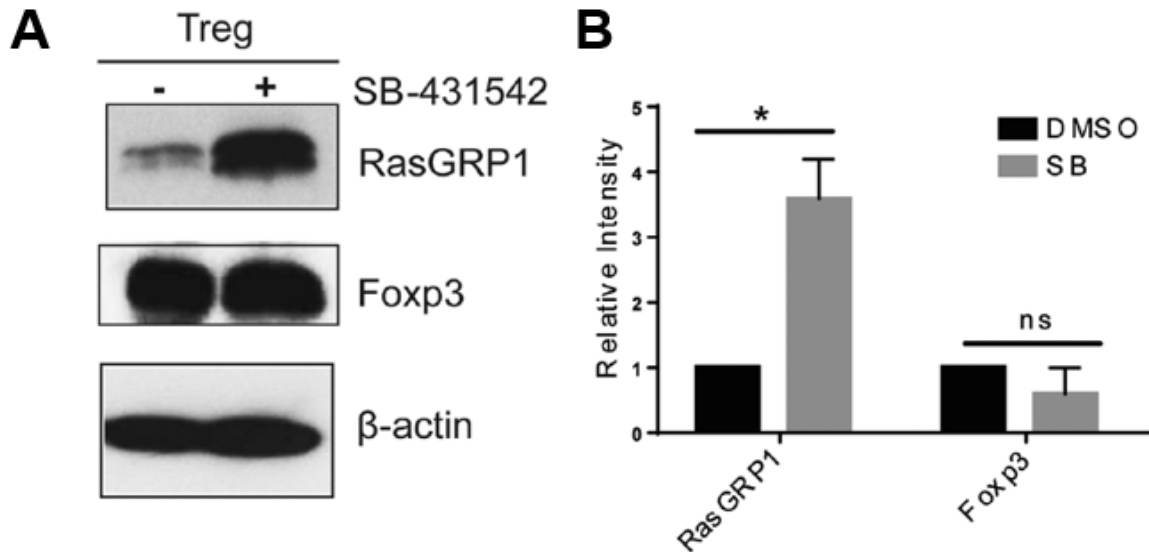
Figure 13. Effect of TGFβRI Signaling Inhibition on RasGRP1 and Foxp3 Expression in Tregs. (A) Tregs were expanded ex vivo for 7 days in the presence of IL-2. After 7 days, cells were re-stimulated on anti-CD3 and anti-CD28 coated plates in the presence of IL-2 and a TGF-β type I kinase inhibitor (SB-431542) or DMSO control. Cells were harvested 5 days after stimulation and lysed in SDS sample buffer. Representative blot from three independent experiments **(B)** Relative intensity of RasGRP1 and Foxp3 in SB-431542 treated Tregs compared to normalized DMSO control. β-actin was used as a loading control. Four spleens were pooled for each independent experiment. Data are shown as mean + SD (n=3) and are representative of three independent experiments. Student's t-test, where p = 0.01 to 0.05 is denoted as *. RasGRP1 p = 0.016; Foxp3 ns p = 0.157. (Takami and Cunha et al. 2018).

RasGRP1 is Critical for PICA Cell Death in Conventional T Cells

RasGRP1 has been shown to transduce apoptotic signals in B cells (Stang et al. 2009). Moreover, sustained ERK signaling can promote cell death (Yang et al. 2011; Martin et al. 2006). Therefore, we hypothesized that conventional T cells are susceptible to PICA because of the increase in RasGRP1 after TCR stimulation, which leads to sustained ERK activation and apoptosis. To test this, we cultured CD4 conventional T cells isolated from the spleens of RasGRP1 knockout or littermate control mice with plate-bound anti-CD3/anti-CD28 antibody stimulation. After 4 days, cells were stained with 7AAD (DNA dye) and Annexin V to detect phosphatidylserine that is only present on the external leaflet of the plasma membrane when cells undergo apoptosis. Cells

that are negative for 7AAD and Annexin V are live cells, cells that are Annexin V$^+$ and 7AAD$^-$ are starting to undergo apoptosis, and cells that are AnnexinV$^+$7AAD$^+$ are dead. If downregulation of RasGRP1 is important for survival under PICA inducing conditions, then RasGRP1 deficient conventional T cells would become resistant to PICA and have a higher frequency of live cells. As predicted, RasGRP1-deficient conventional T cells showed a greater increase in survival, with an increase in live cells after 4 days of stimulation and a decrease in the percentage of AnnexinV$^+$ and 7AAD$^+$ cells (Figure 14A and B). These cells were resistant to PICA and had increased cell counts compared to control cells that underwent apoptosis (Figure 14C). These data show that RasGRP1 expression is required for PICA in conventional T cells and suggest that reduced expression of RasGRP1 by tTregs is a mechanism by which tTregs resist PICA. Since low expression of RasGRP1 in tTregs requires TGF-β signaling, the data illustrate that TGF-β acts as a survival factor in tTregs to resist PICA.
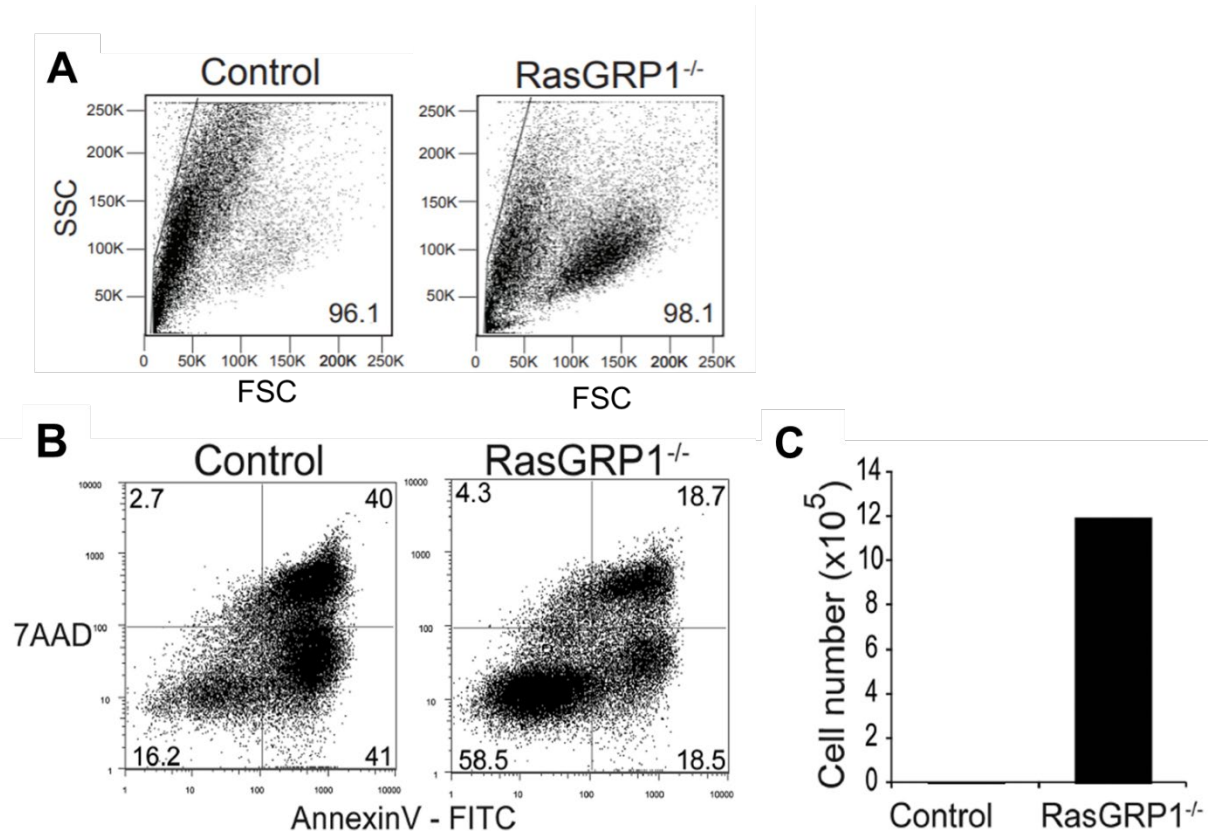
Figure 14. Comparison of Cell Survival Between RasGRP1 Sufficient and Deficient Stimulated Conventional CD4 T Cells. Conventional CD4+T cells (CD4$^+$CD25$^-$ T cells) isolated from the spleens of *RasGRP1* knockout mice (*Rasgrp1−/−*) or littermate controls, (*Rasgrp1*+/+ or +/−) were stimulated with plate-bound anti-CD3/anti-CD28 antibodies supplemented with IL-2 (PICA conditions). Cells were harvested at day 4. **(A)** Gating strategy by flow cytometry after 4 days. **(B)** Cells were stained with Annexin V (FITC) and 7AAD and analyzed by flow cytometry. **(C)** The total number of live cells was determined by Trypan blue exclusion on day 4. The data are representative of four independent experiments, where two or three spleens were pooled for each. Data collected by Dr. Mariko Takami. (Takami and Cunha et al. 2018).

Conclusions from Section 1

Tregs and conventional T cells have differences in the RasGRP1/MAPK pathway

downstream of TCR activation. The data suggest that Tregs have reduced MAPK

activation and ERK signaling compared to conventional T cells, in part due to reduced

RasGRP1 expression. Tregs utilize autocrine TGF-β signaling to suppress RasGRP1

expression, and addition of TGF-β inhibits RasGRP1 upregulation after stimulation.

RasGRP1 deficient T cells have reduced apoptosis during PICA stimulation. These

data suggest that RasGRP1 expression is repressed in Tregs by TGF-β signaling, which renders Tregs resistant to apoptosis. This also suggests that reduced RasGRP1 expression by addition of exogenous TGF-β can promote survival of conventional T cells during PICA stimulation (Figure 15).
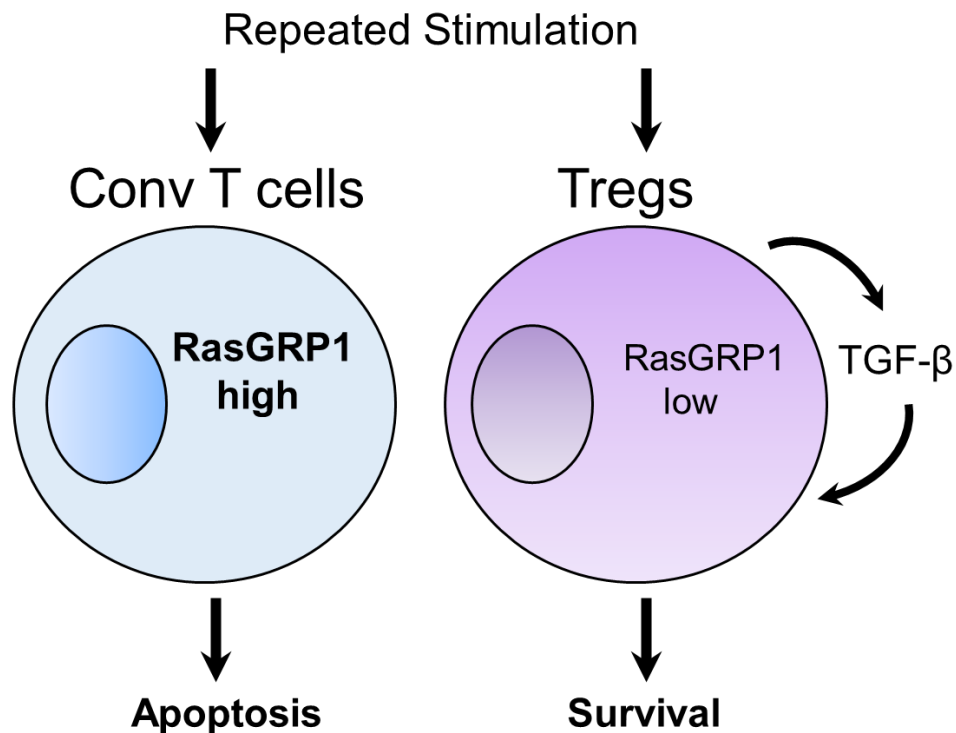
Figure 15. Model of PICA and RasGRP1 in Conv T Cells and Tregs. With repeated stimulation, conventional T cells express high levels of the RasGRP1, while Tregs maintain low levels of RasGRP1. TGF-β signaling suppresses RasGRP1 in Tregs, and addition of TGF-β to conventional T cells suppresses RasGRP1. High RasGRP1 leads to cell death in conventional T cells during PICA, while Tregs resist PICA and maintain low RasGRP1 expression.

Section 2: The Role of FoxO3 in Conventional and Treg Survival and Activation

FoxO3 is Highly Expressed in PICA Conditions and Reduced by TGF-β

The strength of TCR stimulation as well as the duration can dictate survival or death of conventional T cells. We previously established that stimulation of conventional T cells with plate-bound anti-CD3/anti-CD28 antibodies in the presence of

IL-2 (PICA conditions) causes expansion of T cells followed by massive apoptosis by day 4 after stimulation (Singh et al. 2010). However, conventional T cells that are stimulated with plate-bound anti-CD3 and <u>soluble</u> anti-CD28 antibodies proliferate, with little to no cell death. Additionally, addition of TGF-β to plate-bound stimulated conventional T cells rescues them from apoptosis (Takami, Love, and Iwashima 2012). Our previous data demonstrated that there are fundamental differences in T cell activation signaling processes when CD28 is engaged by plate-bound vs soluble form (Singh et al. 2010). PICA (plate-bound) cell death requires Bim, a known pro-apototic molecule in T cells (O'Connor et al. 1998). Bim$^{-/-}$ conventional T cells have dramatically reduced apoptosis compared to wild-type littermates (Singh et al. 2010). Additionally, TGF-β, which rescues T cells from apoptosis, also reduces Bim expression (Takami, Love, and Iwashima 2012). Therefore, conventional T cell death requires Bim, and TGF-β reduces Bim as one mechanism to prevent apoptosis.

We sought to determine signaling pathways that are distinct between stimulation conditions that promote survival (soluble anti-CD28) vs death (plate-bound anti-CD28) of conventional T cells. We examined expression of the Forkhead transcription family member FoxO3, as FoxO3 is sufficient to trigger apoptosis in T cells, and FoxO3 positively regulates transcription of Bim (Stahl et al. 2002; You et al. 2006). Since FoxO3 expression controls Bim and apoptosis, we hypothesized that plate-bound stimulation induces higher FoxO3 than soluble anti-CD28 stimulation. In resting conventional T cells (day 0), we found little to no FoxO3 expression (Figure 16). When cells were stimulated with plate-bound anti-CD3/28 stimulation, FoxO3 was substantially increased compared to no stimulation or soluble anti-CD28 stimulation. Addition of

TGF-β, which renders T cells resistant to PICA cell death, caused a marked decreased

in FoxO3 expression in plate-bound stimulation, while causing no obvious change

during soluble stimulation (Figure 16). Together, the data indicate that FoxO3 is strongly

upregulated during plate-bound PICA stimulation, and that total FoxO3 expression is

reduced by TGF-β.
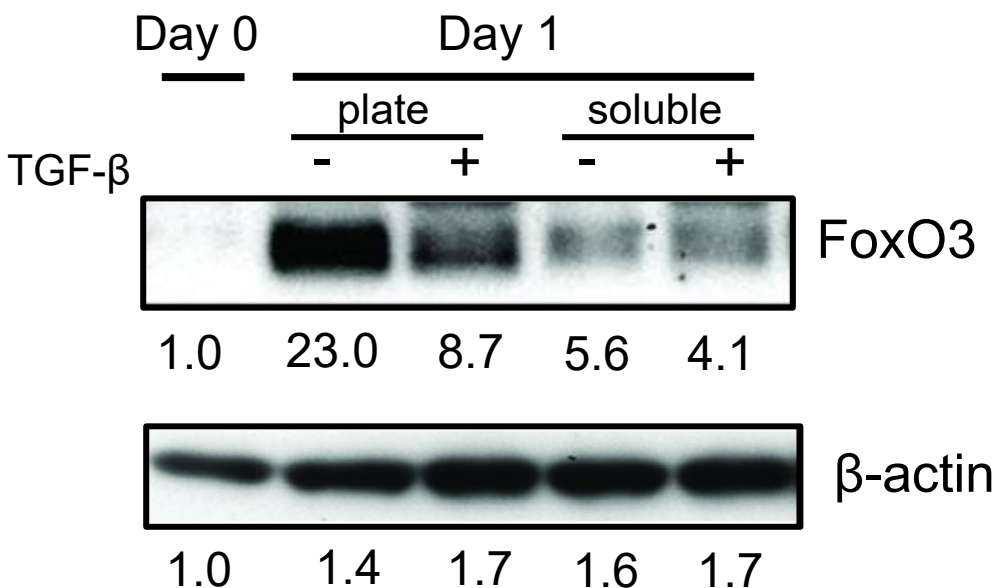
Figure 16. Effect of TGF-β on FoxO3 Expression in Stimulated Conventional T Cells.
Splenic conventional T cells (4+25-) were left unstimulated (day 0), or stimulated for 1 day with plate-bound anti-CD3 along with either plate-bound or soluble anti-CD28 antibodies, +/- TGF-β. Cells were harvested 1 day after stimulation and lysed in SDS sample buffer for western blot analysis. Relative intensity of each band (below each lane) was determined against day 0 data normalizing to β-actin (loading control). Data collected by Dr. Mariko Takami. (Takami, Love, and Iwashima 2012).

Role of FoxO3 in Conventional T Cell Survival

Because FoxO3 was strongly upregulated during PICA stimulation but not

soluble stimulation, we hypothesized that FoxO3 signaling upstream of Bim and

RasGRP leads to apoptosis. FoxO3 can upregulate transcription of Bim, and FoxO3 has

a putative binding site in the RasGRP promoter, which could point to FoxO3 directly

controlling expression of RasGRP. To test this hypothesis, we generated mice that lack

FoxO3 in T cells (CD4 CRE FoxO3 flox/flox). We sorted FoxO3$^{-/-}$ or WT splenic conventional T cells from these mice, and performed plate-bound anti-CD3/anti-CD28 PICA stimulation conditions for 2 or 4 days. If FoxO3 is required for cell death during PICA, then FoxO3$^{-/-}$ T cells should have increased survival. When we stimulated WT conventional T cells in PICA conditions, cells were activated and started to proliferate by 2 days (Figure 17A). As expected by day 4, WT T cells underwent massive apoptosis, with a large frequency staining double positive for Annexin V and 7AAD (dead cells) (Figure 17A and B). When we stimulated conventional T cells from FoxO3$^{-/-}$ mice, we observed a similar activation and cell death pattern as WT cells. Counting live cells on days 2 and 4 showed that the WT and FoxO3$^{-/-}$ T cells expanded and then underwent apoptosis in a similar manner (Figure 17C). The data suggest that FoxO3 is not required for cell death during PICA stimulation.

Figure 17. Survival of FoxO3 Deficient and WT Conventional T Cells During PICA Stimulation. Splenic conventional T cells (4+25-) from FoxO3-/- or WT littermates were stimulated for 2 or 4 days with plate-bound anti-CD3/anti-CD28 antibodies with IL-2. At indicated time points cells were harvested and counted, then analyzed by flow cytometry. **(A, B)** Cells were stained with Annexin V and 7AAD and fluorescence intensity was determined by flow cytometry **(B)** Summary of frequency of 7AAD+/Annexin V+ double positive cells **(C)** Total live cell counts were determined by trypan blue exclusion. **(B, C)** Each dot represents one mouse. Data are representative from 3 independent experiments for Day 2, and 6 independent experiments for Day 4.

FoxO3 and TGF-β Crosstalk in Survival

Since FoxO3 is not required for apoptosis, we next wondered if FoxO3 has a role in T cell survival in response to TGF-β signaling. Although not fully understood in T cells, in other cell types FoxO3 is critical for mediating TGF-β signaling. In neuroepithelial cells and ovarian cancer cells, FoxO3 physically interacts with SMAD transcription factors to control TGF-β mediated transcriptional processes (Seoane et al. 2004; Fu and Peng 2011). If FoxO3 is critical for mediating TGF-β signaling in conventional T cells is unknown. Therefore, we asked if FoxO3$^{-/-}$ conventional CD4 T cells were able to respond to TGF-β signaling and survive during PICA stimulation, similar to WT cells. After two days, both WT and FoxO3$^{-/-}$ T cells appeared activated and had little cell death, as evident by majority of cells staining negative for Annexin V and 7AAD (Figure 18A and B). The overall cell number and survival of WT and FoxO3$^{-/-}$ T cells were similar, whether TGF-β was present or not (Figure 18B and C). As seen previously (Figure 17), by 4 days of stimulation WT and FoxO3$^{-/-}$ T cells both underwent massive apoptosis in the absence of TGF- β. However, after 4 days with TGF-β, FoxO3$^{-/-}$ T cells showed a dramatic defect in responsiveness to TGF-β. FoxO3$^{-/-}$ T cells had a higher frequency of 7AAD+/AnnexinV+ dead cells compared to WT T cells with TGF-β (Figure 18A and B). In accordance with higher cell death, FoxO3$^{-/-}$ T cells also had lower overall cell numbers with TGF-β on day 4 compared to WT littermates (Figure 18C). The data suggest that FoxO3 is not critical for initial activation of conventional T cells (Day 2), but is important for responsiveness to TGF-β and survival of T cells after activation.
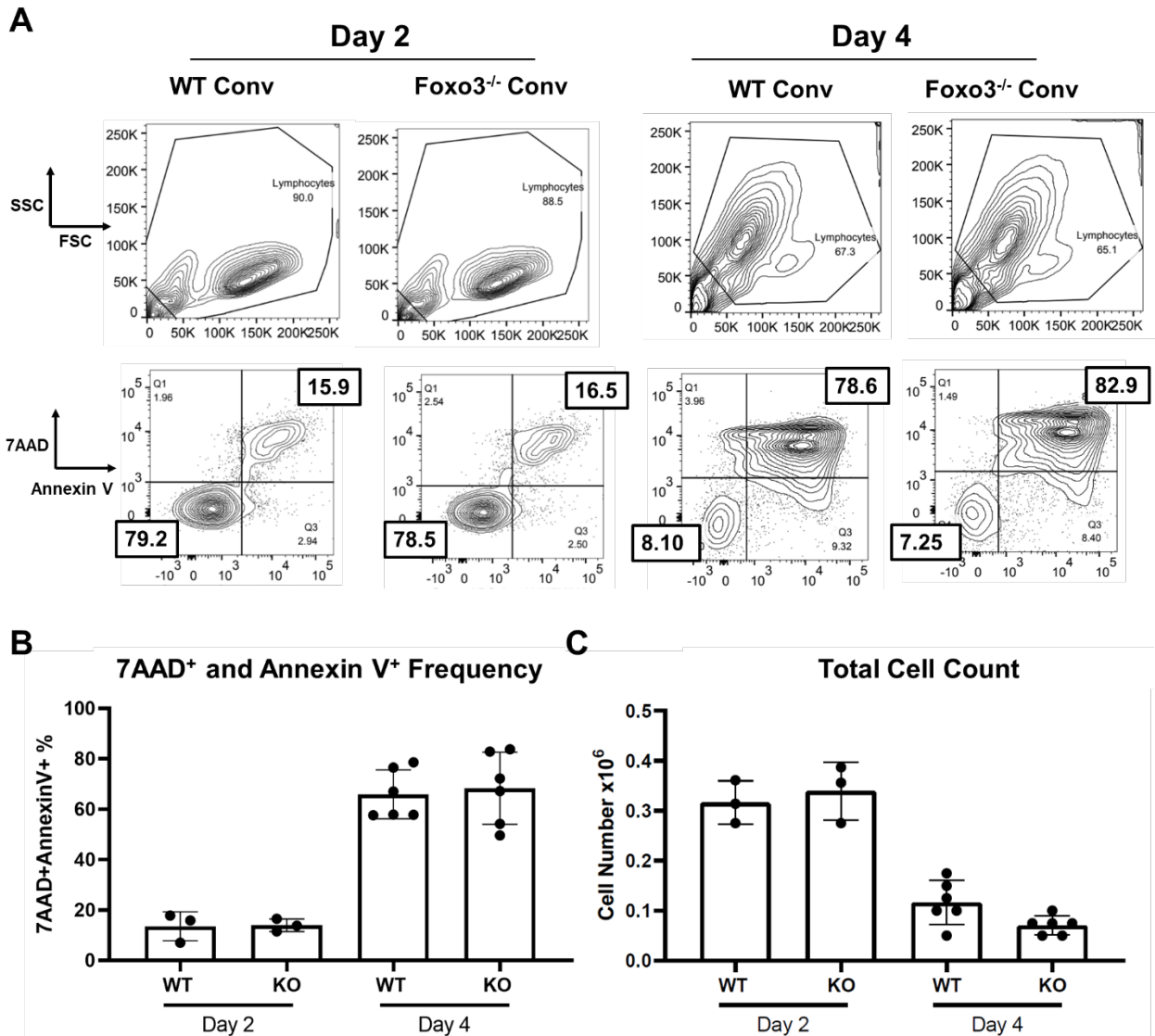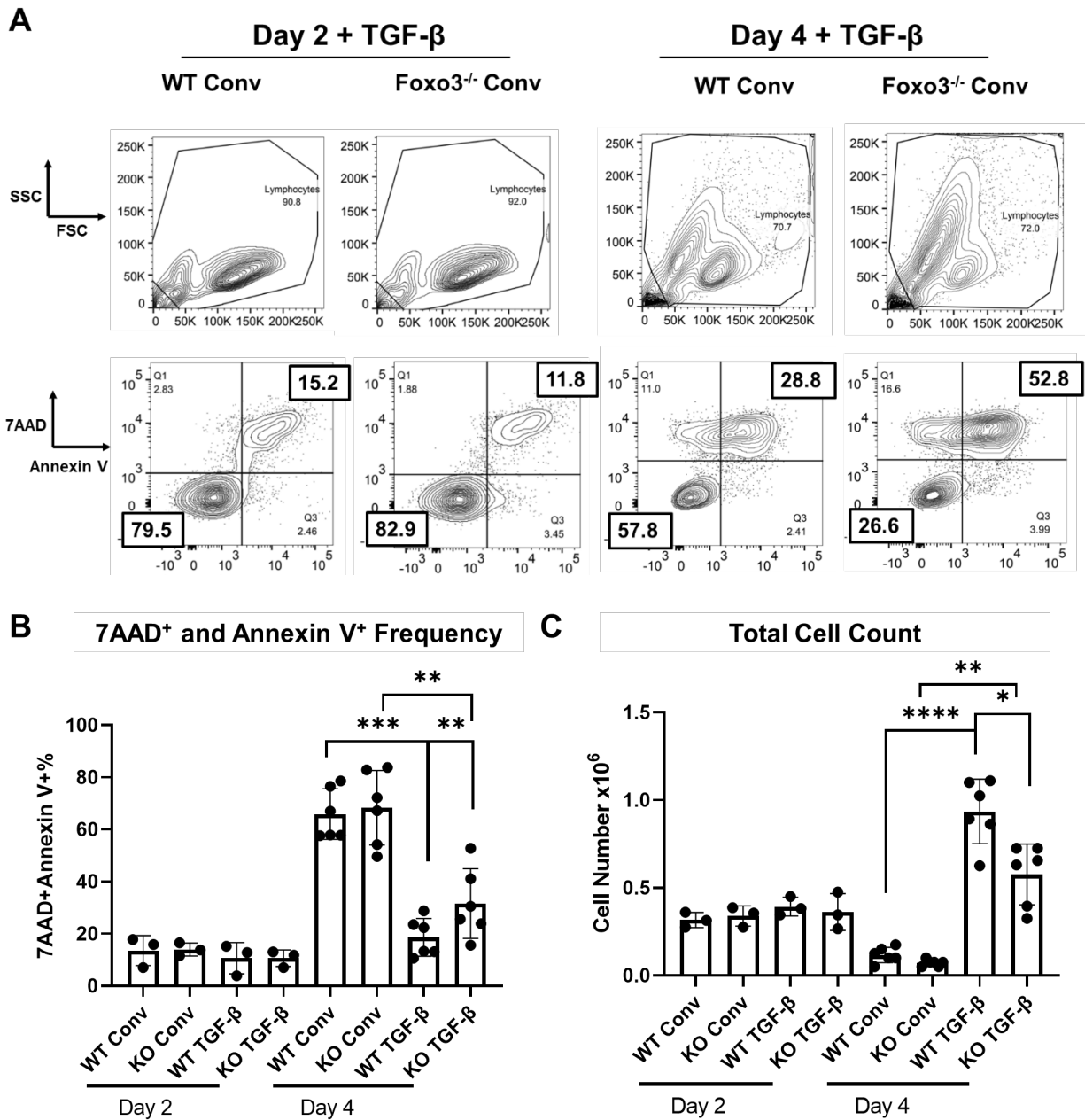
Figure 18. Effect of TGF-β Addition on Survival of FoxO3 Deficient and WT Conventional T Cells During PICA Stimulation. Splenic conventional T cells (4+25-) from FoxO3^{-/-} or WT littermates were stimulated for 2 or 4 days with plate-bound anti-CD3/anti-CD28 antibodies with IL-2, +/- TGF-β. At indicated time points cells were harvested and counted, then analyzed by flow cytometry. **(A, B)** Cells were stained with Annexin V and 7AAD and fluorescence intensity was determined by flow cytometry **(B)** Summary of frequency of 7AAD+/Annexin V+ double positive cells **(C)** Total live cell counts were determined by trypan blue exclusion. **(B, C)** Each dot represents one mouse. Data are representative from 3 independent experiments for Day 2, and 6 independent experiments for Day 4. Statistical significance determined by paired Student's *t* test, with sex-matched age-matched littermates compared for each treatment. p<0.001 denoted as ***, p = 0.001 to 0.01 denoted as **, and p = 0.01 to 0.05 denoted as *.

FoxO3 in Treg Survival

In addition to its role in the differentiation and apoptosis of T cells, FoxO3 and FoxO1 have also been shown to have a critical role in Treg development and function (Kerdiles et al., 2010). Tregs rely on TGF-β signaling to survive PICA stimulation, which suggests there could be crosstalk between FoxO3 and TGF-β signaling pathways. Therefore, we asked if Tregs from WT or FoxO3$^{-/-}$ mice differed in their survival during PICA stimulation. As mentioned previously, splenic Tregs (CD4^{+}25^{-}) become activated and expand robustly during plate-bound stimulation. We compared survival of WT or FoxO3$^{-/-}$ Tregs during plate bound PICA stimulation, with the expectation that if FoxO3 is critical for Treg survival, then FoxO3$^{-/-}$ Tregs should have a defect in proliferation and greater cell death than WT Tregs. When we compared the activation status (by FSC/SSC) and the frequency of 7AAD+/Annexin V+ cells on Day 4, WT and FoxO3$^{-/-}$ Tregs both had similar phenotypes, with majority of cells surviving (double negative for 7AAD/Annexin V) (Figure 19A).   When we examined the frequency of dead cells by flow cytometry, Tregs and conventional T cells had a similar frequency of dead cells (Figure 19B). Although there was some variation among mice, littermates were comparable in their survival (Figure 19A and B) and total cell count on day 4 (Figure 19 C). These data suggest that FoxO3 is not required for Treg survival during PICA stimulation. Based on previous data that FoxO1 and FoxO3 are critical for Treg development and function, FoxO1 could be the dominant transcription factor that allows crosstalk with TGF-β signaling and survival of Tregs during PICA.

A

Day 4

WT Treg   **Foxo3⁻/⁻ Treg**

B 7AAD⁺ and Annexin V⁺ Frequency

C Treg Cell Count
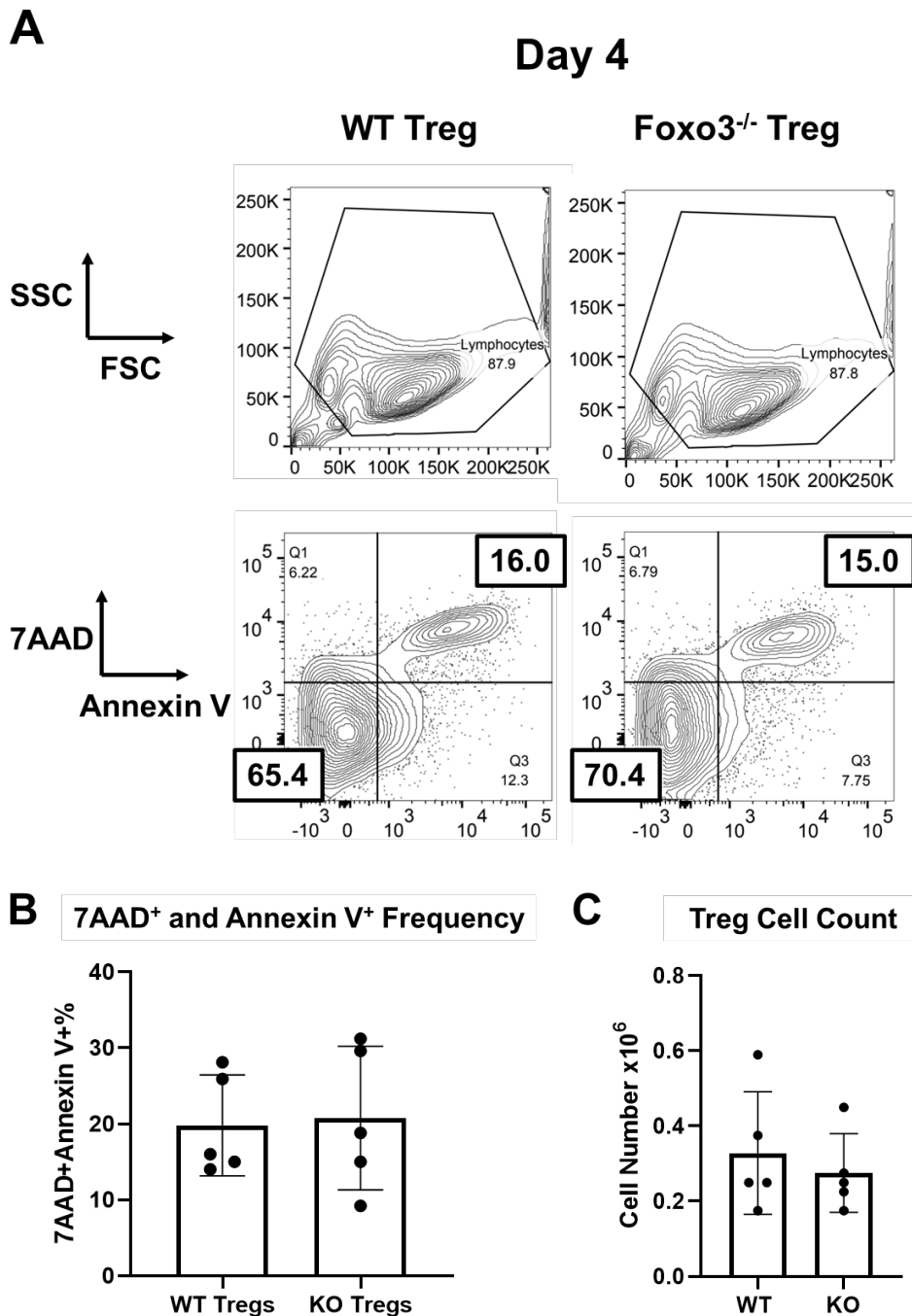
Figure 19. Survival of WT and FoxO3⁻/⁻ Tregs During PICA Stimulation. Splenic Tregs (4+25+) were stimulated for 4 days with plate-bound anti-CD3/anti-CD28 antibodies with IL-2. Four days after stimulation cells were harvested and counted, then analyzed by flow cytometry. **(A, B)** Cells were stained with Annexin V and 7AAD and fluorescence intensity was determined by flow cytometry **(B)** Summary of frequency of 7AAD+/Annexin V+ double positive cells **(C)** Total live cell counts were determined by trypan blue exclusion. **(B, C)** Each dot represents one mouse. Data are representative from 5 independent experiments.

Phenotype of FoxO3$^{-/-}$ Tregs after PICA Stimulation

      Tregs from WT and FoxO3$^{-/-}$ mice had similar survival after PICA stimulation, but we wondered if they were phenotypically similar in expression of canonical Treg markers. Tregs express high levels of the IL-2 receptor α chain CD25, as well as the master transcription factor Foxp3. The Ikaros family member Helios is also a transcription factor that has been shown to play a role in Treg stability and function. Six days after PICA expansion of Tregs, we compared the expression of these markers between WT and FoxO3$^{-/-}$ cells (Figure 20). WT and FoxO3$^{-/-}$ deficient Tregs had high levels of Foxp3, CD25 and Helios, indicative of a canonical phenotype. We did not observe differences in expression between WT and FoxO3$^{-/-}$ littermates, suggesting that expanded Tregs from FoxO3$^{-/-}$ mice are similar to WT in terms of expressing proteins that are critical for Treg function.
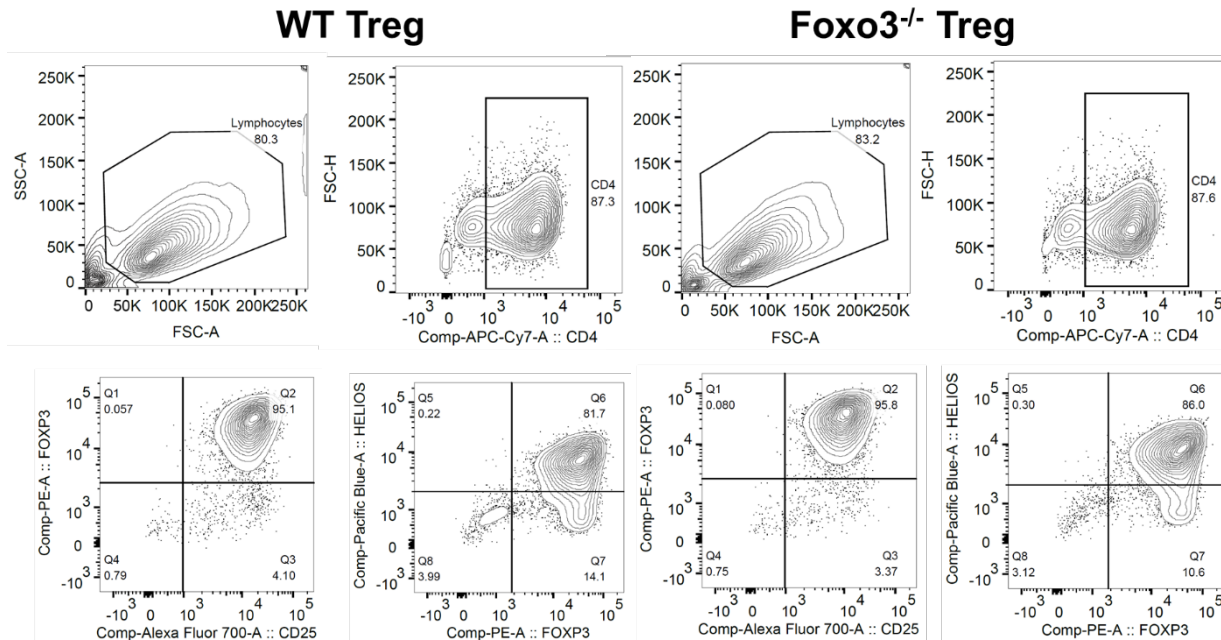
Figure 20. Phenotype of WT and FoxO3$^{-/-}$ Tregs after PICA Expansion. Splenic Tregs (4+25+) were stimulated for 6 days with plate-bound anti-CD3/anti-CD28 antibodies with IL-2. Six days after stimulation, cells were harvested then analyzed by flow cytometry. Cells were gated on by FSC/SSC, live cells, single cells, CD4+, then CD25, Foxp3 and Helios expression was determined. Data are representative of multiple mice from 2 independent experiments.

Conclusions from FoxO3 KO CD4 T Cells and Tregs During PICA Stimulation

During CD3/CD28 plate-bound PICA stimulation, FoxO3 is not required for

apoptosis, as FoxO3 deficient conventional T cells undergo massive apoptosis by day

4, similar to WT littermates. However, FoxO3 is critical for conventional T cells to

survive and expand when TGF-β is added to PICA conditions. With TGF-β, FoxO3

deficient conventional T cells have enhanced apoptosis compared to WT cells. This

suggests that there is a crosstalk between FoxO3 and TGF-β signaling to mediate

survival during continuous stimulation. When Tregs are isolated from the spleens of

FoxO3 deficient mice and subjected to PICA stimulation, they survive and expand

similarly to WT Tregs, and also express canonical Treg markers CD25, Foxp3 and

Helios. These data suggest that FoxO3 is not required for Treg expansion and

phenotype. Therefore, FoxO3 is critical for responding to TGF-β signaling in conventional T cells, but not for TGF-β signaling during Treg expansion. This demonstrates a distinct role of FoxO3 in conventional T cells compared to Tregs in responsiveness to TGF-β signaling.

Phenotype of T Cells in FoxO3$^{-/-}$ Mice in the Intestines and Periphery

Since we saw differences in FoxO3 deficient T cells during PICA stimulation, we sought to examine FoxO3 deficient T cells in a more physiological setting. Since coating antibodies onto plates (PICA stimulation) and stimulating T cells *in vitro* does not perfectly mimic continuous stimulation that occurs with APC-T cell interactions, we sought to examine the phenotype of FoxO3 deficient T cells *in vivo*. One location we can study T cells that are continuously stimulated (similar to PICA conditions) is in the gut. Immune cells in the gut are constantly exposed to a variety of antigens, including bacteria and food antigens. Constitutive sensing of these commensal antigens is thought to play a homeostatic role, as most Th1 and Th17 cells in the intestines develop from signals derived from the microbiota (Gaboriau-Routhiau et al. 2009; Ivanov et al. 2008; Hegazy et al. 2017). Therefore, we examined the activation status and phenotypes of FoxO3$^{-/-}$ intraepithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL) in the small intestines. We noticed a striking phenotype in the expression of the activation and memory marker, CD44. FoxO3 deficient CD8 T cells in the IEL and LPL had a higher frequency of CD44 high cells, compared to WT littermates (Figure 21A). For CD4 T cells, FoxO3$^{-/-}$ mice had significantly higher frequency of CD44 high cells in the LPL compared to WT, with similar levels of CD44 in the IEL (Figure 21A

and B). This suggests that FoxO3 may block CD44 expression and/or promote death of

CD44 high cells, as mice deficient in FoxO3 have enhanced CD44 high frequency.

Figure 21. CD44 Expression in IEL and LPL of WT and FoxO3$^{-/-}$ T Cells. IEL and LPL cells were isolated from the small intestines of adult WT or FoxO3$^{-/-}$ littermates. Cells were directly stained for CD44 expression after isolation. Cells were gated on CD45, CD3, then CD4 or CD8. **(A)** Frequency of CD44 high, medium and low expressing CD4 and CD8 T cells in IEL and LPL. **(B)** Summary of CD44 frequency for 3 independent experiments. Student's *t* test, with sex-matched age-matched littermates compared for each treatment. p = 0.001 to 0.01 denoted as **, and p = 0.01 to 0.05 denoted as *.

We wanted to determine if this CD44 high phenotype was specific to the intestines, or if T cells in the lymph node also showed differences. If the differences in CD44 expression is only in sites where continuous stimulation occurs, then in the mesenteric lymph node (MLN) we should not see differences between WT and KO T cells. Indeed, in the MLN, we see that the levels of CD44 are similar between WT and KO T cells (Figure 22). This suggests that FoxO3 only controls CD44 expression in sites where T cells are actively stimulated in the gut, but not in the MLN where T cells are not constantly stimulated (in the absence of infection).
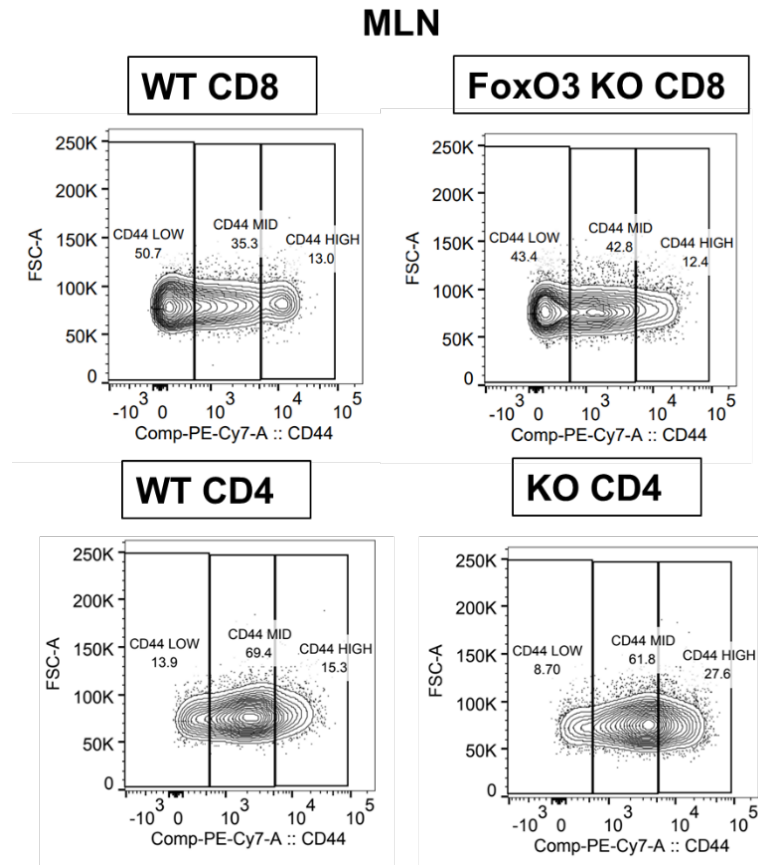
Figure 22. CD44 Expression in MLN of WT and FoxO3$^{-/-}$ T Cells. MLN cells were isolated from adult WT or FoxO3$^{-/-}$ littermates. Cells were directly stained for CD44 expression after isolation. Cells were gated on CD45, CD3, then CD4 or CD8. Data are representative of 4 independent experiments with age-matched, sex-matched littermates.

We sought to determine if the CD44 high phenotype in FoxO3 deficient mice was unique to CD44, or if other T cell markers were enhanced in KO IEL and LPL. Therefore, we examined other markers of activation and memory, CD69 and CD25. CD69 was originally identified as an early T cell marker of activation and is also expressed by a large proportion of tissue memory CD4 and CD8 T cells in mice and humans (Kumar et al. 2017). CD25 is highly expressed upon T cell activation, and is not classically associated with memory, but has been found on some subsets of memory T cells (Herndler-Brandstetter et al. 2005). We examined CD25 and CD69 levels in WT and FoxO3 KO mice, and compared the CD4 and CD8 T cells in the MLN and gut fractions (Figure 23). In the LPL, CD25 expression was relatively low for both WT and KO CD4 and CD8 T cells (Figure 23A). Majority of the CD4 and CD8 T cells in the LPL expressed CD69 for both WT and KO mice. We saw similar trends for CD25 and CD69 in the IEL (data not shown). In the MLN, we see low expression of CD25 in WT and KO CD4 and CD8 T cells (Figure 23B). CD69 expression is markedly lower in the MLN than the LPL, and there were no significant differences between WT and KO mice. Together, the data suggest that FoxO3 KO mice have enhanced CD44 expression in the IEL and LPL, but not in the peripheral organs such as the MLN and spleen. This is unique to CD44, as CD25 and CD69 expression were similar at these sites between WT and KO.

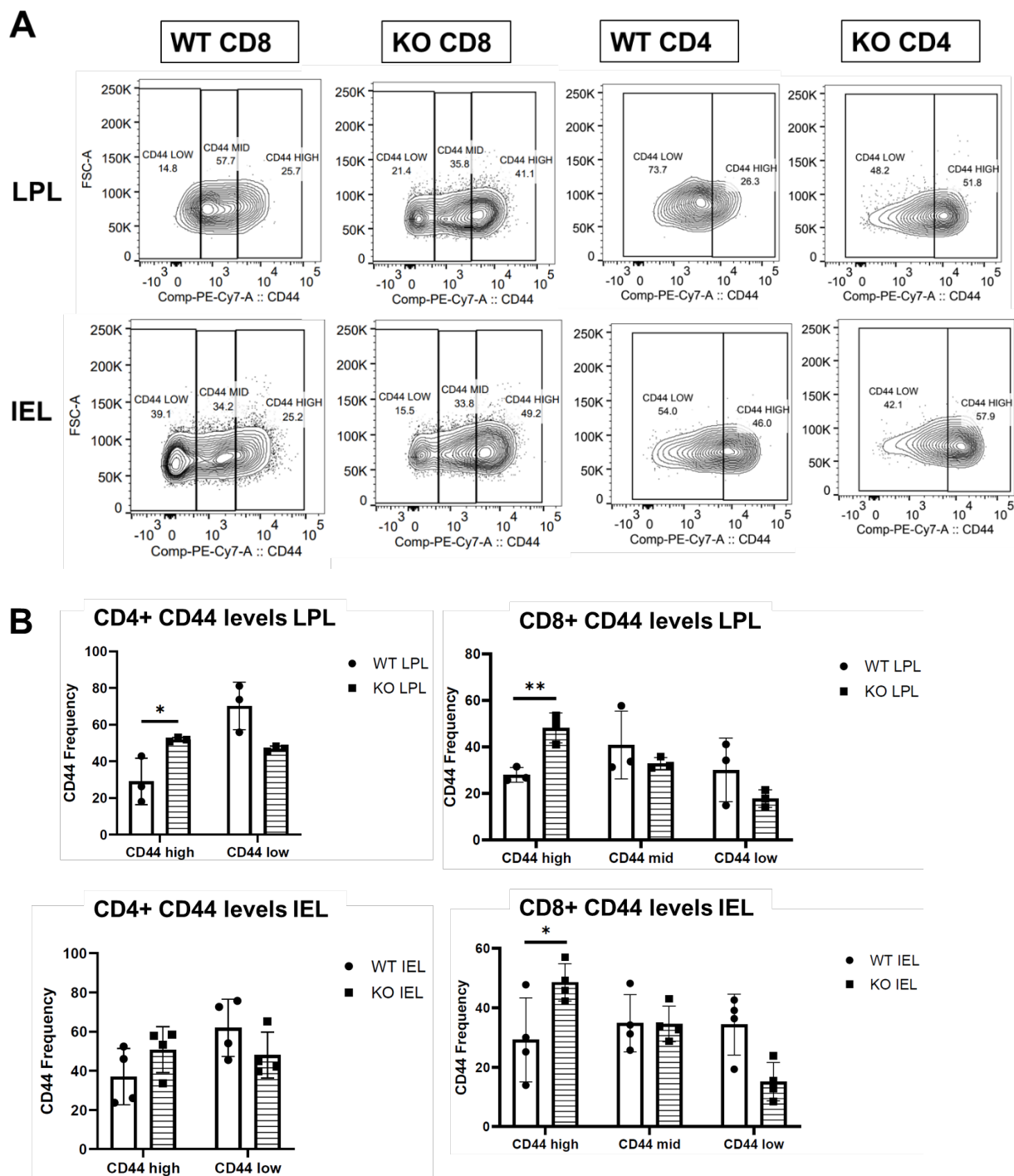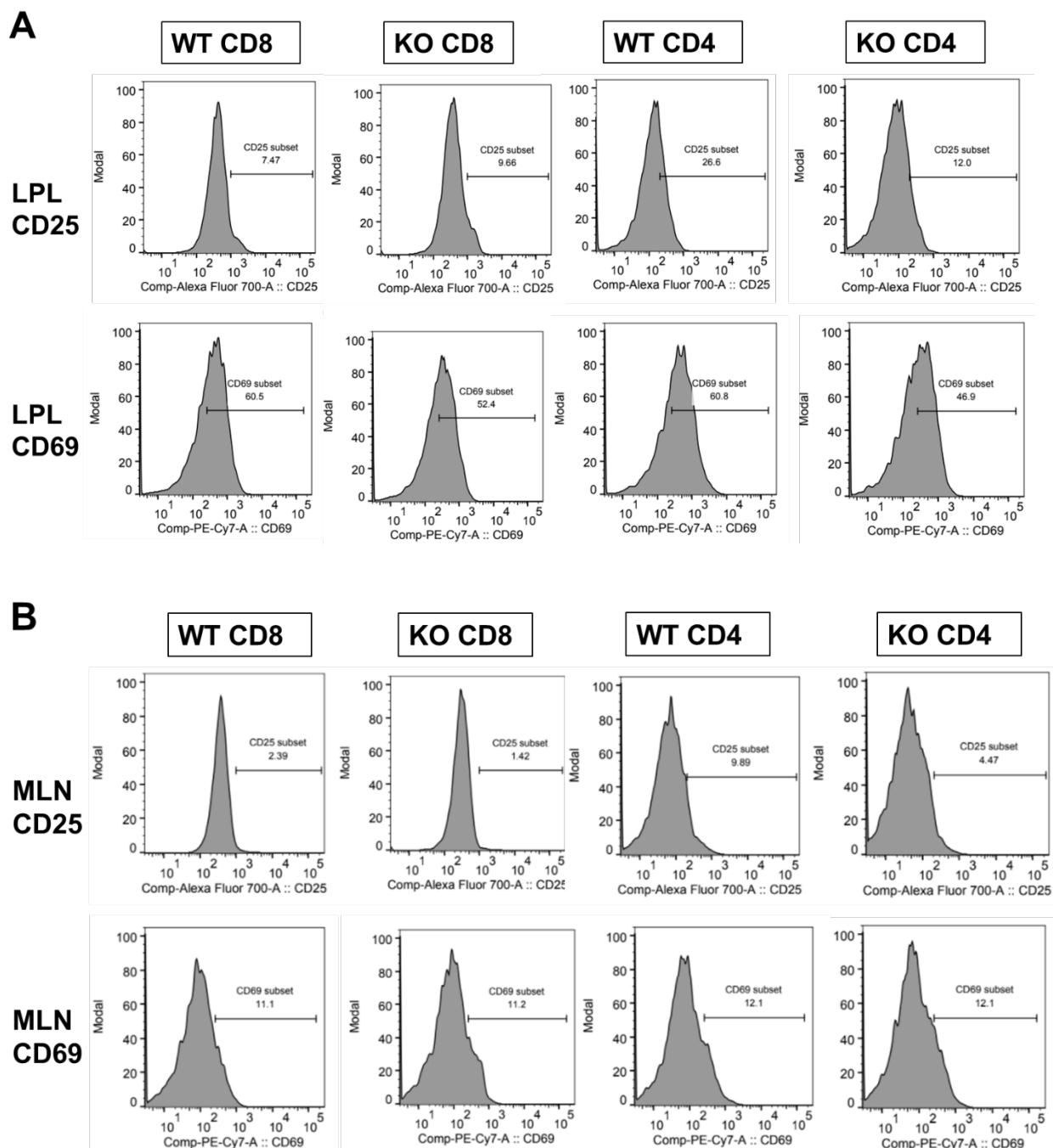**Figure 23. CD25 and CD69 expression in WT and FoxO3$^{-/-}$ T Cells.** IEL and LPL cells were isolated from the small intestines of adult WT or FoxO3$^{-/-}$ littermates. MLN cells were also harvested from the same mice. Cells were directly stained for CD25 and CD69 expression after isolation. Cells were gated on CD45, CD3, then CD4 or CD8. **(A)** Histograms showing CD69 and CD25 expression in CD4 and CD8 T cells in IEL and LPL. **(B)** Histograms showing CD69 and CD25 expression in CD4 and CD8 T cells in the MLN. Data are representative of 3 independent experiments with age-matched, sex matched littermates.

Activation-induced CD44 Expression in FoxO3 KO T Cells

FoxO3 KO mice showed enhanced CD44 expression in the IEL and LPL, where T cells are continuously stimulated. One hypothesis that could explain this phenotype is that FoxO3 signaling suppresses CD44 after T cell activation. Therefore, in FoxO3 deficient T cells, there is hyper CD44 expression after activation compared to WT littermates. To test this hypothesis, we isolated CD4 and CD8 T cells from WT or KO littermates, and stimulated them for one day with anti-CD3/CD28 antibodies to promote T cell activation. We then examined CD44 expression before and after activation. If FoxO3 suppresses CD44 expression, then FoxO3 KO mice should have enhanced CD44 after activation compared to WT T cells. In resting conventional CD4 T cells in the spleen, we see that the levels of CD44^{high}CD62L^{low} cells are similar between WT and FoxO3 KO (Figure 24A). These are classified as CD4 memory T cells, as they express CD44 without activation. When we stimulated conventional CD4 T cells for two days, we see that FoxO3 KO and WT CD4 T cells upregulate CD44 similarly (Figure 24B). We see that CD25 expression (another marker of activation) is also similar between WT and KO mice after activation. In CD8 T cells, we also see similar levels of CD44 expression in WT and KO mice 8 hours and 24 hours after stimulation (data not shown). This suggests that FoxO3 signaling is not required for CD44 suppression in activated T cells.
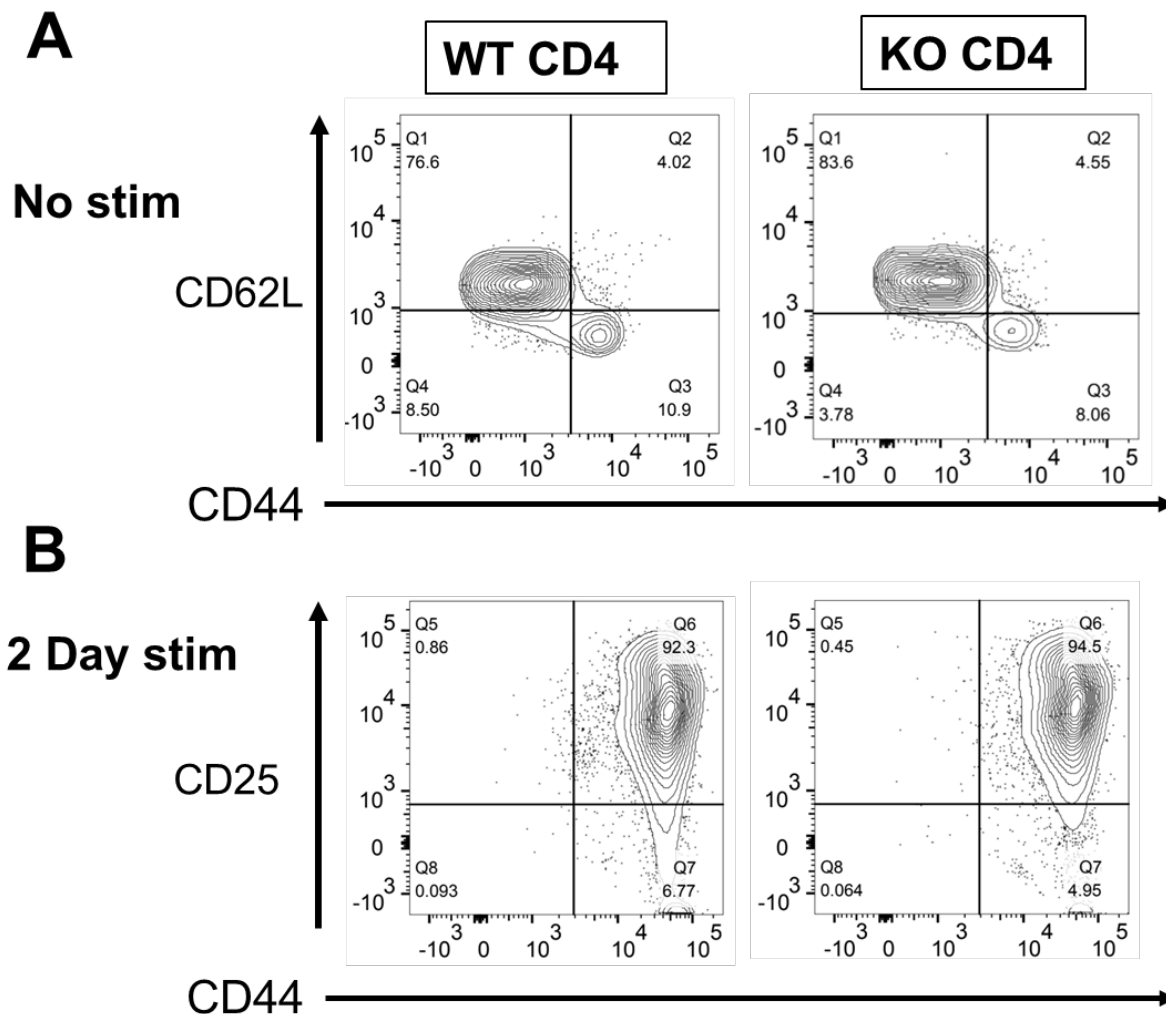
Figure 24. Activation Phenotype of WT and FoxO3$^{-/-}$ CD4 T Cells. CD4$^+$25$^-$ splenic T cells were isolated from WT and FoxO3$^{-/-}$ littermates. **(A)** Cells were unstimulated and directly stained for CD44 and CD62L expression. Cells are gated on live, single, CD4 cells **(B)** CD4$^+$25$^-$ T cells were stimulated with plate-bound CD3/CD28 expression for 2 days in the presence of IL-2. After stimulation, cells were stained for flow cytometry analysis, gating strategy the same as in A. Data are representative of 3 independent experiments, using age-matched sex-matched littermates.

CD8 T Cells in PICA Stimulation

We have found that CD4 conventional T cells undergo massive apoptosis during

PICA stimulation, and that TGF-β can rescue cell death. We have also found that TGF-

β and FoxO3 signaling crosstalk in conventional CD4 T cells to promote survival.

Because CD4 and CD8 T cells differ in their effector functions, we wondered if CD8 T

cells would also respond similarly to CD4 T cells during PICA stimulation. First, we compared the proliferation and survival of the CD8 T cells to CD4 T cells overtime in our PICA assay by looking at cell counts after 2 and 4 days (Figure 25). We saw a striking difference in the expansion of CD8 T cells compared to CD4 conv T cells during PICA. On day 2 after stimulation with IL-2, there are about 0.3-0.4 million CD4 T cells, while there are about 1 million CD8 T cells (Figure 25A). TGF-β addition does not dramatically alter the CD4 or CD8 cell numbers at this time point. However, on day 4, there are approximately 0.1 million CD4 T cells and about 3-6 million CD8 T cells that survive. The addition of TGF-β on day 4 dramatically increases survival of CD4 T cells (about 6-8-fold increase in cell number, ~0.8 million T cells). TGF-β also enhances CD8 T cell survival, about 2-3-fold, ~5-15 million (Figure 25A). When we examine 7AAD/Annexin V staining on day 4, with IL-2 CD4 T cells have 60-80% double positive dead cells, while CD8 T cells have 40-65% dead cells (Figure 25B). TGF-β addition decreases cell death in both CD4 and CD8 T cells. Together, these data suggest that CD8 T cells have enhanced proliferation and survival compared to CD4 T cells, both with and without TGF-β.

A

B
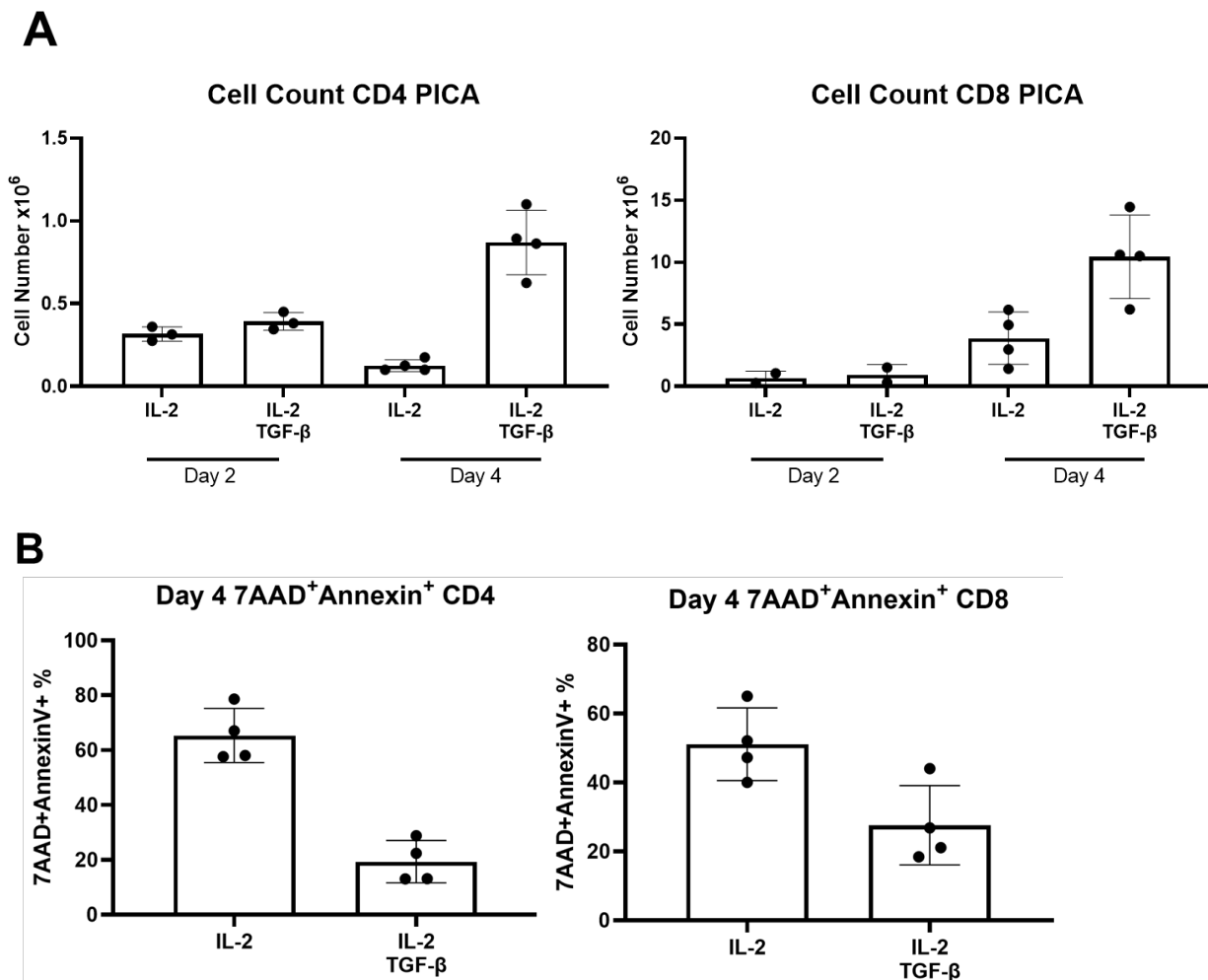
Figure 25. Comparing Survival of CD4 and CD8 Conv T During PICA. CD4+25- and CD8+ splenic T cells were isolated from WT mice. $1.5x10^5$ cells were stimulated on day 0 with standard PICA stimulation (plate-bound anti-CD3/28 + IL-2), +/- TGF-β. After 2 or 4 days, cells were collected and **(A)** counted by tryphan blue exclusion. **(B)** On day 4 cells were stained for 7AAD and Annexin V and analyzed by flow cytometry. Data are from 4 independent experiments.

In CD4 T cells, we observed that FoxO3 deficient CD4s have decreased survival with TGF-β addition (Figure 18). Therefore, we tested if FoxO3 KO CD8 T cells have a defect in responsiveness to TGF-β signaling on Day 4, or if this defect is specific to CD4 conventional T cells. We found that CD8 T cells in WT and FoxO3 KO mice have similar cell counts on day 4 with IL-2 (Figure 26A). With TGF-β addition, we see that there is a similar increase in survival for both FoxO3 KO and WT CD8 T cells. When we

stain for 7AAD/Annexin V to look at dead cells on day 4, we see that with IL-2 alone,

~50-66% of the cells are dead in both WT and KO (Figure 26B). With TGF-β, we see a

similar reduction in cell death between WT and KO. Therefore, FoxO3 is not required in

CD8 T cells for TGF-β-mediated cell survival.

A

B
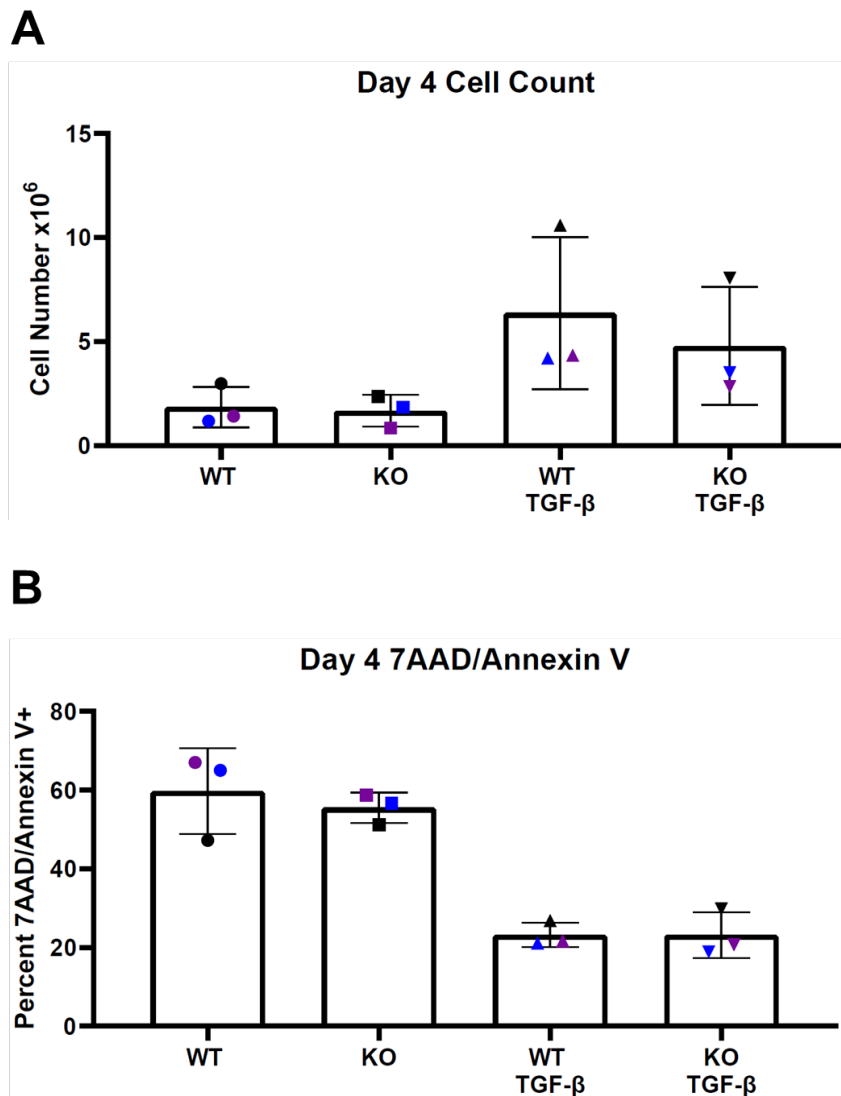
Figure 26. FoxO3$^{-/-}$ CD8 T Cells During PICA Stimulation. CD8+ splenic T cells were isolated from FoxO3$^{-/-}$ or WT littermates. 1.5x10^5 cells were stimulated on day 0 with standard PICA stimulation (plate-bound anti-CD3/28 + IL-2), +/- TGF-β. After 4 days, cells were collected from plates **(A)** Cells were counted by tryphan blue exclusion. **(B)** Cells were stained with Annexin V and 7AAD and fluorescence intensity was determined by flow cytometry. 7AAD$^+$Annexin V$^+$ indicates frequency of dead cells. Each dot represents one mouse. Age-matched sex-matched littermates are depicted with the same color. A and B, n.s.

Conclusions from Section 2

For the first time, we have shown that FoxO3 and TGF-β crosstalk in conventional CD4 T cells to promote survival during continuous stimulation. Although FoxO3 has been shown to promote T cell apoptosis, during PICA stimulation, FoxO3 deficient T cells do not show enhanced survival. However, when TGF-β is added to PICA stimulation, FoxO3 KO mice cannot survive as well as WT controls. This suggests that TGF-β signaling and FoxO3 signaling crosstalk to promote T cell survival during AICD. To examine continuous stimulation in a more physiological setting, we examined CD44 T cells in the gut of FoxO3 KO mice, as these T cells are repeatedly stimulated by bacteria and food antigens. We saw that FoxO3 KO mice have enhanced CD44 expression compared to WT littermates. This is specific to CD44 and not other markers of T cell activation and memory, as CD25 and CD69 expression is similar between WT and KO. FoxO3 KO and WT mice upregulate CD44 expression to a similar extent after stimulation, suggesting that FoxO3 deficiency does not affect activation-induced CD44 expression. Additionally, we found that CD8 T cells proliferate and survive at significantly higher rates than CD4 T cells during PICA stimulation. FoxO3 is not required for TGF-β mediated cell survival in CD8 T cells, but is critical for CD4 T cell survival with TGF-β.

Section 3: Investigating AICD in CAR T Cells and the Role of TGF-β Signaling

Creating an Experimental System to Study AICD in CAR T Cells

Similar to conventional T cells during PICA, CAR T cells undergo AICD after repeated antigen stimulation *in vitro* and *in vivo* (Gargett et al. 2016; Künkele et al. 2015). CAR T cells generally possess a tumor antigen-specific binding domain

generated from a single chain variable fragment, the signaling component of the T cell

antigen receptor (cytoplasmic region of TCR ζ chain) and co-stimulatory molecules (e.g.

CD28, 4-1BB). The CAR T cell construct used in our study was generously gifted by the

Nishimura lab. These CD19-specific CAR T cells have the cytoplasmic region of the

TCR ζ chain, as well as the costimulatory molecule CD28 (Figure 27). The construct

also contains a truncated CD34 protein (truncation prevents signaling). CD34 is not

expressed on T cells, so expression of the truncated CD34 is a very useful way to

identify the CAR$^+$ T cells by flow cytometry and to column sort them using anti-CD34

magnetic beads. Additionally, CD34 is expressed at a 1:1 ratio with the CAR construct,

which makes it a useful marker and analogous to the amount of CAR protein made.
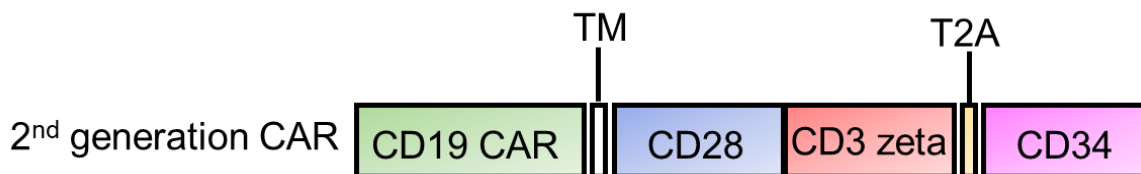
Figure 27. CD19 CAR Construct. This second-generation CAR contains a single-chain
variable fragment specific to CD19. This is followed by a transmembrane domain (TM), and the
costimulatory molecule CD28, followed by the CD3 zeta chain. The CD3 zeta chain is followed
by the T2A self-cleaving peptide, then a truncated CD34 protein. This truncated CD34 cannot
signal, and is therefore usual for detecting T cells with CAR on the surface.

Recent work by others has shown that CD28-based CAR T cells undergo

apoptosis when repeatedly activated by antigen (Gargett et al. 2016; Guedan et al.

2018; Künkele et al. 2015). Additionally, replacing the cytoplasmic region of CD28 with

other co-stimulatory molecules made the CAR T cells more resistant to AICD (Guedan

et al. 2018). These data suggest that CD28-mediated co-stimulation in CAR T cells may

promote apoptosis to a greater extent than other co-stimulators. Therefore, we

hypothesize that the signaling mechanisms involved in PICA cell death could play a significant role in CAR T cell apoptosis.

In CAR T cell activation, CD3 and a co-stimulatory domain such as CD28 are co-engaged, which is similar to plate bound activation in PICA. Thus, CAR T cells provide an excellent model system to study apoptotic signaling processes that occur during and the role of TGF-β in CAR T cell survival. To begin to address the role of PICA in CAR T cells, we developed an *in vitro* system to study AICD of CD19-specific CAR T cells stimulated with the Raji B cell lymphoma line, which express high levels of CD19. We hypothesized that stimulation of CAR T cells with low numbers of Raji B cells, then the CAR T cells would survive and proliferate robustly, while large numbers of Raji B cells would cause AICD and decreased survival. We predict that high numbers of Raji B cells mimic continuous strong TCR stimulation with plate-bound anti-CD3/CD28 during PICA, while low numbers of Raji B cells mimic lower strength stimulation that promotes expansion and survival.

We first tested a range of Raji B cell numbers to determine the conditions to induce AICD. CAR T cells and Raji B cells were placed in the top of a transwell to promote cell contact and media was replaced each day underneath the transwells to ensure CAR T cell survival was not impacted by lack of nutrients. At the end of 5 days, CAR T cells were counted. CAR T cells cultured with fewer Raji B cells (1:1 and 1:10 CAR to Raji), expanded and survived, while CAR T cells cultured with higher numbers of Raji B cells (1:20 and 1:40) showed decreased cell numbers and enhanced apoptosis (Figure 28A). We saw this phenomenon for multiple donors, where CAR T cells cultured at 1:1 expanded after 5 days, while 1:20 CAR to Raji conditions showed decreased

survival (Figure 28B). These data supported previous reports that repeated antigen stimulation can cause CAR T cell death (Gargett et al, 2016) and gave us a model to study the signaling mechanisms controlling CAR T cell death.
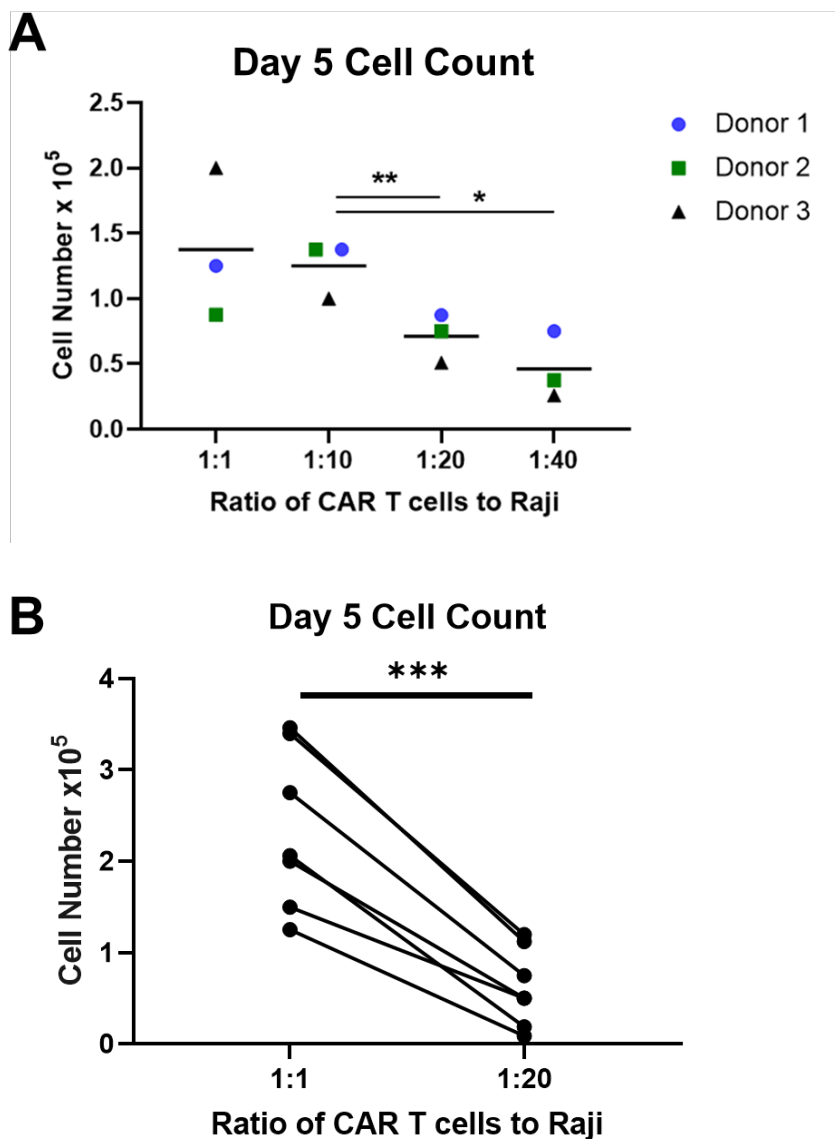
Figure 28. Survival of CAR T cells after Raji Co-culture. CAR T cells (5×10^4) were stimulated for 5 days with irradiated Raji B cells. CAR T cells and Raji B cells were cultured in the top of a transwell (0.4 μm) to promote cell contact. Media underneath the transwell was changed daily, leaving CAR T cells and Raji B cells undisturbed. After 5 days, cell counts were determined by tryphan blue exclusion. Each dot represents a different CAR T cell donor. Statistical significance determined by paired Student's *t* test (A) ** p= 0.0064, * p=0.0192. (B) Each donor for 1:1 and 1:20 is connected by a single line. *** p=0.0001.

CAR T Cells that Survive High Dose Antigen are Predominantly Foxp3$^+$

We sought to determine the phenotype of CAR T cells that survive high dose Raji stimulation. In our PICA model of AICD in mouse T cells, conventional CD4$^+$Foxp3$^-$ cells undergo massive apoptosis, while CD4$^+$CD25$^+$Foxp3$^+$ cells survive and expand robustly. Therefore, we hypothesized that Foxp3$^+$ CAR T cells would be more resistant to AICD with high Raji numbers compared to Foxp3$^-$ T cells. To test this hypothesis, we stained CAR T cells after 5 days of culture with a low ratio of CAR T cells to Raji B cells (1:1 CAR to Raji) or a high ratio (1:20 CAR to Raji). We expected that if Foxp3$^+$ cells are resistant to AICD, we should observe a higher frequency of Foxp3$^+$ surviving cells in 1:20 stimulation conditions compared to 1:1 CAR to Raji stimulation after 5 days. The gating strategy is shown (Figure 29) for the 1:1 condition on day 5, where CAR T cells are gated on by live, single cells (Raji B cells are irradiated on day 0, and thus are dead in the culture or killed by CAR T cells). CARs are then identified by CD34 expression, and further subdivided into CD4 and CD8 subsets.
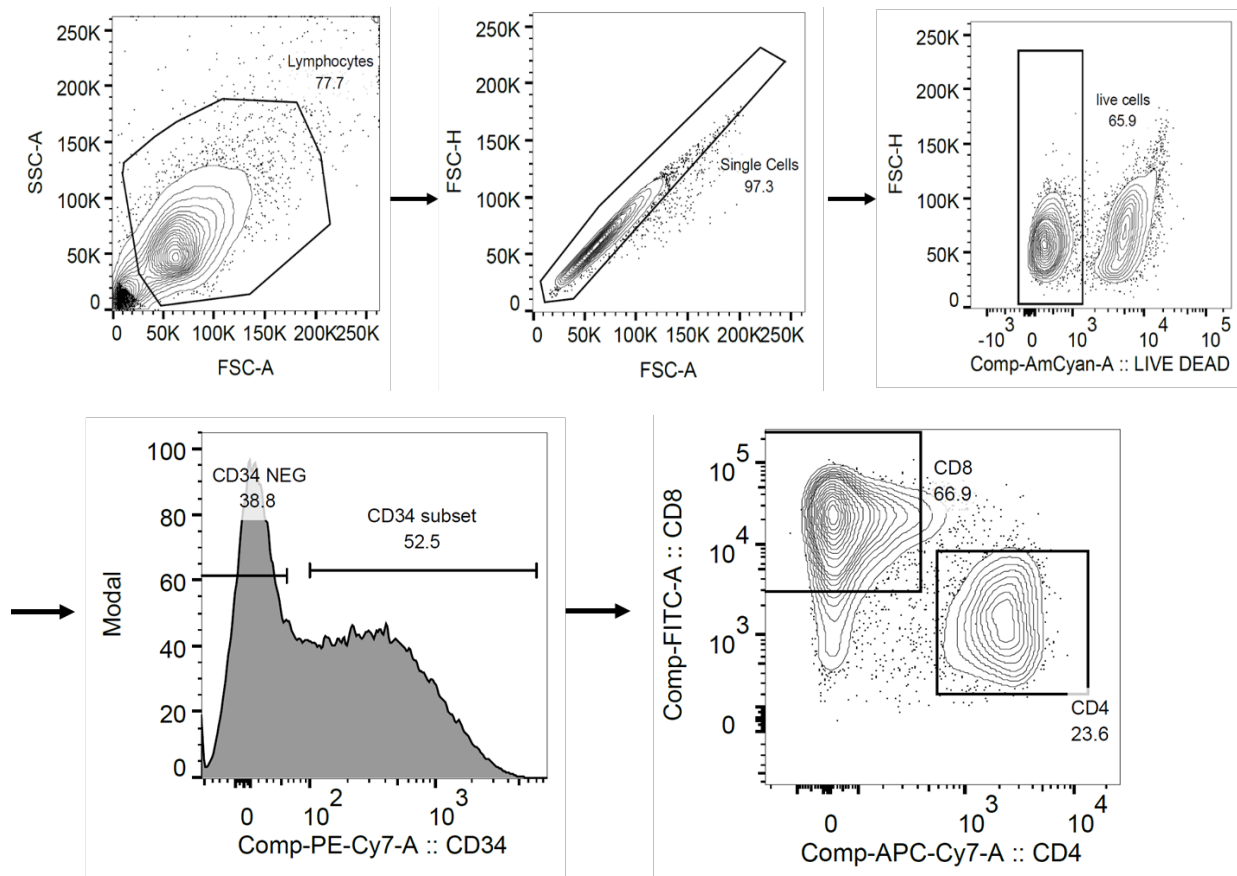
Figure 29. Representative Gating Strategy for Day 5 Stimulated CAR T Cells. CAR T cells ($5x10^4$) were stimulated for 5 days with irradiated Raji B cells. After 5 days, cells were stained for CD34 (marker for CAR), CD4, CD8, CD25, and Foxp3 and subjected to flow cytometry. Cells were gated on by the lymphocyte gate, then single, live cells (top row). Live, single cells were gated for CD34 expression (marker of CAR transduction), then the CARs were separated by CD4 and CD8 expression, before examining CD25 and Foxp3. Data are representative of 1:1 culture of CAR T cells to Raji.

When we stimulated CAR T cells at a 1:1 ratio with Raji B cells, CAR T cells expressed CD25 expression (indicative of activation), with a low frequency of Foxp3+ cells (Figure 30A). However, at 1:20 CAR to Raji B cell ratios, the surviving CAR T cells had a high frequency of CD25+Foxp3+ cells. We saw this trend of high Foxp3 expression in 1:20 ratios for multiple donors, for both CD4 and CD8 CAR T cells (Figure 30B).
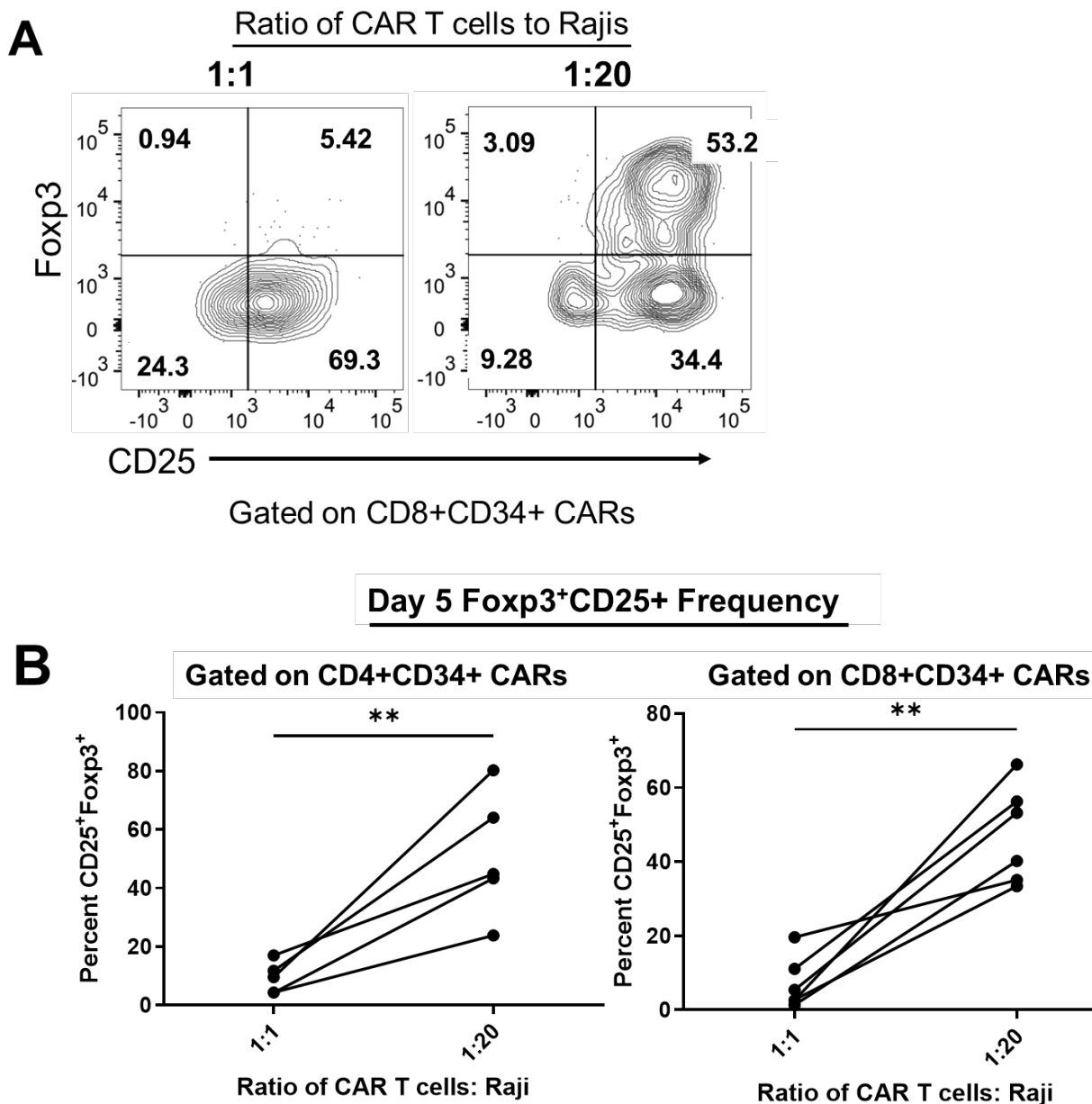
Figure 30. Expression of Foxp3 and CD25 in Raji Stimulated CAR T Cells. CAR T cells (5x10^{4}) were stimulated for 5 days with irradiated Raji B cells. After 5 days, cells were stained for CD34 (marker for CAR), CD4, CD8, CD25, and Foxp3 and subjected to flow cytometry. **(A)** Expression of CD25 and Foxp3 is shown after gating on live, CD8+CD34+ CAR T cells. **(B)** Percent CD25+Foxp3+ CAR T cells after culture with 1:1 or 1:20 CAR to Raji ratios. Each donor represents a single dot, and lines are connecting the same donor that was stimulated with 1:1 or 1:20 CAR to Raji conditions (CD4 left, CD8 right). Statistical significance determined by paired Student's t test, CD4s left ** p=0.0098, CD8s right **p=0.0018.

This suggests that continuous high dose stimulation of CAR T cells promotes AICD of Foxp3⁻ but not Foxp3⁺ CAR T cells. This could occur through *de novo* induction of Foxp3 expression during high dose Raji stimulation, or through survival of Foxp3⁺ cells. The data also suggest that with low numbers of Raji B cells, Foxp3⁻ cells are predominantly expanding and surviving.

CAR Expression is Required for Foxp3 High Phenotype

Since we saw a dramatic difference in the phenotype of CAR T cells when stimulated by low numbers of Raji B cells vs high numbers, we sought to determine what is causing Foxp3⁺ T cell survival. The difference between the low dose (1:1 CAR to Raji) and high dose (1:20 CAR to Raji) is the high number of Raji B cells present in the 1:20 culture. Although the Raji B cells are irradiated and therefore do not divide, they are still highly metabolically active. It could be that the Raji cells are secreting a molecule that promotes Foxp3 expression, or preferentially promotes survival of Foxp3⁺ CAR T cells. If this was the case, then CD34⁻ (CAR negative) T cells cultured in the 1:20 Raji conditions would also be Foxp3⁺. This would indicate that Foxp3 expression is independent of CAR activation. To test this, we performed the co-culture of Rajis with T cell donors that contained CD34⁺ CAR T cells, as well as CD34⁻ T cells that were not successfully transduced with the CAR (or did not have detectable CAR surface expression). After 5 days, we compared the frequency of Foxp3⁺CD25⁺ cells in CD34⁺ CARs vs CD34⁻ T cells from the same culture. If CAR T cell activation by Raji B cells is required for Foxp3 expression or survival of Foxp3⁺ cells, then Foxp3 expression should be present only in CD34⁺ cells. If the culture conditions (large number of tumor cells) are promoting induction of Foxp3 and/or survival of Foxp3⁺ cells, then both CD34⁺

CARs and CD34⁻ T cells should express Foxp3. After 5 days, CD34⁺ CAR T cells had a significant proportion of Foxp3⁺ high T cells, while CD34⁻ T cells in the same culture had low levels of Foxp3 (Figure 31 A and B). Therefore, CAR expression is required for the survival of Foxp3⁺ cells. This suggests that the culture conditions or any molecules produced from culturing T cells 1:20 with Raji are not the cause of Foxp3 survival, as CD34⁻ T cells that survive are Foxp3⁻.
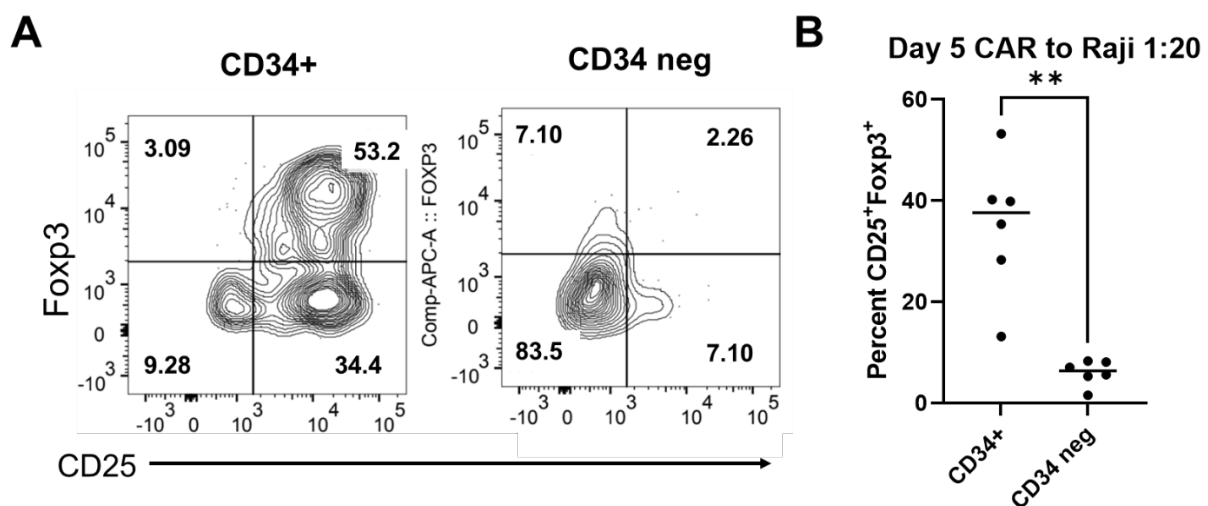
Figure 31. Expression of Foxp3 and CD25 in CD34+ CAR T Cells Compared to CD34- T cells. CAR T cells along with CD34- T cells from the same donor were stimulated for 5 days with irradiated Raji B cells (1:20 CAR to Raji). After 5 days, cells were stained for CD34 (marker for CAR), CD8, CD25, and Foxp3 and subjected to flow cytometry. **(A)** Expression of CD25 and Foxp3 is shown after gating on live, CD8+CD34+ CARs or CD8+CD34- T cells. **(B)** Summary of frequency of CD25+Foxp3+ cells after the 5 day culture. Each dot represents a different donor. Statistical significance determined by paired Student's *t* test, ** p=0.0031.

CAR Recognition of Tumor Antigen is Required for Foxp3 Expression and Decreased Survival

Since we determined that CAR expression is required for Foxp3 high phenotype in surviving T cells during 1:20 stimulation of CARs to Raji, we next asked if recognition of tumor antigen is required for the Foxp3 high phenotype and decreased survival. To answer this question, we cultured CD19 specific CAR T cells with a CD19 negative

tumor cell line and compared the survival and CD25/Foxp3 expression to the 1:20 CAR with CD19$^+$ Raji conditions. The CD19 negative cell line used was Jurkat T cells, which are a T cell lymphoma cell line. We irradiated the Jurkat T cells or Raji B cells on day 0, then cultured the CD19 CAR T cells at a 1:1 or 1:20 ratio with Jurkat T cells or Raji B cells. If the CAR binding CD19 antigen is required for Foxp3$^+$CD25$^+$ expression on day 5, then CARs cultured with Jurkat T cells at 1:20 should have decreased Foxp3$^+$CD25$^+$ frequency compared to CARs cultured with Raji B cells at 1:20. When we cultured CAR T cells alone with no tumor cells, CAR T cells at 1:1 with Jurkat T cells or 1:1 with Raji B cells, we see a low frequency of Foxp3+ cells (Figure 32A). With 1:20 Raji B cells as seen previously, many of the surviving CAR T cells are Foxp3$^+$CD25$^+$ high. However, with 1:20 CAR to Jurkat T cells, we see that there is only a very low level of Foxp3, and no CD25 expression. These data suggests that CD25$^+$Foxp3$^+$ high cells that survive the 1:20 culture of CAR to Raji require engagement of the CAR with CD19. It should be noted that for the 1:1 CAR to Raji culture, some donors showed CD25 expression, while others did not. However, as described previously (Figure 30), only in the 1:20 CAR to Raji conditions do we see high co-expression of CD25 and Foxp3 (Figure 32).

We also examined by cell count the number of CD19 CAR T cells that survived and found that CAR T cells alone (no tumor cells) do not proliferate (Figure 32B). As expected, when CAR T cells are cultured at a ratio of 1:1 with Raji B cells, we see proliferation indicated by a higher cell number on day 5. When CARs are cultured 1:20 with Raji B cells, we see a dramatic decrease in survival. However, when CAR T cells are cultured at 1:1 or 1:20 with CD19 negative Jurkat T cells, we see the same number of CAR T cells for each donor as when CAR T cells are alone (Figure 32B).
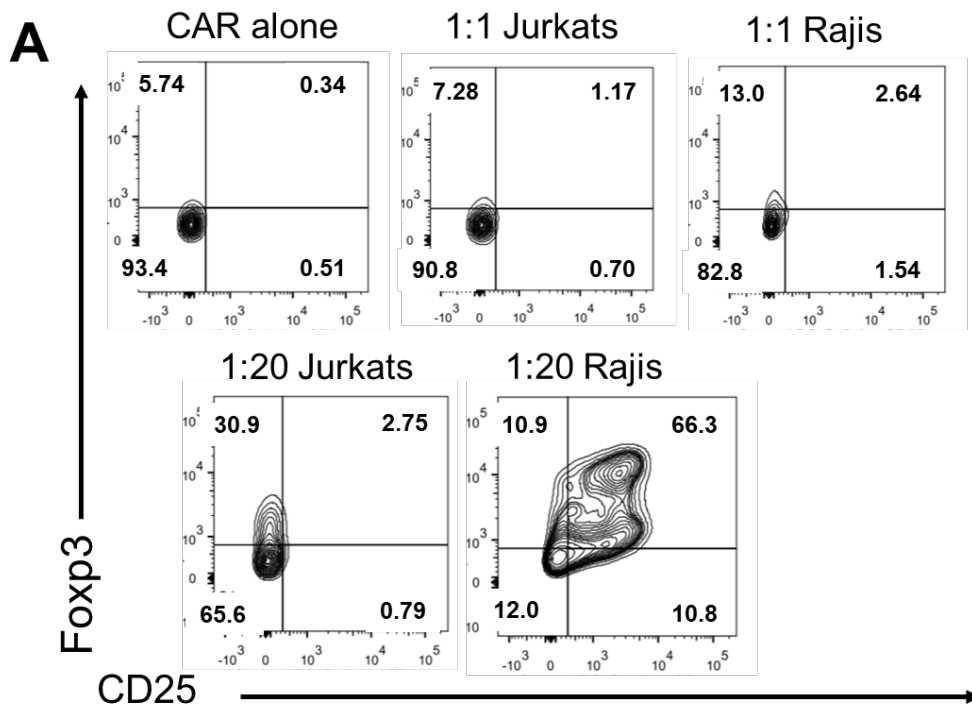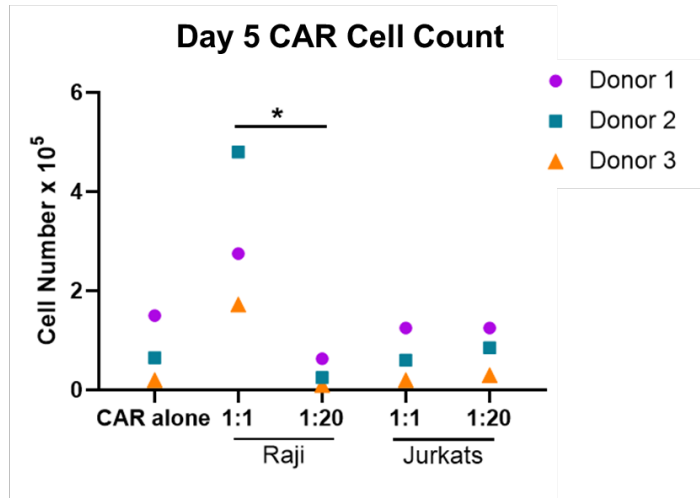
Figure 32. Phenotype and Survival of CAR T Cells after 5 Day Culture with Raji or Jurkat Cell Lines. CD34+ CAR T cells were stimulated for 5 days with irradiated Raji B cells (1:1 or 1:20 CAR to Raji), or with irradiated Jurkat T cells (1:1 or 1:20). After 5 days, cells were counted then stained for CD34 (marker for CAR), CD8, CD25, and Foxp3 and subjected to flow cytometry. **(A)** Expression of CD25 and Foxp3 is shown after gating on live, CD8+CD34+ CARs. Data are representative of 3 independent experiments with 3 unique donors. **(B)** CAR cell counts after the 5 day culture. Each dot represents a different donor. Statistical significance determined by ratio paired Student's *t* test, * p=0.0366.

Together, these data suggest a fundamental difference in response when CD19

CARs recognize lower number of antigen positive tumor cells (1:1 CAR to Raji)

compared to large numbers of cells (1:20 CAR to Raji). At the 1:1 ratio of CAR to Raji,

CAR T cells expand, and the surviving population is Foxp3$^-$. At the 1:20 ratio of Raji B

cells, the CAR T cells undergo massive apoptosis, and surviving cells are majority

CD25$^+$Foxp3$^+$. This phenotype is dependent on continuous stimulation with CD19$^+$ Raji

tumor cells and requires CAR engagement with CD19.

Effect of TGF-β Treatment on CAR Survival

TGF-β has pleotropic effects on T cell functions and survival, depending on the

context. TGF-β can suppress the activation and proliferation of T cells, especially naïve

T cells. On the other hand, TGF-β can promote the generation and survival of memory T

cells (Filippi et al. 2008). During our PICA stimulations that promote AICD of T cells, we

found that TGF-β rescues conventional T cells from death (Singh et al. 2010; Takami,

Love, and Iwashima 2012). Therefore, we hypothesized that TGF-β would promote

survival of CAR T cells that undergo continuous stimulation with Raji cells at high

concentrations (1:20 CAR to Raji).

We stimulated CAR T cells at 1:1 or 1:20 (CAR to Raji) for 5 days in our transwell

system that we established. We assessed the effect of TGF-β addition on CAR T cell

survival after 5 days. As expected, when CAR T cells were cultured at 1:1 ratio with

Raji B cells, they expanded robustly (50,000 cells starting on day 0 to over 300,000 cells

on day 5) (Figure 33). When TGF-β was added to the culture at a 1:1 ratio, there was a

significant suppression of T cell proliferation and survival. When CAR T cells were

cultured with Raji B cells at a 1:20 ratio (CAR to Raji), there is reduced survival

compared to 1:1, as shown previously. However, when TGF-β is added to the 1:20

culture, we see enhanced survival compared to 1:20 alone. This suggests that TGF-β

has differential effects on CAR T cells, depending on how they are engaged with

antigen. TGF-β inhibits proliferation of CAR T cells at low ratios of Raji B cells, but can

promote cell survival when CAR T cells are continuously stimulated with large numbers

of Raji B cells.
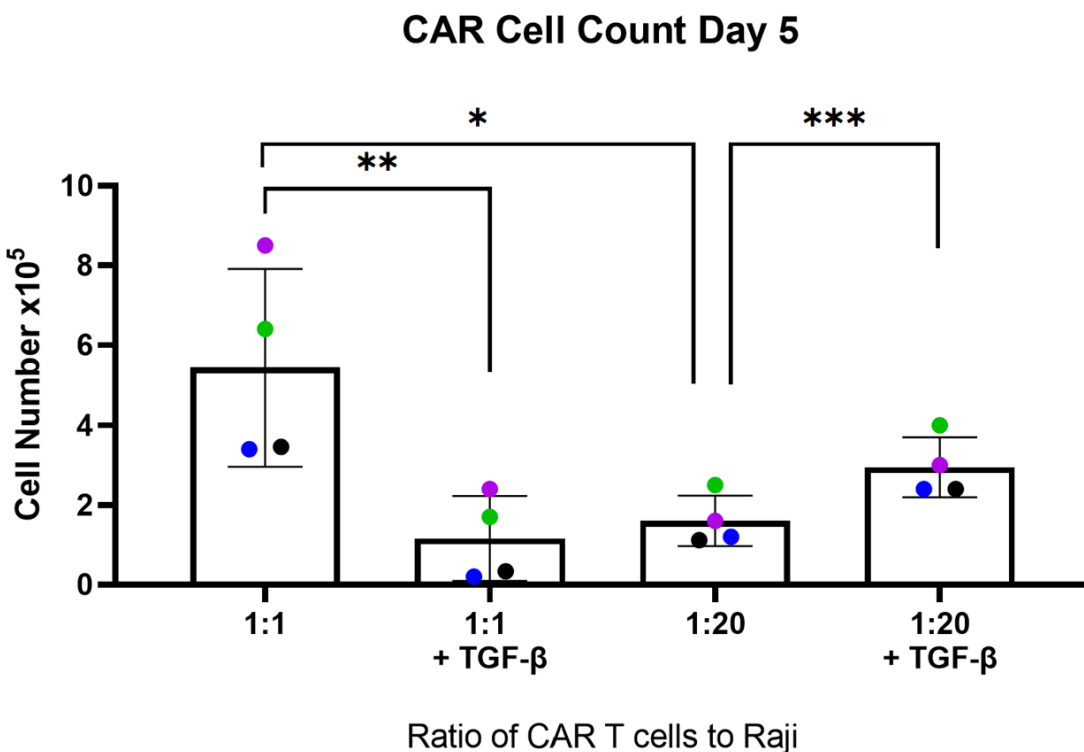
Figure 33. Effect of TGF-β on CAR Survival after Raji Stimulation. CD34+ CAR T cells were stimulated for 5 days with irradiated Raji B cells (1:1 or 1:20 CAR to Raji). In conditions with TGF-β, 10 ng/mL was added on days 1 and 3 after stimulation. Cells were counted by tryphan blue exclusion after 5 days. Paired T test, * p=0.0391, ** p=0.009, ***p=0.0003.

Phenotype of CAR T Cells after TGF-β Treatment

In the TME, TGF-β has been shown to negatively regulate T cell responses,

including suppressing T cell inflammatory cytokine production, and upregulating

checkpoint markers such as CTLA-4 and PD-1 (Leach, Krummel, and Allison 1996;

Maeda and Shiraishi 1996; Thomas and Massagué 2005). TGF-β has also been shown to promote survival of memory T cells in the TME (Robertson 2001; Webb et al. 2014). Based on these data, we hypothesized that CAR T cells treated with TGF-β that survive after 5 days would have phenotypic differences compared to no treatment. We examined expression of CD25, CTLA-4 and Foxp3. CD25 can be suppressed by TGF-β signaling in the TME, which is believed to be a mechanism by which TGF-β suppresses IL-2 dependent growth of T cells (Maeda and Shiraishi 1996). TGF-β signaling can induce Foxp3 expression, and Foxp3 can upregulate CTLA-4 expression, as seen in Tregs in the TME (Fontenot, Gavin, and Rudensky 2003; Zheng et al. 2007). Therefore, if TGF-β negatively regulates T cell function, we should see an increase in expression of Foxp3 and CTLA-4, as well as a decrease in CD25 expression. CAR T cells on day 5 that were cultured with Rajis at 1:20 had high expression of CTLA-4 and CD25, as well as a proportion of cells that expressed Foxp3 (Figure 34). In the 1:20 conditions with TGF-β addition, we see for CD4 T cells similar levels of CTLA-4 and Foxp3 expression, and a reduction in CD25 expression (top row). In CD8 T cells, we see in TGF-β treated conditions enhanced CTLA-4 expression, a reduction in CD25 expression, and similar levels of Foxp3 (bottom row). This suggests that with high numbers of Raji B cells, we see enhanced CAR T cell survival, but changes in protein expression that can diminish CAR T cell function.

Figure 34. Phenotype of CAR T Cells with TGF-β Treatment. CD34+ CAR T cells were stimulated for 5 days with irradiated Raji B cells (1:1 or 1:20 CAR to Raji). In conditions with TGF-β, 10 ng/mL was added on days 1 and 3 after stimulation. After 5 days, cells were stained for live/single cells, CD34, CD4, CD8, CD25, CTLA-4, and Foxp3. Histograms depict expression of CTLA-4, CD25 and Foxp3 after cells were gated on for CD34+ CAR expression, then CD4 (top row) and CD8 (bottom row). Surface expression was determined for CD25, and intracellular staining was performed for CTLA-4 and Foxp3. Data are representative of 3 different donors.

Effect of Caspase Inhibitor on CAR T Cell Survival and Phenotype

In our mouse model with PICA stimulation, conventional CD4$^+$25$^-$ cells expand

for 3 days with plate-bound anti-CD3/CD28 stimulation, then undergo massive

apoptosis by day 4 (Singh et al. 2010). Because the caspase family of proteins is critical

for apoptosis, our lab tested if caspase activity is required for PICA cell death, using a

pan-caspase inhibitor. Addition of the pan-caspase inhibitor 2 days after activation

dramatically reduced cell death by day 4 (~79% dead with no inhibitor and DMSO solvent control, and ~36% dead with the addition of the inhibitor) (Singh et al. 2010). These data suggest that caspase activation is required for apoptosis during PICA. Based on these data, and since we observed decreased CAR survival at 1:20 CAR to Raji compared to 1:1 with Raji, we hypothesized that CAR T cells were undergoing caspase-mediated cell death with high Raji numbers (1:20 CAR to Raji). To test this, we cultured CAR T cells with the addition of the caspase inhibitor at day 1 and day 3 after CAR T cells were mixed with Raji cells. If CAR T cells are undergoing caspase-mediated apoptosis, then addition of the inhibitor should increase cell survival with 1:20 CAR to Raji conditions. As seen previously, CAR T cells proliferate robustly when cultured with Raji B cells at a 1:1 ratio (Figure 35A). At 1:20 CAR to Raji, we see decreased survival. Addition of the caspase inhibitor increased cell survival compared to the 1:20 with DMSO control. Together, the data suggest that caspase activation is contributing to cell death in our cultures. Other mechanisms are also contributing to decreased survival, as the inhibitor cannot fully rescue to the levels of the 1:1 CAR to Raji. When we examined the phenotype of the surviving CAR T cells, we see that unlike TGF-β treatment, addition of the caspase inhibitor did not significantly alter expression of CTLA-4, CD25 and Foxp3 (Figure 35B). These data suggest that caspase activation is leading to apoptosis, while inhibiting caspase activity does not alter the phenotype of the CAR T cells that survive.

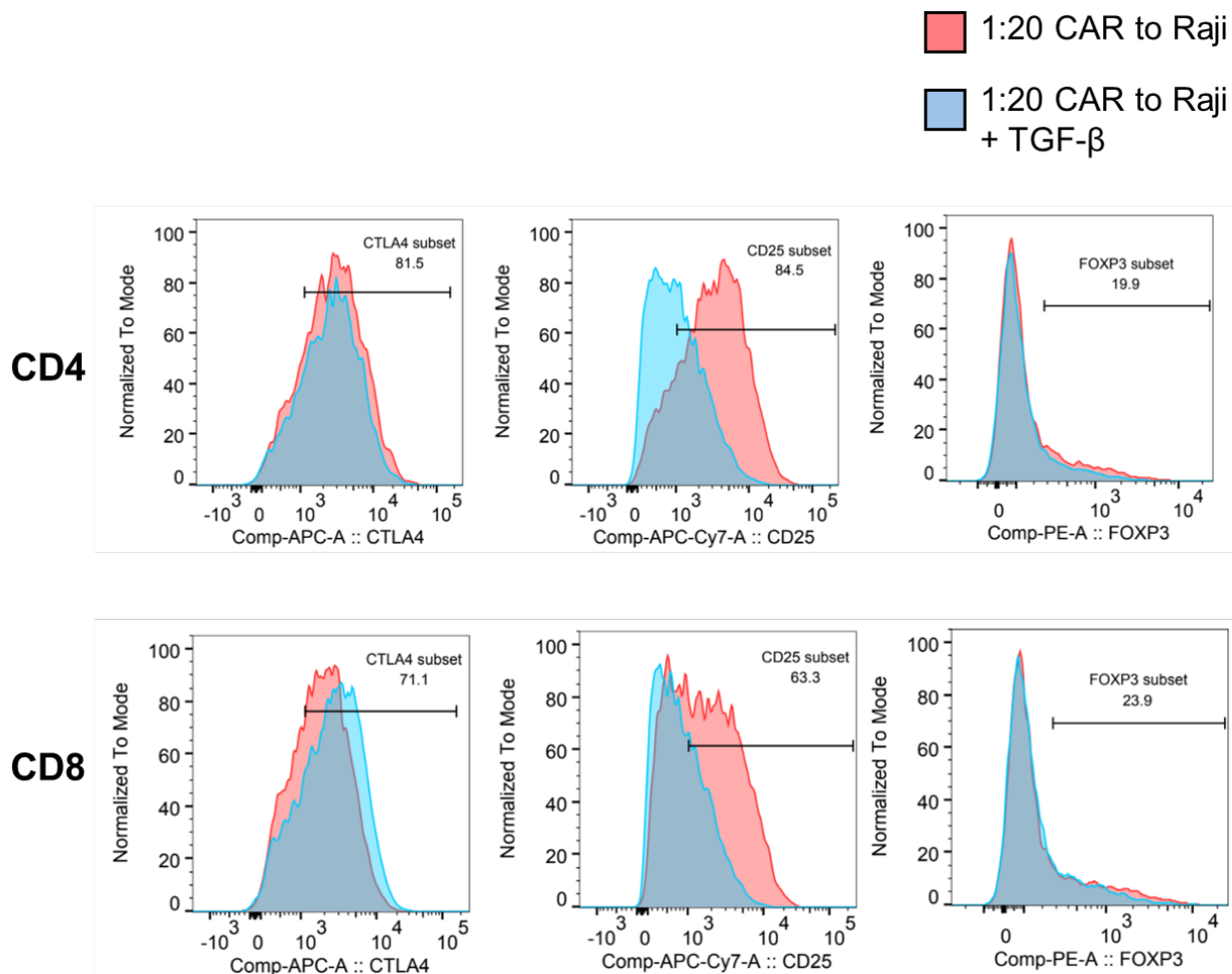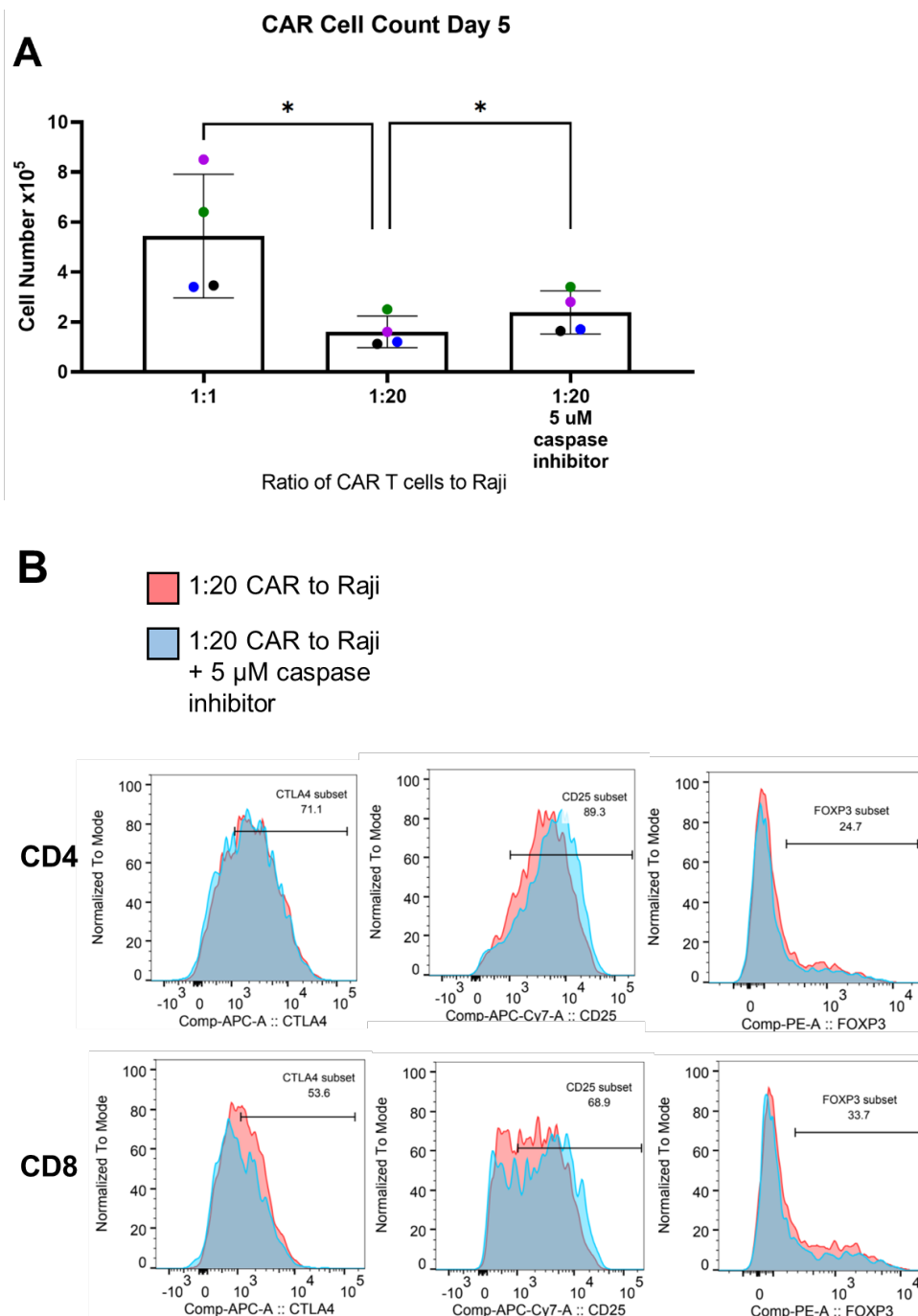**Figure 35. Survival and Phenotype of CAR T Cells with Caspase Inhibitor.** CD34+ CAR T cells were stimulated for 5 days with irradiated Raji B cells (1:1 or 1:20 CAR to Raji). In conditions with caspase inhibitor, 5 µM of the pan-caspase inhibitor z-VAD-fmk or DMSO controls was added on days 1 and 3 after stimulation. After 5 days **(A)** CAR T cells were counted **(B)** Cells were stained for CD34, CD4, CD8, CD25, CTLA-4, and Foxp3. Histograms depict expression of CTLA-4, CD25 and Foxp3 after cells were gated on for CD34+ CAR expression, then CD4 (top row) and CD8 (bottom row). Surface expression was determined for CD25, and intracellular staining was performed for CTLA-4 and Foxp3. Representative of 4 donors. Paired T test, 1:1 vs 1:20 * p=0.0391, 1:20 vs caspase * p=0.0187.

Conclusions from Section 3

We determined that CAR T cell survival, phenotypes and functions are dependent on the number of tumor cells in the environment. With a low number of CD19+ Raji tumor cells (1:1 CAR to Raji), CAR T cells can proliferate and survive robustly. The phenotype of these cells is primarily Foxp3$^-$. When CAR T cells are cultured with high numbers of Raji B cells (1:20 CAR to Raji or greater), we see a significant reduction in the survival of CAR T cells. Compared the 1:1 culture condition, in the 1:20 ratio we see a significant increase in the frequency of CD25$^+$Foxp3$^+$ CAR T cells. CAR expression is required for this CD25$^+$Foxp3$^+$ phenotype, as CD34 negative cells are primarily Foxp3 negative. Functionally, we see that TGF-β alters the phenotype of CAR T cells, increasing checkpoint molecule CTLA-4 and suppressing CD25 expression. These data suggest that when CAR T cells encounter a large number of tumor cells and continuous stimulation, this causes apoptosis of T cells. TGF-β can promote survival from this apoptosis, but functionally it could negatively affect the T cell functionality. Caspase inhibition can promote some level of survival, without changing CAR phenotypes.

CHAPTER FOUR

DISCUSSION

Introduction

Tregs play a pivotal role in maintaining immunological self-tolerance and
homeostasis. While conventional T cells are continuously stimulated only during times
of infection or disease, Tregs are constantly stimulated by self-antigens. Tregs are
primarily self-reactive and are continuously suppressing T cell responses to self-
antigens as well as antigens derived from food and commensal bacteria. Continuous
stimulation of T cells normally results in cell death (AICD), so how do Tregs survive?
Our previous data demonstrated that Tregs ($CD4^+25^+$) and conventional T cells
($CD4^+25^-$) have dramatically different responses to continuous stimulation with plate-
bound anti-CD3/CD28 antibodies. Conventional T cells expand for 2-3 days, then
undergo massive apoptosis by day 4, while Tregs continuously expand over 7000 fold
within 10 days (Singh et al. 2010). The goal of this study is to determine signaling
mechanisms that control AICD and T cell survival during continuous stimulation.

Summary of Data

Our data demonstrate that Tregs can survive continuous PICA stimulation, in part
due to substantially reduced expression of proteins in the ERK signaling/RasGRP1
signaling pathways (Figure 10). While conventional T cells strongly upregulate
RasGRP1 expression and p-ERK minutes after stimulation, Tregs maintain low
expression of these proteins (Figure 11). When RasGRP1$^{-/-}$ conventional T cells are

stimulated with PICA conditions, we see a significant increase in cell survival, suggesting that high expression of RasGRP1 in conventional T cells leads to enhanced ERK signaling and cell death (Figure 14). Expression of RasGRP1 in B cells was shown to be sufficient to promote apoptosis, suggesting that high expression of this pathway can promote apoptosis in T cells (Stang et al. 2009). Furthermore, we see that Tregs maintain low RasGRP1 expression up to a week after stimulation (Figure 11). Our data shows that autocrine TGF-β signaling protects Tregs from AICD (Figure 13), and addition of TGF-β to conventional T cells can promote survival. TGF-β signaling suppresses RasGRP1 expression, which we propose as a novel target of TGF-β signaling in T cells.

To investigate mechanisms of T cell death in PICA stimulation, we examined FoxO3 signaling. The role of FoxO3 in T cells is context dependent, as FoxO3 signaling can promote apoptosis of conventional and memory T cells, but FoxO3 is also critical for differentiation of Tregs and Th1 cells. We discovered for the first time a crosstalk between TGF-β signaling and FoxO3 in conventional T cells. During continuous stimulation with PICA conditions, TGF-β rescues conventional T cell death. CD4 conventional FoxO3 KO T cells have a significant defect in survival with TGF-β addition (Figure 18). Regulatory T cells do not require FoxO3 for their expansion during plate-bound stim, or expression of canonical Treg markers (Figures 19 and 20). *In vivo,* FoxO3 KO T cells in the gut were found to have enhanced expression of CD44, a marker of T cell activation and memory (Figure 21).

To investigate signaling mechanisms of AICD in a more physiological setting, we studied AICD in tumor-specific CAR T cells. We discovered that CAR T cells respond

fundamentally differently to low numbers of tumor targets compared to large numbers of

tumor targets that cause continuous stimulation of CAR T cells. We found that

continuous stimulation with a large number of tumor cells (CAR to Raji 1:20) causes

decreased survival of CAR T cells compared to 1:1 stimulation (Figure 28). The

surviving CAR T cells are primarily CD25$^+$Foxp3$^+$ (Figure 30). Additionally, TGF-β or a

pan-caspase inhibitor can enhance survival of CAR T cells, but TGF-β alters the

surviving CAR T cell phenotype (Figures 33-35). Together, these data show that

signaling mechanisms involved in PICA during plate-bound stimulation can occur in

AICD of tumor-specific CAR T cells, and could therefore be targeted therapeutically.

RasGRP1 Signaling in T Cell Function and Survival

In conventional T cells, we found that RasGRP1 is highly expressed after

activation, and RasGRP1 signaling can promote apoptosis. How does enhanced

RasGRP1 expression cause apoptosis in conventional T cells? This warrants further

investigation, as it would shed light on signaling mechanisms that can promote AICD

during times such as memory formation or in the tumor microenvironment. RasGRP1

enhances ERK activation, and ERK has multiple targets including Fos and Elk-1 that

transcriptionally regulate IL-2 production and T cell activation. Zhu et al found that ERK

activation is induced prior to the onset of AICD caused by anti-CD3 stimulation of T cells

(Zhu et al. 1999). When ERK activation was inhibited with a chemical inhibitor,

apoptosis was also inhibited, which suggests that ERK signaling is required for AICD.

Furthermore, inhibition of ERK signaling also inhibited expression of Fas ligand.

Interactions between Fas/FasL are required for AICD, as this promotes the caspase

signaling cascade leading to apoptosis (Refaeli et al. 1998). Mice deficient in Fas (*lpr*)

develop a lymphoproliferative disorder, and this is believed to occur because of a lack of AICD of lymphocytes (Hewicker, Kromschröder, and Trautwein 1990; Cohen and Eisenberg 1991). We also found that Fas deficient conventional CD4 T cells have significantly enhanced survival during PICA conditions, suggesting that Fas is required for AICD (Singh et al. 2010). Therefore, enhanced RasGRP1 and ERK activation in conventional T cells could lead to enhanced FasL expression and apoptosis. The target of ERK that leads to FasL upregulation is unknown.

Based on our model, low RasGRP1/ERK activation in Tregs could therefore prevent upregulation of FasL and prevent apoptosis. This could be tested by overexpressing RasGRP1 in Tregs and determining if FasL and apoptosis is enhanced. Additionally, RasGRP1$^{-/-}$ conventional T cells may have enhanced survival due to a reduction in FasL, which has yet to be determined. This is supported by the *in vivo* observation that RasGRP1$^{-/-}$ mice develop a lymphoproliferative disorder and autoimmunity including autoantibodies, which could be due to a lack of AICD of T cells (Daley et al. 2013). Additionally in patients with lupus, there have been 13 splice variants for RasGRP1 detected, which resulted in reduced RasGRP1 expression (Yasuda et al. 2007). Patients with these splice variants have lymphoproliferative disorders. Another target of ERK is Bim, which has been shown to promote apoptosis of T cells (Stang et al. 2009). In our PICA model of apoptosis, conventional T cells deficient in Bim had enhanced survival (83% dead in WT mice vs 42% dead in Bim deficient T cells on day 4) (Singh et al. 2010). Therefore, we propose a model where high expression of RasGRP1 in conventional T cells enhances ERK signaling, which enhances FasL and Bim expression to promote apoptosis.

Control of RasGRP1 Expression

How RasGRP1 expression is regulated in conventional T cells is unknown. RasGRP1 signaling is thought to be critical for initial T cell activation, as ERK upregulates IL-2 transcription (Koike et al. 2003). RasGRP1 was shown to be the major Ras activator, responsible for downstream signaling that leads to ERK activation (Warnecke et al. 2012). Our data show that in conventional CD4 T cells, RasGRP1 expression is dramatically increased 3 days after stimulation, and this high expression is stable for 7 days post-stimulation (Figure 11). Further research will need to uncover how expression of RasGRP1 is regulated, and if T cells require sustained RasGRP1 signaling to promote proliferation and other effector functions. In a recent study, Riese et al investigated the role of RasGRP1/ERK signaling on CD8 T cell proliferation and cytokine production. They used mice deficient in a negative regulator of RasGRP1 activation (DGKζ), which resulted in robust Ras/ERK signaling and higher p-ERK after T cell activation (Riese et al. 2011). DGKζ deficient mice had decreased numbers of splenic CD8+ T cells, which based on our model could be due to enhanced RasGRP1/ERK signaling leading to enhanced T cell apoptosis. Additionally, these deficient mice produced enhanced IL-2 and IFN-γ and had higher rates of proliferation compared to WT mice after proliferation. These data suggest that RasGRP1 has a role in promoting effector functions, but sustained expression can trigger apoptosis. Investigating how RasGRP1 expression is sustained in T cells and what causes the switch between proliferation/cytokine production to apoptosis would be of great interest.

Crosstalk between TCR/RasGRP1 Pathway and TGF-β Signaling

How do Tregs maintain low RasGRP1 expression when given the same stimuli as conventional T cells? Our data suggests that Tregs utilize autocrine TGF-β signaling to suppress RasGRP1 expression, as addition of TGF-β can promote survival and suppress RasGRP1 expression in conventional T cells (Figure 11). Additionally, blocking autocrine TGF-β signaling enhances RasGRP1 expression in Tregs. This suggests that RasGRP1/ERK signaling pathway is cross talking with the TGF-β signaling pathway. Our lab previously identified Foxp3 binding sites in the RasGRP1 promoter (Dr. Mariko Takami, unpublished). Using a ChIP assay, Dr. Takami identified Forkhead consensus sites upstream of RasGRP1 that Foxp3 targets. It is unknown if binding of Foxp3 at these sites positively or negatively regulates RasGRP1 expression. Our hypothesis based on our results is that autocrine TGF-β signaling in Tregs promotes Foxp3 binding to the RasGRP1 promoter and suppressing RasGRP1 expression. Indeed, we saw that expanded Tregs have a significantly decreased level of RasGRP1 mRNA compared to expanded conventional T cells (Figure 10). This suggests that autocrine TGF-β signaling could suppress RasGRP1 expression at the level of transcription. An interesting implication of this data is that TGF-β signaling and low RasGRP1 expression in Tregs could be a primary mechanism that suppresses IL-2 production. Conventional T cells secrete IL-2 after activation, however Tregs do not produce their own IL-2 (Thornton and Shevach 1998). Hickman et al demonstrated that enhancing PKC and Ras pathways caused Tregs to produce IL-2 and proliferate at higher rates (Hickman et al. 2006). This suggests that Tregs could utilize autocrine TGF-β signaling to actively suppress the RasGRP1/ERK pathway to suppress IL-2

production and proliferation. Indeed, when TGF-β signaling was blocked in Tregs (Figure 13), not only was RasGRP1 expression enhanced, but the Tregs appeared to proliferate more and become more metabolically active, similar to conventional T cells (visual observation). High RasGRP1 expression in conventional T cells may enhance IL-2 and proliferation, but also can lead to activation-induced cell death when sustained RasGRP1 expression promotes apoptotic pathways. TGF-β is also known to be important in maintaining naïve T cell homeostasis and survival (Ouyang et al. 2013). In naïve T cells, TGF-β signaling could mediate suppression of RasGRP1 and ERK signaling to promote survival while also limiting cell proliferation in the absence of antigen stimulation.

TGF-β Promotes Survival of T Cells during AICD

TGF-β has been found to have pleiotropic effects on T cell function, depending on the context and the lifecycle of the T cell. During thymic selection, TGF-β promotes CD8 and Treg differentiation and survival (Li, Sanjabi, and Flavell 2006; Yongzhong Liu et al. 2008). In naïve T cells TGF-β promotes the differentiation of pTregs and Th17 cells, while inhibiting Th1 and Th2 differentiation (Gorelik, Fields, and Flavell 2000; Veldhoen et al. 2006). TGF-β also actively suppresses the proliferation of naïve and effector T cells (Shull et al. 1992). However during memory formation, TGF-β promotes the survival of antigen-specific memory T cells (Ma and Zhang 2015). How this cytokine can perform these context dependent functions remains unknown. Our data show that TGF-β promotes survival of CD4 and CD8 T cells that undergo continuous stimulation, which we believe mimics the process of antigen-specific T cells that survive continuous stimulation after the resolution of infection. Based on the signaling pathways that TGF-β

targets, this could also dramatically alter the metabolic state of T cells to promote

memory survival. Based on our data, the following model could be tested. During naïve

T cell homeostasis, TGF-β promotes survival of naïve T cells, and suppresses

RasGRP1 and glycolytic metabolic pathways to prevent proliferation in the absence of

antigen. During an infection, continuous TCR stimulation shuts down TGF-β signaling

processes, and allows T cells to upregulate RasGRP1, IL-2 production, and effector

functions. After resolution of infection, TGF-β signaling suppresses RasGRP1

expression, glycolytic activity, and promotes survival of memory T cells (Figure 36).
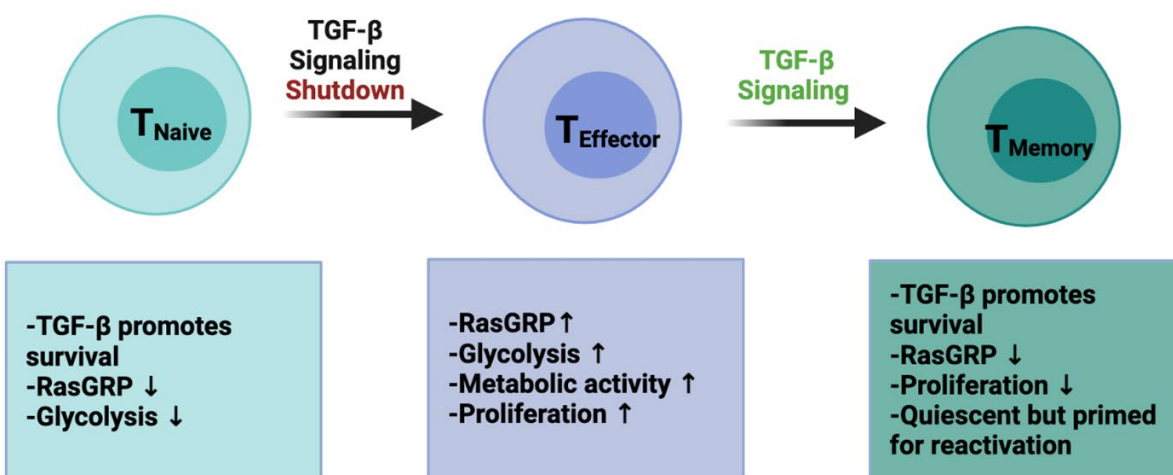
Figure 36. Proposed Model of RasGRP and TGF-β Signaling in T cell Lifecycle. In naïve T
cells, TGF-β is known to promote survival. Because naïve T cells have low levels of proliferation
and glycolysis, we propose that RasGRP1 signaling is low in naïve T cells to maintain
homeostatic proliferation. Once T cells are activated during infection, TGF-β signaling is known
to be significantly reduced, which is believed to promote high proliferation rates, glycolysis and
from our data high RasGRP1 expression. During memory formation, TGF-β signaling is known
to promote survival. Memory T cells are quiescent and have reduced proliferation, which we
propose is mediated in part by low RasGRP1 signaling.

Differences in CD4 and CD8 T Cells during PICA

During an infection, antigen-specific CD8 and CD8 T cells proliferate robustly and

perform cytolytic functions as well as secrete inflammatory cytokines and help activate

B cells to promote memory and antibody production. After the pathogen has been cleared, AICD occurs to reduce the size of the memory T cell pool and keep only a small number of memory T cells (5-10%), ready to fight future infection (Yang Liu and Janeway 1990; Webb, Morris, and Sprent 1990; Kawabe and Ochi 1991). During our model of AICD *in vitro* (PICA stimulation conditions), we see a striking difference between CD4 and CD8 T cell survival (Figure 25). On day 0, 150,000 cells were plated for both CD4 and CD8. With IL-2 alone, CD4 T cells have ~0.1 million cells alive by day 4, while CD8 T cells have between 3-6 million. TGF-β enhances both CD4 and CD8 survival, however there are ~0.8 million surviving CD4s, and ~10 million surviving CD8 T cells (Figure 25). This demonstrates that during continuous PICA stimulation, CD8 T cells divide more rapidly than CD4 T cells. Because CD4 T cells have very little survival in the absence of TGF-β, CD4 T cells may heavily rely on TGF-β signaling during memory formation. CD8 T cells have significantly higher cell numbers on day 4 after PICA stimulation than CD4s, as well as decreased frequency of dead cells. This suggests that CD8 T cells are more resistant to death during continuous stimulation, and may not rely on exogenous TGF-β as heavily as CD4 T cells for survival. This could be tested by directly comparing antigen-specific CD4 and CD8 T cell survival in the absence of TGF-β signaling during memory formation.

Crosstalk between FoxO3 and TGF-β Signaling

During PICA stimulation, we observed that FoxO3 deficient CD4 T cells have a defect in survival with exogenous TGF-β addition (Figure 18). This shows for the first time that in conventional T cells, FoxO3-TGF-β signaling pathways crosstalk to promote survival. In T cells, it is currently not known how FoxO3, which is controlled by TCR

stimulation and PI3K pathways, can crosstalk with the TGF-β signaling pathway. In non-lymphoid cells such as keratinocytes, FoxO3 can directly interact with smad transcription factors to mediate TGF-β signaling (Seoane et al. 2004; Gomis et al. 2006). Further investigation is needed to see if FoxO3 also binds smads in T cells to help mediate TGF-β signaling. In Tregs, FoxO1 and FoxO3 were shown to transcriptionally promote Foxp3 expression, which also suggests that FoxO3 could mediate TGF-β signaling in conventional T cells. Previous work from our lab showed that TGF-β does not upregulate Foxp3 during PICA stimulation of conventional CD4 T cells (Takami, Love, and Iwashima 2012). Therefore, we hypothesize that FoxO3 is targeting TGF-β signaling processes independent of Foxp3 expression to promote survival.

FoxO3 was found to have binding sites in the RasGRP1 promoter, although it is unknown if FoxO3 enhances or suppresses RasGRP1 expression in T cells (Joseph et al. 2016). Since TGF-β reduces RasGRP1 expression, one possibility is that after TGF-β addition, FoxO3 binds to the RasGRP1 promoter (alone or in conjunction with smads) and suppresses expression of RasGRP1. Based on our model, reduced RasGRP1 expression could subsequently promote survival of the conventional T cells. During T cell activation, FoxO3 has also been shown to promote transcription of the pro-apoptotic protein Bim (Stahl et al. 2002). Our data suggests that FoxO3 is not required for death during PICA stimulation (Figure 17). FoxO3 still could be contributing to death, but in the absence of FoxO3 other apoptotic signaling pathways are still sufficient for death. We previously showed that during PICA stimulation, TGF-β can reduce expression of Bim, which we predict supports survival of T cells and blocks apoptosis (Takami, Love, and

Iwashima 2012). Since FoxO3 is critical for promoting survival with TGF-β signaling, we hypothesize that FoxO3 promotes Bim and RasGRP in the absence of TGF-β signaling, and suppresses their expression when TGF-β signaling is active. These differential effects of FoxO3 could be mediated through the interaction of FoxO3 with distinct transcription factors in the presence or absence of TGF-β signaling. Further research is needed to determine if FoxO3 has distinct binding partners (activators/repressors) to initiate these different outcomes, and/or if FoxO3 can bind to different sites in the promoters of these genes to affect transcription.

CD44 High T Cells in FoxO3 Deficient Mice

To study T cells that are continuously stimulated *in vivo*, we looked at the small intestine intraepithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL). T cells in the gut are constantly exposed to commensal and food antigens, and therefore we reasoned this would mimic our *in vitro* PICA continuous plate-bound stimulation. When we examined the gut IEL and LPL, we found that FoxO3 deficient T cells have enhanced levels of CD44 (Figure 21). This was specific to CD44 and not for other activation markers, CD25 and CD69 (memory and activation marker) (Figure 23). Additionally, as reported previously, (Dejean et al. 2009) we saw no differences in the MLN and spleen in CD44 expression (Figures 22 and 24). Therefore, CD44^{high} T cells in FoxO3 KO mice are specific to the gut. When examining these CD44^{high} cells, we gated on CD45 (hematopoietic marker), CD3 (marker for T cells) and then looked at CD44 expression. In the gut, CD3 T cells contain a significant fraction of γδ TCR CD3$^+$ T cells as well as αβ CD3$^+$ T cells. We are actively investigating if both populations have CD44 high expression, or if this is specific to one TCR subset. Although the distinct functions

of γδ and αβ T cells in the gut are still being actively investigated, γδ T cells classically respond to antigens that include lipids and heat shock proteins, rather than peptide antigens like αβ T cells. Therefore, it would be interesting to determine if FoxO3 deficiency affects one subset or both.

CD44 is highly expressed upon T cell activation, and is maintained in memory T cells (Budd et al. 1987). Our data show that CD69, a tissue-retention marker, is also expressed on these T cells (Figure 23). This could suggest that these CD44 high T cells are memory T cells, but since CD44 and CD69 are also expressed upon T cell activation, more research is needed to determine if these are effector T cells or more memory type T cells. These T cells could also be tissue-resident or effector/central memory T cells. Additionally, CD44 has been shown to be critical for Th1 memory effector T cell survival (Baaten et al. 2010). It would be interesting to determine functionally what type of CD4 Th cells are CD44 high in the FoxO3 KO and WT mice, as well as the functionality of the CD8 T cells that have high CD44 expression. *In vitro*, we determined that FoxO3 KO splenic T cells have similar levels of CD44 expression upon T cell activation, suggesting that FoxO3 deficiency does not alter activation-induced CD44 expression in the periphery (Figure 24). More research will need to be conducted to determine if FoxO3 affects CD44 expression differently in the gut compared to the peripheral T cell populations.

Crosstalk between TGF-β Signaling and CD44?

CD44 is not only a marker of T cell activation and memory, but also can promote AICD of T cells. Crosslinking CD44 with antibodies or with its ligand HA can promote FasL expression and lead to AICD (Nakano et al. 2007). TGF-β signaling can protect T

cells from AICD, and there is high TGF-β in the gut environment. Our data show that FoxO3 deficient CD4 T cells have a defect in responding to TGF-β signaling *in vitro*, and we see that FoxO3 T cells have high CD44 expression in the gut. One intriguing hypothesis based on these data is that TGF-β signaling suppresses CD44 expression to protect T cells from AICD, especially in the gut environment. If this is the case, then FoxO3 KO T cells would maintain high CD44 expression due to their defect in responding to TGF-β signaling. To test this, we can determine if TGF-β suppresses CD44 expression in peripheral and gut T cells, and determine if FoxO3 KO T cells have a defect in reducing CD44 expression in response to TGF-β. Further investigation is needed to determine if there is a link between CD44 expression or survival of CD44 cells and TGF-β signaling.

CAR T Cell AICD

CAR T cells are efficiently generated and activated by tumor antigens, and have been very effective for the treatment of some cancers, particularly hematologic cancers. One problem, however, is that CAR T cells often do not persist *in vivo*, especially for solid tumors where CARs have been shown to undergo AICD (H. Wang et al. 2021; Tschumi et al. 2018). Currently, knowledge of the mechanisms of CAR T cell death and strategies to promote CAR T cell survival are limited. We developed a system to study AICD in CAR T cells by stimulating CARs with a high number of antigen positive tumor cells (1:20 CAR to Raji tumor cells). In our system, we see that when CAR T cells are given an equal number of Rajis (1:1), the cells proliferate robustly, and have low expression of Foxp3 (Figure 30). When CAR T cells are stimulated 1:20 with Raji, we

see that there is significantly decreased survival, and a proportion of the surviving CAR T cells are CD25$^+$Foxp3$^+$ (Figures 29 and 30).

Our data demonstrate that CAR T cells that are continuously stimulated (1:20 CAR to Raji) are phenotypically distinct from CARs that receive less stimulation (1:1 CAR to Raji). What does the phenotype of CD25$^+$Foxp3$^+$ mean for CAR T cell functionality? CD25 is the alpha chain of the IL-2 receptor, and IL-2 is critical for T cell activation, proliferation, and survival (Morgan, Ruscetti, and Gallo 1976; Taniguchi et al. 1983). In the 1:1 condition with Raji, some donors show CD25 expression while others do not. In the 1:20 CAR to Raji conditions, almost all cells are CD25+. We predict that this is due to the continuous stimulation of the CAR T cells, and is indicative of strong T cell activation, since CD25 is upregulated in T cells upon stimulation (Cerdan et al. 1992). CD25 expression and IL-2 signaling is critical for AICD, and blocking CD25 prevents AICD in T cells (Richter et al. 2009; Lenardo 1991). Therefore, CAR T cells with high CD25 expression during continuous stimulation with Rajis may promote IL-2-dependent AICD. This could be tested by blocking IL-2 or CD25 in our system and measuring survival of CARs. CAR T cells with Rajis at a 1:1 ratio are for the most part able to kill Raji targets after 1 day (observations under microscope). So, we predict based on our data that CD25 expression is up days 1-2 post-activation, then goes back down by day 5. We predict that since CARs with 1:20 are continuously stimulated (and unable to kill all the Rajis), their CD25 expression is sustained. To test this hypothesis, we would need to determine the kinetics of CD25 expression with low and high dose Raji, and also determine if IL-2 signaling and CD25 expression is required for decreased survival of CARs at the 1:20 ratio.

Foxp3 Expression in CAR T Cells

A significant portion of surviving CAR T cells after stimulation with a ratio of 1:20 CAR:Rajis are Foxp3$^+$. Foxp3 is the canonical transcription factor that controls Treg development and function (Fontenot, Gavin, and Rudensky 2003; Hori, Nomura, and Sakaguchi 2003). However, Foxp3 is not only expressed in Tregs, but is upregulated in human T cells upon T cell activation. During continuous stimulation of conventional CD4 T cells with plate-bound anti-CD3 and soluble anti-CD28, a majority of T cells upregulate Foxp3 one day post-activation (J. Wang et al. 2007). If T cells are maintained with antibody stimulation for 7 days, almost 100% of T cells express Foxp3 at the 7-day time point. This suggests that continuous stimulation of T cells promotes Foxp3 expression. Wang et al sought to determine if this expression of Foxp3 was stable or transient. If the stimulated conventional CD4 T cells are rested without stimulation for 7 additional days in IL-2, Foxp3 expression was dramatically reduced. However, stimulated Tregs maintain high, stable Foxp3 expression after resting for 7 days. In our CAR system, we see high Foxp3 expression when CAR T cells are stimulated at the 1:20 ratio (CAR:Raji) (Figure 30). It would be of interest to test if this expression of Foxp3 is stable, or if it is transient and due to activation. If Foxp3 is stable, that would suggest that continuous antigen stimulation can program T cells to become more "Treg-like" and could diminish their effector cytokine production and functionality. However, if Foxp3 is transient, these T cells could potentially still elicit effector functions and kill tumor targets. This could help explain a mechanism by which CAR T cells have difficulty surviving and promoting anti-tumor immunity in solid tumors. If majority of CAR T cells in the TME undergo AICD after repeated stimulation and the surviving T cells

express Foxp3 *in vivo*, we could test if these CAR T cells are functionally impaired upon tumor re-challenge *ex vivo.* Additionally, we could track Foxp3 expression in CAR T cells in the TME and determine the kinetics of Foxp3 and CD25 expression, and see if this affects functionality.

RasGRP1 in CAR T Cell Death?

Our data suggest that CAR T cells can undergo AICD when continuously stimulated by tumor cells. Because we determined that RasGRP1 can promote AICD in mouse conventional T cells (Figure 14), sustained RasGRP1 signaling may also promote AICD of CAR T cells. The kinetics of RasGRP1 expression and ERK signaling is unknown in CAR T cells, but based on our mouse T cell data (Figure 11), we would expect RasGRP1 expression to be low in unstimulated CAR T cells, and to be expressed by day 3 after continuous stimulation. We hypothesize that this sustained RasGRP1 signaling could promote Fas/FasL mediated apoptosis, as CAR T cells have been shown to undergo AICD by Fas/FasL-mediated signaling during continuous stimulation (Tschumi et al. 2018). In the situation where CAR T cells are stimulated with low numbers of Raji (1:1), CAR T cells are not continuously stimulated due to their ability to kill the Raji tumor targets after ~1 day. We would expect CAR T cells stimulated with these conditions to maintain lower levels of RasGRP1 than the 1:20 conditions, due to the lack of continuous stimulation.

Our data support the hypothesis that inhibition of RasGRP1 signaling would enhance CAR T cell survival during continuous stimulation. To test this, we constructed CAR T cells that contained a DN RasGRP1, with a point mutation R271E that has been shown to prevent Ras activation and subsequent MAPK/ERK signaling in B cells

(Guilbault and Kay 2004). A self-cleaving P2A peptide followed by DN RasGRP1 was inserted after CD3 zeta in the CAR construct. The CAR, DN RasGRP1, and truncated CD34 marker were all under control of the same promoter, so if CD34 is expressed, so is the CAR and the DN RasGRP1. We were able to express these CARs in Jurkat T cells and show that they are functional after Raji stimulation (by activation marker and cytokine expression). However, due to the size of the CAR construct with the inserted RasGRP1, it was very difficult to transduce primary T cells and we were unable to obtain a sufficient number of CARs to test in our assays. If this transduction could be further optimized, we would expect that DN RasGRP1 CARs would survive high dose Raji stimulation better than CAR alone. Additionally, if we put a different marker on the DN RasGRP1 CAR (such as CD14 rather than CD34), we could co-inject the DN RasGRP1 CAR or regular CAR into NSG mice engrafted with human B cell tumors. We could then test if DN RasGRP1 CARs have enhanced survival and functionality *in vivo* compared to control CARs. Optimizing CAR T cell construction is of great interest, as the choice of costimulatory domains, transmembrane domains and other features of the CARs have been shown to affect survival and functionality. For instance, 4-1BB CARs show enhanced survival and decreased AICD compared to CD28 based CARs (Guedan et al. 2018). Therefore, combining DN RasGRP1 CARs with 4-1BB co-stimulation could promote greater T cell survival in solid tumor models.

Role of TGF-β and Caspase Signaling in CAR T Cell Survival

In our model of PICA with continuous stimulation of conventional T cells, TGF-β can rescue cell death (Takami, Love, and Iwashima 2012). Our data demonstrate that addition of TGF-β to CAR T cells can also enhance survival when stimulated with high

numbers of tumor cells (1:20 CAR to Raji) (Figure 33). However, when stimulated with low numbers of tumor cells (1:1), TGF-β mediates T cell suppression. How does TGF-β signaling cause massive suppression of T cells at the 1:1 ratio but survival at 1:20 CAR to Raji ratio? This is currently unknown. Because there are so many signaling pathways that can be affected by TGF-β signaling in T cells, RNA seq analysis could be beneficial for determining pathways that are altered in CAR T cells treated with TGF-β at 1:1 compared to 1:20 CAR to Raji.

When we examined expression of activation markers, we saw that TGF-β enhances CTLA-4 expression and suppresses CD25 expression compared to 1:20 CAR to Raji with no TGF-β addition (Figure 34). So, although the CAR T cells are surviving better with TGF-β, upregulation of the checkpoint inhibitor CTLA-4 could prevent efficient tumor killing and cytokine production. Additionally, suppression of CD25 could prevent IL-2 signaling and proliferation. Blocking TGF-β signaling in tumor-specific T cells can enhance anti-tumor immunity (Gorelik and Flavell 2000; Maeda and Shiraishi 1996), and our data suggest that this could be mediated through enhancing CD25 expression and suppressing CTLA-4 expression.

When CAR T cells were treated with a pan-caspase inhibitor at the 1:20 CAR to Raji, we also saw enhanced survival of CAR T cells (Figure 35). Our data suggest that decreased survival of CAR T cells we see with 1:20 (CAR to Raji) is mediated at least in part by caspase-dependent apoptosis. However, unlike addition of TGF-β, the CAR T cells were phenotypically similar to 1:20 with DMSO control. We see similar levels of CTLA-4, CD25, and Foxp3. This suggests that caspase inhibition does not alter the phenotype of CAR T cells, at least for the markers tested. The effect of caspase

inhibitors on improving CAR T cell survival *in vivo* is unknown. More rigorous studies would need to occur, measuring how long survival lasts, and if the CAR T cells are functionally different after treatment.

Concluding Remarks

T cells must integrate a variety of signals to promote their activation, differentiation, proliferation, and survival. Integrating these signals controls the balance between Tregs and conventional T cells, during states of homeostasis and disease. Tregs are constantly exposed to self-antigens and commensals, yet Treg populations remained balanced in healthy individuals. We propose that Tregs are able to resist AICD during repeated stimulation through autocrine TGF-β signaling and the suppression of the RasGRP1/MAPK pathways. After infection or immunization, T cells are activated and proliferate to help clear the pathogen and form memory. During the resolution of infection, AICD reduces the numbers of effector T cells and promotes memory formation. Our results show that high RasGRP1 signaling can promote apoptosis during AICD. TGF-β is crucial for protecting T cells from AICD. We found that TGF-β suppresses RasGRP1 expression and FoxO3 and TGF-β signaling pathways integrate to promote T cell survival (Figure 37). Understanding the mechanisms that control AICD in T cells is critical for our understanding of memory formation after infection. Additionally, understanding these signaling pathways in tumor-specific T cells like CAR T cells can inform strategies to enhance survival and persistence.
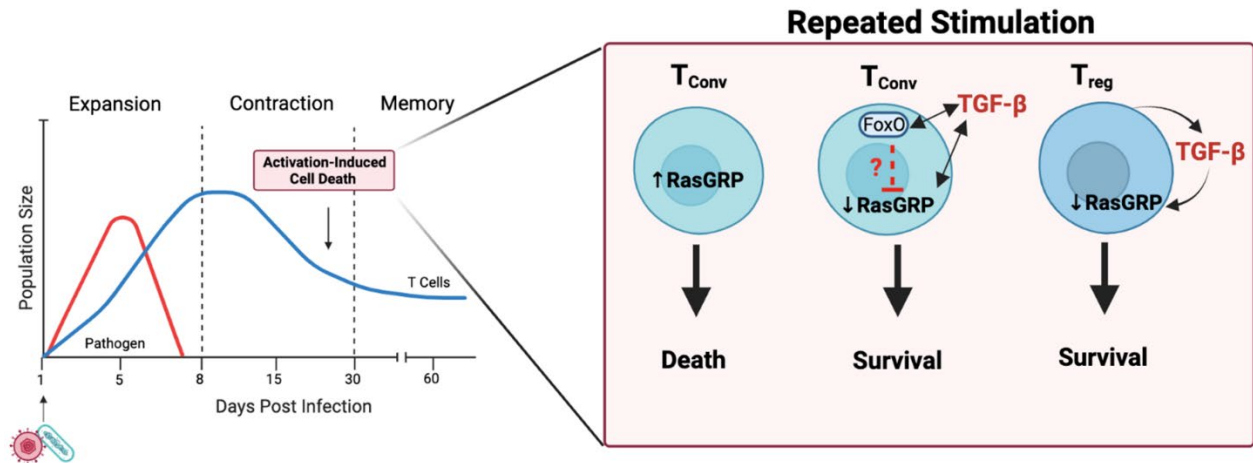
Figure 37. Model for Signaling Mechanisms that Control AICD. High RasGRP1 signaling in conventional T cells promotes AICD, while TGF-β can protect T cells from death. TGF-β signaling crosstalks with FoxO3 signaling to promote survival, and TGF-β suppresses RasGRP1 expression. Tregs are able to resist AICD through autocrine TGF-β signaling, which suppresses RasGRP1 expression.

REFERENCE LIST

Aaltonen, Johanna, Petra Björses, Jaakko Perheentupa, Nina Horelli-Kuitunen, Aarno Palotie, Leena Peltonen, Yeon Lee Su, et al. 1997. "An Autoimmune Disease, APECED, Caused by Mutations in a Novel Gene Featuring Two PHD-Type Zinc-Finger Domains." *Nature Genetics* 17 (4): 399–403. https://doi.org/10.1038/ng1297-399.

Abbas, Abul K., Kenneth M. Murphy, and Alan Sher. 1996. "Functional Diversity of Helper T Lymphocytes." *Nature*. https://doi.org/10.1038/383787a0.

Abdollah, Shirin, Marina Macías-Silva, Tomoo Tsukazaki, Hidetoshi Hayashi, Liliana Attisano, and Jeffrey L. Wrana. 1997. "TβRI Phosphorylation of Smad2 on Ser465 and Ser467 Is Required for Smad2-Smad4 Complex Formation and Signaling." *Journal of Biological Chemistry* 272 (44): 27678–85. https://doi.org/10.1074/jbc.272.44.27678.

Ahmadzadeh, Mojgan, and Steven A. Rosenberg. 2005. "TGF-B1 Attenuates the Acquisition and Expression of Effector Function by Tumor Antigen-Specific Human Memory CD8 T Cells." *The Journal of Immunology* 174 (9): 5215–23. https://doi.org/10.4049/jimmunol.174.9.5215.

Allison, James P., Arthur A. Hurwitz, and Dana R. Leach. 1995. "Manipulation of Costimulatory Signals to Enhance Antitumor T-Cell Responses." *Current Opinion in Immunology* 7 (5): 682–86. https://doi.org/10.1016/0952-7915(95)80077-8.

Andreasen, Susanne Ørding, Jeanette Erbo Christensen, Ole Marker, and Allan Randrup Thomsen. 2000. " Role of CD40 Ligand and CD28 in Induction and Maintenance of Antiviral CD8 + Effector T Cell Responses ." *The Journal of Immunology* 164 (7): 3689–97. https://doi.org/10.4049/jimmunol.164.7.3689.

Anel, Alberto, Michel Buferne, Claude Boyer, Anne-Marie -M Schmitt-Verhulst, and Pierre Golstein. 1994. "T Cell Receptor-induced Fas Ligand Expression in Cytotoxic T Lymphocyte Clones Is Blocked by Protein Tyrosine Kinase Inhibitors and Cyclosporin A." *European Journal of Immunology* 24 (10): 2469–76. https://doi.org/10.1002/eji.1830241032.

Ariel, A., O. Lider, A. Brill, L. Cahalon, N. Savion, D. Varon, and R. Hershkoviz. 2000. "Induction of Interactions between CD44 and Hyaluronic Acid by a Short Exposure of Human T Cells to Diverse Pro-Inflammatory Mediators." *Immunology* 100 (3): 345–51. https://doi.org/10.1046/j.1365-2567.2000.00059.x.

Aspinall, A. I., A. Pinto, I. A. Auer, P. Bridges, J. Luider, L. Dimnik, K. D. Patel, K. Jorgenson, and R. C. Woodman. 1999. "Identification of New Fas Mutations in a Patient with Autoimmune Lymphoproliferative Syndrome (ALPS) and Eosinophilia." *Blood Cells, Molecules, and Diseases* 25 (4): 227–38. https://doi.org/10.1006/bcmd.1999.0248.

Asseman, Chrystelle, Smita Mauze, Michael W. Leach, Robert L. Coffman, and Fiona Powrie. 1999. "An Essential Role for Interleukin 10 in the Function of Regulatory T Cells That Inhibit Intestinal Inflammation." *Journal of Experimental Medicine* 190 (7): 995–1003. https://doi.org/10.1084/jem.190.7.995.

Atkuri, Kondala R., Leonard A. Herzenberg, and Leonore A. Herzenberg. 2005. "Culturing at Atmospheric Oxygen Levels Impacts Lymphocyte Function." *Proceedings of the National Academy of Sciences of the United States of America* 102 (10): 3756–59. https://doi.org/10.1073/pnas.0409910102.

Baars, Matthijs J.D., Thera Douma, Dimitre R. Simeonov, Darienne R. Myers, Kayla Kulhanek, Saikat Banerjee, Susan Zwakenberg, et al. 2021. "Dysregulated RASGRP1 Expression through RUNX1 Mediated Transcription Promotes Autoimmunity." *European Journal of Immunology* 51 (2): 471–82. https://doi.org/10.1002/eji.201948451.

Baaten, Bas J.G., Cheng Rui Li, Mia F. Deiro, Melissa M. Lin, Phyllis J. Linton, and Linda M. Bradley. 2010. "CD44 Regulates Survival and Memory Development in Th1 Cells." *Immunity* 32 (1): 104–15. https://doi.org/10.1016/j.immuni.2009.10.011.

Baban, Babak, Phillip R. Chandler, Madhav D. Sharma, Jeanene Pihkala, Pandelakis A. Koni, David H. Munn, and Andrew L. Mellor. 2009. "IDO Activates Regulatory T Cells and Blocks Their Conversion into Th17-Like T Cells." *The Journal of Immunology* 183 (4): 2475–83. https://doi.org/10.4049/jimmunol.0900986.

Bajorath, Jürgen, Brad Greenfield, Sandra B. Munro, Anthony J. Day, and Alejandro Aruffo. 1998. "Identification of CD44 Residues Important for Hyaluronan Binding and Delineation of the Binding Site." *Journal of Biological Chemistry* 273 (1): 338–43. https://doi.org/10.1074/jbc.273.1.338.

Bakin, Andrei V., Cammie Rinehart, Anne K. Tomlinson, and Carlos L. Arteaga. 2002. "P38 Mitogen-Activated Protein Kinase Is Required for TGFβ-Mediated Fibroblastic Transdifferentiation and Cell Migration." *Journal of Cell Science* 115 (15): 3193–3206. https://doi.org/10.1242/jcs.115.15.3193.

Banerji, Suneale, Anthony J. Day, Jan D. Kahmann, and David G. Jackson. 1998. "Characterization of a Functional Hyaluronan-Binding Domain from the Human CD44 Molecule Expressed in Escherichia Coil." *Protein Expression and Purification* 14 (3): 371–81. https://doi.org/10.1006/prep.1998.0971.

Barber, Daniel L., E. John Wherry, David Masopust, Baogong Zhu, James P. Allison, Arlene H. Sharpe, Gordon J. Freeman, and Rafi Ahmed. 2006. "Restoring Function in Exhausted CD8 T Cells during Chronic Viral Infection." *Nature* 439 (7077): 682–87. https://doi.org/10.1038/nature04444.

Baskar, Sivasubramanian, Suzanne Ostrand-Rosenberg, Nasrin Nabavi, Lee M. Nadler, Gordon J. Freeman, and Laurie H. Glimcher. 1993. "Constitutive Expression of B7 Restores Immunogenicity of Tumor Cells Expressing Truncated Major Histocompatibility Complex Class II Molecules." *Proceedings of the National Academy of Sciences of the United States of America* 90 (12): 5687–90. https://doi.org/10.1073/pnas.90.12.5687.

Bennett, Craig L., Jacinda Christie, Fred Ramsdell, Mary E. Brunkow, Polly J. Ferguson, Luke Whitesell, Thaddeus E. Kelly, Frank T. Saulsbury, Phillip F. Chance, and Hans D. Ochs. 2001. "The Immune Dysregulation, Polyendocrinopathy, Enteropathy, X-Linked Syndrome (IPEX) Is Caused by Mutations of FOXP3." *Nature Genetics* 27 (1): 20–21. https://doi.org/10.1038/83713.

Bjorkman, P. J., M. A. Saper, B. Samraoui, W. S. Bennett, J. L. Strominger, and D. C. Wiley. 1987. "Structure of the Human Class I Histocompatibility Antigen, HLA-A2." *Nature* 329 (6139): 506–12. https://doi.org/10.1038/329506a0.

Boehme, S A, and M J Lenardo. 1996. "TCR-Mediated Death of Mature T Lymphocytes Occurs in the Absence of P53." *Journal of Immunology (Baltimore, Md. : 1950)* 156 (11): 4075–78. http://www.ncbi.nlm.nih.gov/pubmed/8666771.

Brabletz, T, I Pfeuffer, E Schorr, F Siebelt, T Wirth, and E Serfling. 1993. "Transforming Growth Factor Beta and Cyclosporin A Inhibit the Inducible Activity of the Interleukin-2 Gene in T Cells through a Noncanonical Octamer-Binding Site." *Molecular and Cellular Biology* 13 (2): 1155–62. https://doi.org/10.1128/mcb.13.2.1155.

Brentjens, Renier J., Jean Baptiste Latouche, Elmer Santos, Francesc Marti, Michael C. Gong, Clay Lyddane, Philip D. King, et al. 2003. "Eradication of Systemic B-Cell Tumors by Genetically Targeted Human T Lymphocytes Co-Stimulated by CD80 and Interleukin-15." *Nature Medicine* 9 (3): 279–86. https://doi.org/10.1038/nm827.

Brichard, Vincent, Aline Van Pel, Thomas Wölfel, Catherine Wölfel, Etienne De Plaen, Bernard Lethé, Pierre Coulie, and Thierry Boon. 1993. "The Tyrosinase Gene Codes for an Antigen Recognized by Autologous Cytolytic T Lymphocytes on HLA-A2 Melanomas." *Journal of Experimental Medicine* 178 (2): 489–95. https://doi.org/10.1084/jem.178.2.489.

Brown, Christine E., Darya Alizadeh, Renate Starr, Lihong Weng, Jamie R. Wagner, Araceli Naranjo, Julie R. Ostberg, et al. 2016. "Regression of Glioblastoma after Chimeric Antigen Receptor T-Cell Therapy." *New England Journal of Medicine* 375 (26): 2561–69. https://doi.org/10.1056/nejmoa1610497.

Bruggen, P. Van Der, C. Traversari, P. Chomez, C. Lurquin, E. De Plaen, B. Van Den Eynde, A. Knuth, and T. Boon. 1991. "A Gene Encoding an Antigen Recognized by Cytolytic T Lymphocytes on a Human Melanoma." *Science* 254 (5038): 1643–47. https://doi.org/10.1126/science.1840703.

Brunet, Anne, Azad Bonni, Michael J. Zigmond, Michael Z. Lin, Peter Juo, Linda S. Hu, Michael J. Anderson, Karen C. Arden, John Blenis, and Michael E. Greenberg. 1999. "Akt Promotes Cell Survival by Phosphorylating and Inhibiting a Forkhead Transcription Factor." *Cell* 96 (6): 857–68. https://doi.org/10.1016/S0092-8674(00)80595-4.

Brunet, Jean Fraṇois, Fraṇois Denizot, Marie Fraṇoise Luciani, Magali Roux-Dosseto, Marie Suzan, Marie Geneviève Mattei, and Pierre Golstein. 1988. "A New Member of the Immunoglobulin Superfamily-CTLA-4." *Nature* 328 (6127): 267–70. https://doi.org/10.1038/328267a0.

Brunkow, Mary E., Eric W. Jeffery, Kathryn A. Hjerrild, Bryan Paeper, Lisa B. Clark, Sue Ann Yasayko, J. Erby Wilkinson, David Galas, Steven F. Ziegler, and Fred Ramsdell. 2001. "Disruption of a New Forkhead/Winged-Helix Protein, Scurfin, Results in the Fatal Lymphoproliferative Disorder of the Scurfy Mouse." *Nature Genetics* 27 (1): 68–73. https://doi.org/10.1038/83784.

Budd, R C, J C Cerottini, C Horvath, C Bron, T Pedrazzini, R C Howe, and H R MacDonald. 1987. "Distinction of Virgin and Memory T Lymphocytes. Stable Acquisition of the Pgp-1 Glycoprotein Concomitant with Antigenic Stimulation." *The Journal of Immunology* 138 (10).

Burchill, Matthew A., Jianying Yang, Kieng B. Va James J. Moon, H. Hamlet Chu, Chan Wang J. Lio, Amanda L. Vegoe, Chyi Song Hsieh, Marc K. Jenkins, and Michael A. Farrar. 2008. "Linked T Cell Receptor and Cytokine Signaling Govern the Development of the Regulatory T Cell Repertoire." *Immunity* 28 (1): 112–21. https://doi.org/10.1016/j.immuni.2007.11.022.

Burchill, Matthew A., Jianying Yang, Christine Vogtenhuber, Bruce R. Blazar, and Michael A. Farrar. 2007. " IL-2 Receptor β-Dependent STAT5 Activation Is Required for the Development of Foxp3 + Regulatory T Cells ." *The Journal of Immunology* 178 (1): 280–90. https://doi.org/10.4049/jimmunol.178.1.280.

Burr, John S., Nigel D. L. Savage, Grace E. Messah, Stephanie L. Kimzey, Andrey S. Shaw, Robert H. Arch, and Jonathan M. Green. 2001. " Cutting Edge: Distinct Motifs Within CD28 Regulate T Cell Proliferation and Induction of Bcl-X L ." *The Journal of Immunology* 166 (9): 5331–35. https://doi.org/10.4049/jimmunol.166.9.5331.

Burtrum, D B, S Kim, E C Dudley, A C Hayday, and H T Petrie. 1996. "TCR Gene Recombination and Alpha Beta-Gamma Delta Lineage Divergence: Productive TCR-Beta Rearrangement Is Neither Exclusive nor Preclusive of Gamma Delta Cell Development." *Journal of Immunology (Baltimore, Md. : 1950)* 157 (10): 4293–96. http://www.ncbi.nlm.nih.gov/pubmed/8906802.

Caldwell, Charles C., Hidefumi Kojima, Dmitriy Lukashev, John Armstrong, Mark Farber, Sergey G. Apasov, and Michail V. Sitkovsky. 2001. "Differential Effects of Physiologically Relevant Hypoxic Conditions on T Lymphocyte Development and Effector Functions." *The Journal of Immunology* 167 (11): 6140–49. https://doi.org/10.4049/jimmunol.167.11.6140.

Cao, Xuefang, Sheng F. Cai, Todd A. Fehniger, Jiling Song, Lynne I. Collins, David R. Piwnica-Worms, and Timothy J. Ley. 2007. "Granzyme B and Perforin Are Important for Regulatory T Cell-Mediated Suppression of Tumor Clearance." *Immunity* 27 (4): 635–46. https://doi.org/10.1016/j.immuni.2007.08.014.

Carpenito, Carmine, Michael C. Milone, Raffit Hassan, Jacqueline C. Simonet, Mehdi Lakhal, Megan M. Suhoski, Angel Varela-Rohena, et al. 2009. "Control of Large, Established Tumor Xenografts with Genetically Retargeted Human T Cells Containing CD28 and CD137 Domains." *Proceedings of the National Academy of Sciences of the United States of America* 106 (9): 3360–65. https://doi.org/10.1073/pnas.0813101106.

Cerdan, C, Y Martin, M Courcoul, H Brailly, C Mawas, F Birg, and D Olive. 1992. "Prolonged IL-2 Receptor Alpha/CD25 Expression after T Cell Activation via the Adhesion Molecules CD2 and CD28. Demonstration of Combined Transcriptional and Post-Transcriptional Regulation." *The Journal of Immunology* 149 (7).

Cerwenka, Adelheid, Heinrich Kovar, Otto Majdic, and Wolfgang Holter. 1996. "Fas- and Activation-Induced Apoptosis Are Reduced in Human T Cells Preactivated in the Presence of TGF-B1." *Journal of Immunology* 156 (2): 459–64.

Chan, Andrew C., Mark Dalton, Robin Johnson, Guang Hui Kong, Tian Wang, Rich Thoma, and Tomohiro Kurosaki. 1995. "Activation of ZAP-70 Kinase Activity by Phosphorylation of Tyrosine 493 Is Required for Lymphocyte Antigen Receptor Function." *EMBO Journal* 14 (11): 2499–2508. https://doi.org/10.1002/j.1460-2075.1995.tb07247.x.

Chang, Chih Hao, Jonathan D. Curtis, Leonard B. Maggi, Brandon Faubert, Alejandro V. Villarino, David O'Sullivan, Stanley Ching Cheng Huang, et al. 2013. "Posttranscriptional Control of T Cell Effector Function by Aerobic Glycolysis." *Cell* 153 (6): 1239. https://doi.org/10.1016/j.cell.2013.05.016.

Chen, Chunxia, Emily A. Rowell, Rajan M. Thomas, Wayne W. Hancock, and Andrew D. Wells. 2006. "Transcriptional Regulation by Foxp3 Is Associated with Direct Promoter Occupancy and Modulation of Histone Acetylation." *Journal of Biological Chemistry* 281 (48): 36828–34. https://doi.org/10.1074/jbc.M608848200.

Chen, Lieping, Stephanie Ashe, William A. Brady, Ingegerd Hellström, Karl Erik Hellström, Jeffrey A. Ledbetter, Patrick McGowan, and Peter S. Linsley. 1992. "Costimulation of Antitumor Immunity by the B7 Counterreceptor for the T Lymphocyte Molecules CD28 and CTLA-4." *Cell* 71 (7): 1093–1102. https://doi.org/10.1016/S0092-8674(05)80059-5.

Chen, Ruey Hwa, Päivi J. Miettinen, E. Miko Maruoka, Lisa Choy, and Rik Derynck. 1995. "A WD-Domain Protein That Is Associated with and Phosphorylated by the Type II TGF-β Receptor." *Nature*. Nature Publishing Group. https://doi.org/10.1038/377548a0.

Chen, Wan Jun, Wenwen Jin, Neil Hardegen, Ke Jian Lei, Li Li, Nancy Marinos, George McGrady, and Sharon M. Wahl. 2003. "Conversion of Peripheral CD4+CD25- Naive T Cells to CD4+CD25+ Regulatory T Cells by TGF-β Induction of Transcription Factor Foxp3." *Journal of Experimental Medicine* 198 (12): 1875–86. https://doi.org/10.1084/jem.20030152.

Chen, Xiaoxi, John J. Priatel, Michael T. Chow, and Hung-Sia Teh. 2008. "Preferential Development of CD4 and CD8 T Regulatory Cells in RasGRP1-Deficient Mice." *The Journal of Immunology* 180 (9): 5973–82. https://doi.org/10.4049/jimmunol.180.9.5973.

Chong, Elise A., J. Joseph Melenhorst, Simon F. Lacey, David E. Ambrose, Vanessa Gonzalez, Bruce L. Levine, Carl H. June, and Stephen J. Schuster. 2017. "PD-1 Blockade Modulates Chimeric Antigen Receptor (CAR)-Modified T Cells: Refueling the CAR." *Blood*. American Society of Hematology. https://doi.org/10.1182/blood-2016-09-738245.

Cohen, P. L., and R. A. Eisenberg. 1991. "Lpr and Gld: Single Gene Models of Systemic Autoimmunity and Lymphoproliferative Disease." *Annual Review of Immunology*. https://doi.org/10.1146/annurev.iy.09.040191.001331.

Correia, J. J., B. M. Chacko, S. S. Lam, and K. Lin. 2001. "Sedimentation Studies Reveal a Direct Role of Phosphorylation in Smad3:Smad4 Homo- and Hetero-Trimerization." *Biochemistry* 40 (5): 1473–82. https://doi.org/10.1021/bi0019343.

Cottrez, Françoise, and Hervé Groux. 2001. "Regulation of TGF-β Response During T Cell Activation Is Modulated by IL-10." *The Journal of Immunology* 167 (2): 773–78. https://doi.org/10.4049/jimmunol.167.2.773.

Coulie, Pierre G., Vincent Brichard, Aline Van Pel, Thomas Wolfel, Jörg Schneider, Catia Traversari, Stes Mattei, et al. 1994. "A New Gene Coding for a Differentiation Antigen Recognized by Autologous Cytolytic T Lymphocytes on HLA-A2 Melanomas." *Journal of Experimental Medicine* 180 (1): 35–42. https://doi.org/10.1084/jem.180.1.35.

Cousens, L P, J S Orange, and C A Biron. 1995. "Endogenous IL-2 Contributes to T Cell Expansion and IFN-Gamma Production during Lymphocytic Choriomeningitis Virus Infection." *Journal of Immunology (Baltimore, Md. : 1950)* 155 (12): 5690–99. http://www.ncbi.nlm.nih.gov/pubmed/7499855.

Curtsinger, J M, C S Schmidt, A Mondino, D C Lins, R M Kedl, M K Jenkins, and M F Mescher. 1999. "Inflammatory Cytokines Provide a Third Signal for Activation of Naive CD4+ and CD8+ T Cells." *Journal of Immunology (Baltimore, Md. : 1950)* 162 (6): 3256–62. http://www.ncbi.nlm.nih.gov/pubmed/10092777.

D'Aloia, Maria Michela, Ilaria Grazia Zizzari, Benedetto Sacchetti, Luca Pierelli, and Maurizio Alimandi. 2018. "CAR-T Cells: The Long and Winding Road to Solid Tumors Review-Article." *Cell Death and Disease*. Nature Publishing Group. https://doi.org/10.1038/s41419-018-0278-6.

D'Cruz, Louise M., and Ludger Klein. 2005. "Development and Function of Agonist-Induced CD25+Foxp3+ Regulatory T Cells in the Absence of Interleukin 2 Signaling." *Nature Immunology* 6 (11): 1152–59. https://doi.org/10.1038/ni1264.

Daley, Stephen R., Kristen M. Coakley, Daniel Y. Hu, Katrina L. Randall, Craig N. Jenne, Andre Limnander, Darienne R. Myers, et al. 2013. "Rasgrp1 Mutation Increases Naïve T-Cell CD44 Expression and Drives MTOR-Dependent Accumulation of Helios+ T Cells and Autoantibodies." *ELife* 2013 (2). https://doi.org/10.7554/eLife.01020.

DeGrendele, Heather C., Pila Estess, and Mark H. Siegelman. 1997. "Requirement for CD44 in Activated T Cell Extravasation into an Inflammatory Site." *Science* 278 (5338): 672–75. https://doi.org/10.1126/science.278.5338.672.

Dejean, Anne S, Daniel R Beisner, Irene L Ch'en, Yann M Kerdiles, Anna Babour, Karen C Arden, Diego H Castrillon, Ronald A DePinho, and Stephen M Hedrick. 2009. "Transcription Factor Foxo3 Controls the Magnitude of T Cell Immune Responses by Modulating the Function of Dendritic Cells." *Nature Immunology* 10 (5): 504–13. https://doi.org/10.1038/ni.1729.

Delisle, J. S., M. Giroux, G. Boucher, J. R. Landry, M. P. Hardy, S. Lemieux, R. G. Jones, B. T. Wilhelm, and C. Perreault. 2013. "The TGF-β-Smad3 Pathway Inhibits CD28-Dependent Cell Growth and Proliferation of CD4 T Cells." *Genes and Immunity* 14 (2): 115–26. https://doi.org/10.1038/gene.2012.63.

Desai, Dev M., Marianne E. Newton, Theresa Kadlecek, and Arthur Weiss. 1990. "Stimulation of the Phosphatidyl-Inositol Pathway Can Induce T-Cell Activation." *Nature* 348 (6296): 66–69. https://doi.org/10.1038/348066a0.

Dhein, Jens, Henning Walczak, Caroline Bäumlert, Klaus Michael Debatint, and Peter H. Krammer. 1995. "Autocrine T-Cell Suicide Mediated by APO-1/(Fas/CD95)." *Nature* 373 (6513): 438–41. https://doi.org/10.1038/373438a0.

Dijkers, Pascale F., Rene H. Medema, Cornelieke Pals, Lolita Banerji, N. Shaun B. Thomas, Eric W.-F. Lam, Boudewijn M. T. Burgering, et al. 2000. " Forkhead Transcription Factor FKHR-L1 Modulates Cytokine-Dependent Transcriptional Regulation of P27 KIP1 ." *Molecular and Cellular Biology* 20 (24): 9138–48. https://doi.org/10.1128/mcb.20.24.9138-9148.2000.

Djenidi, Fayçal, Julien Adam, Aïcha Goubar, Aurélie Durgeau, Guillaume Meurice, Vincent de Montpréville, Pierre Validire, Benjamin Besse, and Fathia Mami-Chouaib. 2015. " CD8 + CD103 + Tumor–Infiltrating Lymphocytes Are Tumor-Specific Tissue-Resident Memory T Cells and a Prognostic Factor for Survival in Lung Cancer Patients ." *The Journal of Immunology* 194 (7): 3475–86. https://doi.org/10.4049/jimmunol.1402711.

Djuretic, Ivana M., Ditsa Levanon, Varda Negreanu, Yoram Groner, Anjana Rao, and K. Mark Ansel. 2007. "Transcription Factors T-Bet and Runx3 Cooperate to Activate Ifng and Silence Il4 in T Helper Type 1 Cells." *Nature Immunology*. Nature Publishing Group. https://doi.org/10.1038/ni1424.

Do, Yoonkyung, Prakash S. Nagarkatti, and Mitzi Nagarkatti. 2004. "Role of CD44 and Hyaluronic Acid (HA) in Activation of Alloreactive and Antigen-Specific T Cells by Bone Marrow-Derived Dendritic Cells." *Journal of Immunotherapy* 27 (1): 1–12. https://doi.org/10.1097/00002371-200401000-00001.

Donkor, Moses K., Abira Sarkar, Peter A. Savage, Ruth A. Franklin, Linda K. Johnson, Achim A. Jungbluth, James P. Allison, and Ming O. Li. 2011. "T Cell Surveillance of Oncogene-Induced Prostate Cancer Is Impeded by T Cell-Derived TGF-B1 Cytokine." *Immunity* 35 (1): 123–34. https://doi.org/10.1016/j.immuni.2011.04.019.

Dower, Nancy A., Stacey L. Stang, Drell A. Bottorff, Julius O. Ebinu, Peter Dickie, Hanne L. Ostergaard, and James C. Stone. 2000. "RasGRP Is Essential for Mouse Thymocyte Differentiation and TCR Signaling." *Nature Immunology* 1 (4): 317–21. https://doi.org/10.1038/79766.

Doyle, Carolyn, and Jack L. Strominger. 1987. "Interaction between CD4 and Class II MHC Molecules Mediates Cell Adhesion." *Nature* 330 (6145): 256–59. https://doi.org/10.1038/330256a0.

Dubois, C. M., M. H. Laprise, F. Blanchette, L. E. Gentry, and R. Leduc. 1995. "Processing of Transforming Growth Factor B1 Precursor by Human Furin Convertase." *Journal of Biological Chemistry* 270 (18): 10618–24. https://doi.org/10.1074/jbc.270.18.10618.

Dubois, Claire M., François Blanchette, Marie Hélène Laprise, Richard Leduc, Francine Grondin, and Nabil G. Seidah. 2001. "Evidence That Furin Is an Authentic Transforming Growth Factor-B1-Converting Enzyme." *American Journal of Pathology* 158 (1): 305–16. https://doi.org/10.1016/S0002-9440(10)63970-3.

Dummer, Wolfgang, Bettina Ernst, Eric LeRoy, Dong-Sup Lee, and Charles D. Surh. 2001. "Autologous Regulation of Naive T Cell Homeostasis Within the T Cell Compartment." *The Journal of Immunology* 166 (4): 2460–68. https://doi.org/10.4049/jimmunol.166.4.2460.

Ebinu, Julius O., Drell A. Bottorff, Edmond Y.W. Chan, Stacey L. Stang, Robert J. Dunn, and James C. Stone. 1998. "RasGRP, a Ras Guanyl Nucleotide- Releasing Protein with Calcium- and Diacylglycerol-Binding Motifs." *Science* 280 (5366): 1082–86. https://doi.org/10.1126/science.280.5366.1082.

Ebinu, Julius O., Stacey L. Stang, Christine Teixeira, Drell A. Bottorff, Jonathan Hooton, Peter M. Blumberg, Michele Barry, R. Chris Bleakley, Hanne L. Ostergaard, and James C. Stone. 2000. "RasGRP Links T-Cell Receptor Signaling to Ras." *Blood* 95 (10): 3199–3203. https://doi.org/10.1182/blood.v95.10.3199.

Ebisawa, Takanori, Minoru Fukuchi, Gyo Murakami, Tomoki Chiba, Keiji Tanaka, Takeshi Imamura, and Kohei Miyazono. 2001. "Smurf1 Interacts with Transforming Growth Factor-β Type I Receptor through Smad7 and Induces Receptor Degradation." *Journal of Biological Chemistry* 276 (16): 12477–80. https://doi.org/10.1074/jbc.C100008200.

Eisenlohr, L. C., and C. J. Hackett. 1989. "Class II Major Histocompatibility Complex-Restricted T Cells Specific for a Virion Structural Protein That Do Not Recognize Exogenous Influenza Virus. Evidence That Presentation of Labile T Cell Determinants Is Favored by Endogenous Antigen Synthesis." *Journal of Experimental Medicine* 169 (3): 921–31. https://doi.org/10.1084/jem.169.3.921.

Ernst, Bettina, Dong Sup Lee, Jennifer M. Chang, Jonathan Sprent, and Charles D. Surh. 1999. "The Peptide Ligands Mediating Positive Selection in the Thymus Control T Cell Survival and Homeostatic Proliferation in the Periphery." *Immunity* 11 (2): 173–81. https://doi.org/10.1016/S1074-7613(00)80092-8.

Eyerich, Stefanie, Kilian Eyerich, Davide Pennino, Teresa Carbone, Francesca Nasorri, Sabatino Pallotta, Francesca Cianfarani, et al. 2009. "Th22 Cells Represent a Distinct Human T Cell Subset Involved in Epidermal Immunity and Remodeling." *Journal of Clinical Investigation* 119 (12): 3573–85. https://doi.org/10.1172/JCI40202.

Faget, Julien, Nathalie Bendriss-Vermare, Michael Gobert, Isabelle Durand, Daniel Olive, Cathy Biota, Thomas Bachelot, et al. 2012. "ICOS-Ligand Expression on Plasmacytoid Dendritic Cells Supports Breast Cancer Progression by Promoting the Accumulation of Immunosuppressive CD4 + T Cells." *Cancer Research* 72 (23): 6130–41. https://doi.org/10.1158/0008-5472.CAN-12-2409.

Falk, Ingrid, Gabi Nerz, Ian Haidl, Anna Krotkova, and Klaus Eichmann. 2001. "Immature Thymocytes That Fail to Express TCRβ and/or TCRγδ Proteins Die by Apoptotic Cell Death in the CD44-CD25- (DN4) Subset." *European Journal of Immunology* 31 (11): 3308–17. https://doi.org/10.1002/1521-4141(200111)31:11<3308::AID-IMMU3308>3.0.CO;2-5.

Fang, Yujiang, Xuhui Chen, Qian Bai, Chenglu Qin, Abdimalik O. Mohamud, Ziwen Zhu, Tyler W. Ball, et al. 2015. "IL-9 Inhibits HTB-72 Melanoma Cell Growth through Upregulation of P21 and TRAIL." *Journal of Surgical Oncology* 111 (8): 969–74. https://doi.org/10.1002/jso.23930.

Filippi, Christophe M., Amy E. Juedes, Janine E. Oldham, Ellie Ling, Lisa Togher, Yufeng Peng, Richard A. Flavell, and Matthias G. Von Herrath. 2008. "Transforming Growth Factor-β Suppresses the Activation of Cd8 + t-Cells When Naive but Promotes Their Survival and Function Once Antigen Experienced: A Two-Faced Impact on Autoimmunity." *Diabetes* 57 (10): 2684–92. https://doi.org/10.2337/db08-0609.

Finotto, Susetta, Markus F. Neurath, Jonathan N. Glickman, Shixin Qin, Hans A. Lehr, Francis H.Y. Green, Kate Ackerman, et al. 2002. "Development of Spontaneous Airway Changes Consistent with Human Asthma in Mice Lacking T-Bet." *Science* 295 (5553): 336–38. https://doi.org/10.1126/science.1065544.

Föger, Niko, Rachid Marhaba, and Margot Zöller. n.d. "CD44 Supports T Cell Proliferation and Apoptosis by Apposition of Protein Kinases." https://doi.org/10.1002/1521-4141.

Fontenot, Jason D., Jeffrey P. Rasmussen, Luke M. Williams, James L. Dooley, Andrew G. Farr, and Alexander Y. Rudensky. 2005. "Regulatory T Cell Lineage Specification by the Forkhead Transcription Factor Foxp3." *Immunity* 22 (3): 329–41. https://doi.org/10.1016/j.immuni.2005.01.016.

Fontenot, Jason D, Marc A Gavin, and Alexander Y Rudensky. 2003. "Foxp3 Programs the Development and Function of CD4+CD25+ Regulatory T Cells." *Nature Immunology* 4 (4): 330–36. https://doi.org/10.1038/ni904.

Förster, Reinhold, Andreas Schubel, Dagmar Breitfeld, Elisabeth Kremmer, Ingrid Renner-Müller, Eckhard Wolf, and Martin Lipp. 1999. "CCR7 Coordinates the Primary Immune Response by Establishing Functional Microenvironments in Secondary Lymphoid Organs." *Cell* 99 (1): 23–33. https://doi.org/10.1016/S0092-8674(00)80059-8.

Freeden-Jeffry, Ursula Von, Nanette Solvason, Maureen Howard, and Richard Murray. 1997. "The Earliest T Lineage-Committed Cells Depend on IL-7 for Bcl-2 Expression and Normal Cell Cycle Progression." *Immunity* 7 (1): 147–54. https://doi.org/10.1016/S1074-7613(00)80517-8.

Freeman, Gordon J., Andrew J. Long, Yoshiko Iwai, Karen Bourque, Tatyana Chernova, Hiroyuki Nishimura, Lori J. Fitz, et al. 2000. "Engagement of the PD-1 Immunoinhibitory Receptor by a Novel B7 Family Member Leads to Negative Regulation of Lymphocyte Activation." *Journal of Experimental Medicine* 192 (7): 1027–34. https://doi.org/10.1084/jem.192.7.1027.

Fu, G., and C. Peng. 2011. "Nodal Enhances the Activity of FoxO3a and Its Synergistic Interaction with Smads to Regulate Cyclin G2 Transcription in Ovarian Cancer Cells." *Oncogene* 30 (37): 3953–66. https://doi.org/10.1038/onc.2011.127.

Fuller, Deirdre M., Minghua Zhu, Xiaohua Song, Chih wen Ou-Yang, Sarah A. Sullivan, James C. Stone, and Weiguo Zhang. 2012. "Regulation of RasGRP1 Function in T Cell Development and Activation by Its Unique Tail Domain." *PLoS ONE* 7 (6): e38796. https://doi.org/10.1371/journal.pone.0038796.

Furuyama, Tatsuo, Toru Nakazawa, Itsuko Nakano, and Nozomu Mori. 2000. "Identification of the Differential Distribution Patterns of MRNAs and Consensus Binding Sequences for Mouse DAF-16 Homologues." *Biochemical Journal* 349 (2): 629–34. https://doi.org/10.1042/0264-6021:3490629.

Gaboriau-Routhiau, Valérie, Sabine Rakotobe, Emelyne Lécuyer, Imke Mulder, Annaïg Lan, Chantal Bridonneau, Violaine Rochet, et al. 2009. "The Key Role of Segmented Filamentous Bacteria in the Coordinated Maturation of Gut Helper T Cell Responses." *Immunity* 31 (4): 677–89. https://doi.org/10.1016/j.immuni.2009.08.020.

Galkina, Elena, Kyriakos Tanousis, Graham Preece, Mauro Tolaini, Dimitris Kioussis, Oliver Florey, Dorian O. Haskard, Thomas F. Tedder, and Ann Ager. 2003. "L-Selectin Shedding Does Not Regulate Constitutive T Cell Trafficking but Controls the Migration Pathways of Antigen-Activated T Lymphocytes." *Journal of Experimental Medicine* 198 (9): 1323–35. https://doi.org/10.1084/jem.20030485.

Gargett, Tessa, Wenbo Yu, Gianpietro Dotti, Eric S Yvon, Susan N Christo, John D Hayball, Ian D Lewis, Malcolm K Brenner, and Michael P Brown. 2016. "GD2-Specific CAR T Cells Undergo Potent Activation and Deletion Following Antigen Encounter but Can Be Protected From Activation-Induced Cell Death by PD-1 Blockade." *Molecular Therapy : The Journal of the American Society of Gene Therapy* 24 (6): 1135–49. https://doi.org/10.1038/mt.2016.63.

Gavin, Marc A., Jeffrey P. Rasmussen, Jason D. Fontenot, Valeria Vasta, Vincent C. Manganiello, Joseph A. Beavo, and Alexander Y. Rudensky. 2007. "Foxp3-Dependent Programme of Regulatory T-Cell Differentiation." *Nature* 445 (7129): 771–75. https://doi.org/10.1038/nature05543.

Gavin, Marc A., Troy R. Torgerson, Evan Houston, Paul DeRoos, William Y. Ho, Asbjørg Stray-Pedersen, Elisabeth L. Ocheltree, Philip D. Greenberg, Hans D. Ochs, and Alexander Y. Rudensky. 2006. "Single-Cell Analysis of Normal and FOXP3-Mutant Human T Cells: FOXP3 Expression without Regulatory T Cell Development." *Proceedings of the National Academy of Sciences of the United States of America* 103 (17): 6659–64. https://doi.org/10.1073/pnas.0509484103.

Geiselhart, Lisa A., Courtney A. Humphries, Theresa A. Gregorio, Sherry Mou, Jeffrey Subleski, and Kristin L. Komschlies. 2001. "IL-7 Administration Alters the CD4:CD8 Ratio, Increases T Cell Numbers, and Increases T Cell Function in the Absence of Activation." *The Journal of Immunology* 166 (5): 3019–27. https://doi.org/10.4049/jimmunol.166.5.3019.

Genestier, Laurent, Shailaja Kasibhatla, Thomas Brunner, and Douglas R. Green. 1999. "Transforming Growth Factor B1 Inhibits Fas Ligand Expression and Subsequent Activation-Induced Cell Death in T Cells via Downregulation of c- Myc." *Journal of Experimental Medicine* 189 (2): 231–39. https://doi.org/10.1084/jem.189.2.231.

Ghassemi, Saba, Felipe Bedoya, Selene Nunez-Cruz, Carl June, Jos Melenhorst, and Michael Milone. 2016. "Shortened T Cell Culture with IL-7 and IL-15 Provides the Most Potent Chimeric Antigen Receptor (CAR)-Modified T Cells for Adoptive Immunotherapy." *The Journal of Immunology* 196 (1 Supplement).

Giannakou, Maria E., Martin Goss, Martin A. Jünger, Ernst Hafen, Sally J. Leevers, and Linda Partridge. 2004. "Long-Lived Drosophila with over-Expressed DFOXO in Adult Fat Body." *Science* 305 (5682): 361. https://doi.org/10.1126/science.1098219.

Gibson, S, A August, Y Kawakami, T Kawakami, B Dupont, and G B Mills. 1996. "The EMT/ITK/TSK (EMT) Tyrosine Kinase Is Activated during TCR Signaling: LCK Is Required for Optimal Activation of EMT." *The Journal of Immunology* 156 (8).

Gilboa, Lilach, Rebecca G. Wells, Harvey F. Lodish, and Yoav I. Henis. 1998. "Oligomeric Structure of Type I and Type II Transforming Growth Factor β Receptors: Homodimers Form in the ER and Persist at the Plasma Membrane." *Journal of Cell Biology* 140 (4): 767–77. https://doi.org/10.1083/jcb.140.4.767.

Gogishvili, Tea, Fred Lühder, Sandra Goebbels, Sandra Beer-Hammer, Klaus Pfeffer, and Thomas Hünig. 2013. "Cell-Intrinsic and -Extrinsic Control of Treg-Cell Homeostasis and Function Revealed by Induced CD28 Deletion." *European Journal of Immunology* 43 (1): 188–93. https://doi.org/10.1002/eji.201242824.

Gómez, J, C Martínez-A, B Fernández, A García, and A Rebollo. 1996. "Critical Role of Ras in the Proliferation and Prevention of Apoptosis Mediated by IL-2." *The Journal of Immunology* 157 (6).

Gomis, Roger R., Claudio Alarcón, Wei He, Qiongqing Wang, Joan Seoane, Alex Lash, and Joan Massagué. 2006. "A FoxO-Smad Synexpression Group in Human Keratinocytes." *Proceedings of the National Academy of Sciences of the United States of America* 103 (34): 12747–52. https://doi.org/10.1073/pnas.0605333103.

Gorelik, Leonid, Patrick E. Fields, and Richard A. Flavell. 2000. "Cutting Edge: TGF-β Inhibits Th Type 2 Development Through Inhibition of GATA-3 Expression." *The Journal of Immunology* 165 (9): 4773–77. https://doi.org/10.4049/jimmunol.165.9.4773.

Gorelik, Leonid, and Richard A. Flavell. 2000. "Abrogation of TGFβ Signaling in T Cells Leads to Spontaneous T Cell Differentiation and Autoimmune Disease." *Immunity* 12 (2): 171–81. https://doi.org/10.1016/S1074-7613(00)80170-3.

Gorham, James D., Jack T. Lin, James L. Sung, Lynnie A. Rudner, and Margaret A. French. 2001. "Genetic Regulation of Autoimmune Disease: BALB/c Background TGF-B1-Deficient Mice Develop Necroinflammatory IFN-γ-Dependent Hepatitis." *The Journal of Immunology* 166 (10): 6413–22. https://doi.org/10.4049/jimmunol.166.10.6413.

Gray, Alane M., and Anthony J. Mason. 1990. "Requirement for Activin a and Transforming Growth Factor-B1 pro-Regions in Homodimer Assembly." *Science* 247 (4948): 1328–30. https://doi.org/10.1126/science.2315700.

Grohmann, Ursula, Ciriana Orabona, Francesca Fallarino, Carmine Vacca, Filippo Calcinaro, Alberto Falorni, Paola Candeloro, et al. 2002. "CTLA-4-Ig Regulates Tryptophan Catabolism in Vivo." *Nature Immunology* 3 (11): 1097–1101. https://doi.org/10.1038/ni846.

Gross, G., T. Waks, and Z. Eshhar. 1989. "Expression of Immunoglobulin-T-Cell Receptor Chimeric Molecules as Functional Receptors with Antibody-Type Specificity." *Proceedings of the National Academy of Sciences of the United States of America* 86 (24): 10024–28. https://doi.org/10.1073/pnas.86.24.10024.

Grossman, William J., James W. Verbsky, Winfried Barchet, Marco Colonna, John P. Atkinson, and Timothy J. Ley. 2004. "Human T Regulatory Cells Can Use the Perforin Pathway to Cause Autologous Target Cell Death." *Immunity* 21 (4): 589–601. https://doi.org/10.1016/j.immuni.2004.09.002.

Guan, Hongbing, Prakash S. Nagarkatti, and Mitzi Nagarkatti. 2011. "CD44 Reciprocally Regulates the Differentiation of Encephalitogenic Th1/Th17 and Th2/Regulatory T Cells through Epigenetic Modulation Involving DNA Methylation of Cytokine Gene Promoters, Thereby Controlling the Development of Experimental Autoimmune Encephalomyelitis." *The Journal of Immunology* 186 (12): 6955–64. https://doi.org/10.4049/jimmunol.1004043.

Guedan, Sonia, Avery D Posey, Carolyn Shaw, Anna Wing, Tong Da, Prachi R Patel, Shannon E McGettigan, et al. 2018. "Enhancing CAR T Cell Persistence through ICOS and 4-1BB Costimulation." *JCI Insight* 3 (1). https://doi.org/10.1172/jci.insight.96976.

Guertin, David A., Deanna M. Stevens, Carson C. Thoreen, Aurora A. Burds, Nada Y. Kalaany, Jason Moffat, Michael Brown, Kevin J. Fitzgerald, and David M. Sabatini. 2006. "Ablation in Mice of the MTORC Components Raptor, Rictor, or MLST8 Reveals That MTORC2 Is Required for Signaling to Akt-FOXO and PKCα, but Not S6K1." *Developmental Cell* 11 (6): 859–71. https://doi.org/10.1016/j.devcel.2006.10.007.

Guilbault, Benoit, and Robert J. Kay. 2004. "RasGRP1 Sensitizes an Immature B Cell Line to Antigen Receptor-Induced Apoptosis." *Journal of Biological Chemistry* 279 (19): 19523–30. https://doi.org/10.1074/JBC.M314273200.

Hager-Theodorides, Ariadne L., Nicola J. Rowbotham, Susan V. Outram, Johannes T. Dessens, and Tessa Crompton. 2007. "β-Selection: Abundance of TCRβ-/Γδ - CD44-CD25- (DN4) Cells in the Foetal Thymus." *European Journal of Immunology* 37 (2): 487–500. https://doi.org/10.1002/eji.200636503.

Hara, Masaki, Cherry I. Kingsley, Masanori Niimi, Simon Read, Stuart E. Turvey, Andrew R. Bushell, Peter J. Morris, Fiona Powrie, and Kathryn J. Wood. 2001. "IL-10 Is Required for Regulatory T Cells to Mediate Tolerance to Alloantigens In Vivo." *The Journal of Immunology* 166 (6): 3789–96. https://doi.org/10.4049/jimmunol.166.6.3789.

Hartsough, Melanie T., and Kathleen M. Mulder. 1995. "Transforming Growth Factor β Activation of P44mapk in Proliferating Cultures of Epithelial Cells." *Journal of Biological Chemistry* 270 (13): 7117–24. https://doi.org/10.1074/jbc.270.13.7117.

Hayashi, Hidetoshi, Shirin Abdollah, Yubin Qiu, Jiexing Cai, Yong Yao Xu, Brian W. Grinnell, Mark A. Richardson, et al. 1997. "The MAD-Related Protein Smad7 Associates with the TGFβ Receptor and Functions as an Antagonist of TGFβ Signaling." *Cell* 89 (7): 1165–73. https://doi.org/10.1016/S0092-8674(00)80303-7.

Heczey, Andras, Chrystal U. Louis, Barbara Savoldo, Olga Dakhova, April Durett, Bambi Grilley, Hao Liu, et al. 2017. "CAR T Cells Administered in Combination with Lymphodepletion and PD-1 Inhibition to Patients with Neuroblastoma." *Molecular Therapy* 25 (9): 2214–24. https://doi.org/10.1016/j.ymthe.2017.05.012.

Hegazy, Ahmed N., Nathaniel R. West, Michael J.T. Stubbington, Emily Wendt, Kim I.M. Suijker, Angeliki Datsi, Sebastien This, et al. 2017. "Circulating and Tissue-Resident CD4+ T Cells With Reactivity to Intestinal Microbiota Are Abundant in Healthy Individuals and Function Is Altered During Inflammation." *Gastroenterology* 153 (5): 1320-1337.e16. https://doi.org/10.1053/j.gastro.2017.07.047.

Hegde, Venkatesh L., Narendra P. Singh, Prakash S. Nagarkatti, and Mitzi Nagarkatti. 2008. "CD44 Mobilization in Allogeneic Dendritic Cell-T Cell Immunological Synapse Plays a Key Role in T Cell Activation." *Journal of Leukocyte Biology* 84 (1): 134–42. https://doi.org/10.1189/jlb.1107752.

Herndler-Brandstetter, Dietmar, Susanne Schwaiger, Ellen Veel, Christine Fehrer, Daniel P. Cioca, Giovanni Almanzar, Michael Keller, et al. 2005. " CD25-Expressing CD8 + T Cells Are Potent Memory Cells in Old Age ." *The Journal of Immunology* 175 (3): 1566–74. https://doi.org/10.4049/jimmunol.175.3.1566.

Hewicker, M., E. Kromschröder, and G. Trautwein. 1990. "Detection of Circulating Immune Complexes in MRL Mice with Different Forms of Glomerulonephritis." *Zeitschrift Fur Versuchstierkunde* 33 (4): 149–56. https://europepmc.org/article/med/2238887.

Hickman, Somia P., Jaeseok Yang, Rajan M. Thomas, Andrew D. Wells, and Laurence A. Turka. 2006. " Defective Activation of Protein Kinase C and Ras-ERK Pathways Limits IL-2 Production and Proliferation by CD4 + CD25 + Regulatory T Cells ." *The Journal of Immunology* 177 (4): 2186–94. https://doi.org/10.4049/jimmunol.177.4.2186.

Higuchi, Kiyoshi, and Osamu Hayaishi. 1967. "Enzymic Formation of D-Kynurenine from d-Tryptophan." *Archives of Biochemistry and Biophysics* 120 (2): 397–403. https://doi.org/10.1016/0003-9861(67)90256-1.

Hirano, Fumiya, Katsumi Kaneko, Hideto Tamura, Haidong Dong, Shengdian Wang, Masao Ichikawa, Cecilia Rietz, et al. 2005. "Blockade of B7-H1 and PD-1 by Monoclonal Antibodies Potentiates Cancer Therapeutic Immunity." *Cancer Research* 65 (3): 1089–96.

Hogquist, Kristin A., Stephen C. Jameson, William R. Heath, Jane L. Howard, Michael J. Bevan, and Francis R. Carbone. 1994. "T Cell Receptor Antagonist Peptides Induce Positive Selection." *Cell* 76 (1): 17–27. https://doi.org/10.1016/0092-8674(94)90169-4.

Hori, Shohei, Takashi Nomura, and Shimon Sakaguchi. 2003. "Control of Regulatory T Cell Development by the Transcription Factor Foxp3." *Science* 299 (5609): 1057–61. https://doi.org/10.1126/science.1079490.

Hosaka, Taisuke, William H. Biggs, David Tieu, Antonia D. Boyer, Nissi M. Varki, Webster K. Cavenee, and Karen C. Arden. 2004. "Disruption of Forkhead Transcription Factor (FOXO) Family Members in Mice Reveals Their Functional Diversification." *Proceedings of the National Academy of Sciences of the United States of America* 101 (9): 2975–80. https://doi.org/10.1073/pnas.0400093101.

Howie, Duncan, Stephen Paul Cobbold, Elizabeth Adams, Annemieke Ten Bokum, Andra Stefania Necula, Wei Zhang, Honglei Huang, et al. 2017. "Foxp3 Drives Oxidative Phosphorylation and Protection from Lipotoxicity." *JCI Insight* 2 (3): 89160. https://doi.org/10.1172/jci.insight.89160.

Hsieh, Chyi Song, Yuqiong Liang, Aaron J. Tyznik, Steven G. Self, Denny Liggitt, and Alexander Y. Rudensky. 2004. "Recognition of the Peripheral Self by Naturally Arising CD25+ CD4+ T Cell Receptors." *Immunity* 21 (2): 267–77. https://doi.org/10.1016/j.immuni.2004.07.009.

Hsieh, Chyi Song, Steven E. Macatonia, Catherine S. Tripp, Stanley F. Wolf, Anne O'Garra, and Kenneth M. Murphy. 1993. "Development of TH1 CD4+ T Cells through IL-12 Produced by Listeria-Induced Macrophages." *Science* 260 (5107): 547–49. https://doi.org/10.1126/science.8097338.

Huber, Samuel, Christoph Schramm, Hans A. Lehr, Amrit Mann, Steffen Schmitt, Christoph Becker, Martina Protschka, Peter R. Galle, Markus F. Neurath, and Manfred Blessing. 2004. " Cutting Edge: TGF-β Signaling Is Required for the In Vivo Expansion and Immunosuppressive Capacity of Regulatory CD4 + CD25 + T Cells ." *The Journal of Immunology* 173 (11): 6526–31. https://doi.org/10.4049/jimmunol.173.11.6526.

Hwangbo, Dae Sung, Boris Garsham, Meng Ping Tu, Michael Palmer, and Marc Tatar. 2004. "Drosophila DFOXO Controls Lifespan and Regulates Insulin Signalling in Brain and Fat Body." *Nature* 429 (6991): 562–66. https://doi.org/10.1038/nature02549.

Iezzi, Giandomenica, Klaus Karjalainen, and Antonio Lanzavecchia. 1998. "The Duration of Antigenic Stimulation Determines the Fate of Naive and Effector T Cells." *Immunity* 8 (1): 89–95. https://doi.org/10.1016/S1074-7613(00)80461-6.

Itoh, M, T Takahashi, N Sakaguchi, Y Kuniyasu, J Shimizu, F Otsuka, and S Sakaguchi. 1999. "Thymus and Autoimmunity: Production of CD25+CD4+ Naturally Anergic and Suppressive T Cells as a Key Function of the Thymus in Maintaining Immunologic Self-Tolerance." *Journal of Immunology (Baltimore, Md. : 1950)* 162 (9): 5317–26. http://www.ncbi.nlm.nih.gov/pubmed/10228007.

Itoh, Naoto, and Shigekazu Nagata. 1993. "A Novel Protein Domain Required for Apoptosis MUTATIONAL ANALYSIS OF HUMAN Fas ANTIGEN*." *THE JOURNAL OF BIOLOGICAL CHEMISTRY*. Vol. 6.

Itoh, Naoto, Shin Yonehara, Ai Ishii, Minako Yonehara, Sei Ichi Mizushima, Masazumi Sameshima, Atsushi Hase, Yoshiyuki Seto, and Shigekazu Nagata. 1991. "The Polypeptide Encoded by the CDNA for Human Cell Surface Antigen Fas Can Mediate Apoptosis." *Cell* 66 (2): 233–43. https://doi.org/10.1016/0092-8674(91)90614-5.

Ivanov, Ivaylo I., Rosa de Llanos Frutos, Nicolas Manel, Keiji Yoshinaga, Daniel B. Rifkin, R. Balfour Sartor, B. Brett Finlay, and Dan R. Littman. 2008. "Specific Microbiota Direct the Differentiation of IL-17-Producing T-Helper Cells in the Mucosa of the Small Intestine." *Cell Host and Microbe* 4 (4): 337–49. https://doi.org/10.1016/j.chom.2008.09.009.

Iwai, Yoshiko, Masayoshi Ishida, Yoshimasa Tanaka, Taku Okazaki, Tasuku Honjo, and Nagahiro Minato. 2002. "Involvement of PD-L1 on Tumor Cells in the Escape from Host Immune System and Tumor Immunotherapy by PD-L1 Blockade." *Proceedings of the National Academy of Sciences of the United States of America* 99 (19): 12293–97. https://doi.org/10.1073/pnas.192461099.

Iwashima, M, B A Irving, N S van Oers, A C Chan, and A Weiss. 1994. "Sequential Interactions of the TCR with Two Distinct Cytoplasmic Tyrosine Kinases." *Science (New York, N.Y.)* 263 (5150): 1136–39. https://doi.org/10.1126/science.7509083.

Jain, Jugnu, Patricia G. McCafffrey, Zoe Miner, Tom K. Kerppola, John N. Lambert, Gregory L. Verdine, Tom Curran, and Anjana Rao. 1993. "The T-Cell Transcription Factor NFATp Is a Substrate for Calcineurin and Interacts with Fos and Jun." *Nature* 365 (6444): 352–55. https://doi.org/10.1038/365352a0.

Jensen, Michael C., Leslie Popplewell, Laurence J. Cooper, David DiGiusto, Michael Kalos, Julie R. Ostberg, and Stephen J. Forman. 2010. "Antitransgene Rejection Responses Contribute to Attenuated Persistence of Adoptively Transferred CD20/CD19-Specific Chimeric Antigen Receptor Redirected T Cells in Humans." *Biology of Blood and Marrow Transplantation* 16 (9): 1245–56. https://doi.org/10.1016/j.bbmt.2010.03.014.

Jiang, Qiong, Wen Qing Li, Robert R. Hofmeister, Howard A. Young, David R. Hodge, Jonathan R. Keller, Annette R. Khaled, and Scott K. Durum. 2004. "Distinct Regions of the Interleukin-7 Receptor Regulate Different Bcl2 Family Members." *Molecular and Cellular Biology* 24 (14): 6501–13. https://doi.org/10.1128/mcb.24.14.6501-6513.2004.

Jiang, Xiaodong, Rachael A. Clark, Luzheng Liu, Amy J. Wagers, Robert C. Fuhlbrigge, and Thomas S. Kupper. 2012. "Skin Infection Generates Non-Migratory Memory CD8 + T RM Cells Providing Global Skin Immunity." *Nature* 483 (7388): 227–31. https://doi.org/10.1038/nature10851.

Jie, Hyun Bae, Patrick J. Schuler, Steve C. Lee, Raghvendra M. Srivastava, Athanassios Argiris, Soldano Ferrone, Theresa L. Whiteside, and Robert L. Ferris. 2015. "CTLA-4+ Regulatory t Cells Increased in Cetuximab-Treated Head and Neck Cancer Patients Suppress Nk Cell Cytotoxicity and Correlate with Poor Prognosis." *Cancer Research* 75 (11): 2200–2210. https://doi.org/10.1158/0008-5472.CAN-14-2788.

Joseph, Julie, Emmanuelle S. Ametepe, Naveen Haribabu, Gerard Agbayani, Lakshmi Krishnan, Alexandre Blais, and Subash Sad. 2016. "Inhibition of ROS and Upregulation of Inflammatory Cytokines by FoxO3a Promotes Survival against Salmonella Typhimurium." *Nature Communications* 7. https://doi.org/10.1038/ncomms12748.

Jordan, Martha S., Alina Boesteanu, Amy J. Reed, Andria L. Petrone, Andrea E. Holenbeck, Melissa A. Lerman, Ali Naji, and Andrew J. Caton. 2001. "Thymic Selection of CD4+CD25+ Regulatory T Cells Induced by an Agonist Self-Peptide." *Nature Immunology* 2 (4): 301–6. https://doi.org/10.1038/86302.

June, C H, J A Ledbetter, M M Gillespie, T Lindsten, and C B Thompson. 1987. "T-Cell Proliferation Involving the CD28 Pathway Is Associated with Cyclosporine-Resistant Interleukin 2 Gene Expression." *Molecular and Cellular Biology* 7 (12): 4472–81. https://doi.org/10.1128/mcb.7.12.4472.

Jünger, Martin A., Felix Rintelen, Hugo Stocker, Jonathan D. Wasserman, Mátyás Végh, Thomas Radimerski, Michael E. Greenberg, and Ernst Hafen. 2003. "The Drosophila Forkhead Transcription Factor FOXO Mediates the Reduction in Cell Number Associated with Reduced Insulin Signaling." *Journal of Biology* 2 (3). https://doi.org/10.1186/1475-4924-2-20.

Kaech, Susan M., and Rafi Ahmed. 2001. "Memory CD8+ T Cell Differentiation: Initial Antigen Encounter Triggers a Developmental Program in Naïve Cells." *Nature Immunology* 2 (5): 415–22. https://doi.org/10.1038/87720.

Kaech, Susan M., and E. John Wherry. 2007. "Heterogeneity and Cell-Fate Decisions in Effector and Memory CD8+ T Cell Differentiation during Viral Infection." *Immunity*. https://doi.org/10.1016/j.immuni.2007.08.007.

Kane, Lawrence P., Joseph Lin, and Arthur Weiss. 2000. "Signal Transduction by the TCR for Antigen." *Current Opinion in Immunology*. Current Biology Ltd. https://doi.org/10.1016/S0952-7915(00)00083-2.

Kappler, John W., Neal Roehm, and Philippa Marrack. 1987. "T Cell Tolerance by Clonal Elimination in the Thymus." *Cell* 49 (2): 273–80. https://doi.org/10.1016/0092-8674(87)90568-X.

Katoh, Shigeki, H. Taniguchi, Y. Matsubara, N. Matsumoto, K. Fukushima, J. Kadota, S. Matsukura, and S. Kohno. 1999. "Overexpression of CD44 on Alveolar Eosinophils with High Concentrations of Soluble CD44 in Bronchoalveolar Lavage Fluid in Patients with Eosinophilic Pneumonia." *Allergy: European Journal of Allergy and Clinical Immunology* 54 (12): 1286–92. https://doi.org/10.1034/j.1398-9995.1999.00277.x.

Kawabe, Yojiro, and Atsuo Ochi. 1991. "Programmed Cell Death and Extrathymic Reduction of Vβ8+CD4+ T Cells in Mice Tolerant to Staphylococcus Aureus Enterotoxin B." *Nature* 349 (6306): 245–48. https://doi.org/10.1038/349245a0.

Kawakami, Y., S. Eliyahu, C. H. Delgado, P. F. Robbins, K. Sakaguchi, E. Appella, J. R. Yannelli, G. J. Adema, T. Miki, and S. A. Rosenberg. 1994. "Identification of a Human Melanoma Antigen Recognized by Tumor- Infiltrating Lymphocytes Associated with in Vivo Tumor Rejection." *Proceedings of the National Academy of Sciences of the United States of America* 91 (14): 6458–62. https://doi.org/10.1073/pnas.91.14.6458.

Kehrl, John H., Lalage M. Wakefield, Anita B. Roberts, Sonia Jakowlew, Melchor Alvarez-Mon, Rik Derynck, Michael B. Sporn, and Anthony S. Fauci. 1986. "Production of Transforming Growth Factor β by Human T Lymphocytes and Its Potential Role in the Regulation of T Cell Growth." *Journal of Experimental Medicine* 163 (5): 1037–50. https://doi.org/10.1084/jem.163.5.1037.

Kenyon, Cynthia, Jean Chang, Erin Gensch, Adam Rudner, and Ramon Tabtiang. 1993. "A C. Elegans Mutant That Lives Twice as Long as Wild Type." *Nature* 366 (6454): 461–64. https://doi.org/10.1038/366461a0.

Kerdiles, Yann M., Daniel R. Beisner, Roberto Tinoco, Anne S. Dejean, Diego H. Castrillon, Ronald A. DePinho, and Stephen M. Hedrick. 2009. "Foxo1 Links Homing and Survival of Naive T Cells by Regulating L-Selectin, CCR7 and Interleukin 7 Receptor." *Nature Immunology* 10 (2): 176–84. https://doi.org/10.1038/ni.1689.

Kerdiles, Yann M, Erica L Stone, Daniel R Beisner, Daniel L Beisner, Maureen A McGargill, Irene L Ch'en, Christian Stockmann, Carol D Katayama, and Stephen M Hedrick. 2010. "Foxo Transcription Factors Control Regulatory T Cell Development and Function." *Immunity* 33 (6): 890–904. https://doi.org/10.1016/j.immuni.2010.12.002.

Kershaw, Michael H., Jennifer A. Westwood, Linda L. Parker, Gang Wang, Zelig Eshhar, Sharon A. Mavroukakis, Donald E. White, et al. 2006. "A Phase I Study on Adoptive Immunotherapy Using Gene-Modified T Cells for Ovarian Cancer." *Clinical Cancer Research* 12 (20 PART 1): 6106–15. https://doi.org/10.1158/1078-0432.CCR-06-1183.

Khaled, Annette R., Kyungjae Kim, Robert Hofmeister, Kathrin Muegge, and Scott K. Durum. 1999. "Withdrawal of IL-7 Induces Bax Translocation from Cytosol to Mitochondria through a Rise in Intracellular PH." *Proceedings of the National Academy of Sciences of the United States of America* 96 (25): 14476–81. https://doi.org/10.1073/pnas.96.25.14476.

Kim, Peter W., Zhen Yu J. Sun, Stephen C. Blacklow, Gerhard Wagner, and Michael J. Eck. 2003. "A Zinc Clasp Structure Tethers Lck to T Cell Coreceptors CD4 and CD8." *Science* 301 (5640): 1725–28. https://doi.org/10.1126/science.1085643.

Kiniwa, Yukiko, Yoshihiro Miyahara, Helen Y. Wang, Weiyi Peng, Guangyong Peng, Thomas M. Wheeler, Timothy C. Thompson, Lloyd J. Old, and Rong Fu Wang. 2007. "CD8+ Foxp3+ Regulatory T Cells Mediate Immunosuppression in Prostate Cancer." *Clinical Cancer Research* 13 (23): 6947–58. https://doi.org/10.1158/1078-0432.CCR-07-0842.

Kischkel, Frank C., Stefan Hellbardt, Iris Behrmann, Matthias Germer, Michael Pawlita, Peter H. Krammer, and Marcus E. Peter. 1995. "Cytotoxicity-Dependent APO-1 (Fas/CD95)-Associated Proteins Form a Death-Inducing Signaling Complex (DISC) with the Receptor." *EMBO Journal* 14 (22): 5579–88. https://doi.org/10.1002/j.1460-2075.1995.tb00245.x.

Kjer-Nielsen, Lars, Michelle A. Dunstone, Lyudmila Kostenko, Lauren K. Ely, Travis Beddoe, Nicole A. Mifsud, Anthony W. Purcell, Andrew G. Brooks, James McCluskey, and Jamie Rossjohn. 2004. "Crystal Structure of the Human T Cell Receptor CD3εγ Heterodimer Complexed to the Therapeutic MAb OKT3." *Proceedings of the National Academy of Sciences of the United States of America* 101 (20): 7675–80. https://doi.org/10.1073/pnas.0402295101.

Klonowski, Kimberly D., Kristina J. Williams, Amanda L. Marzo, David A. Blair, Elizabeth G. Lingenheld, and Leo Lefrançois. 2004. "Dynamics of Blood-Borne CD8 Memory T Cell Migration in Vivo." *Immunity* 20 (5): 551–62. https://doi.org/10.1016/S1074-7613(04)00103-7.

Koike, Toru, Hiroko Yamagishi, Yasue Hatanaka, Atsuki Fukushima, Jing wen Chang, Yan Xia, Mark Fields, Phillip Chandler, and Makio Iwashima. 2003. "A Novel ERK-Dependent Signaling Process That Regulates Interleukin-2 Expression in a Late Phase of T Cell Activation." *Journal of Biological Chemistry* 278 (18): 15685–92. https://doi.org/10.1074/jbc.M210829200.

Konkel, Joanne E., Dunfang Zhang, Peter Zanvit, Cheryl Chia, Tamsin Zangarle-Murray, Wenwen Jin, Songlin Wang, and Wan Jun Chen. 2017. "Transforming Growth Factor-β Signaling in Regulatory T Cells Controls T Helper-17 Cells and Tissue-Specific Immune Responses." *Immunity* 46 (4): 660–74. https://doi.org/10.1016/j.immuni.2017.03.015.

Kramer, Jamie M., Jason T. Davidge, Joseph M. Lockyer, and Brian E. Staveley. 2003. "Expression of Drosophila FOXO Regulates Growth and Can Phenocopy Starvation." *BMC Developmental Biology* 3 (July): 1–14. https://doi.org/10.1186/1471-213X-3-5.

Krummel, Matthew F., and James P. Allison. 1995. "CD28 and CTLA-4 Have Opposing Effects on the Response of T Ceils to Stimulation." *Journal of Experimental Medicine* 182 (2): 459–65. https://doi.org/10.1084/jem.182.2.459.

Kumar, Brahma V, Wenji Ma, Michelle Miron, Amy L Friedman, Yufeng Shen, and Donna L Farber. 2017. "Human Tissue-Resident Memory T Cells Are Defined by Core Transcriptional and Functional Signatures in Lymphoid and Mucosal Sites." *Cell Reports* 20. https://doi.org/10.1016/j.celrep.2017.08.078.

Künkele, Annette, Adam J Johnson, Lisa S Rolczynski, Cindy A Chang, Virginia Hoglund, Karen S Kelly-Spratt, and Michael C Jensen. 2015. "Functional Tuning of CARs Reveals Signaling Threshold above Which CD8+ CTL Antitumor Potency Is Attenuated Due to Cell Fas-FasL-Dependent AICD." *Cancer Immunology Research* 3 (4): 368–79. https://doi.org/10.1158/2326-6066.CIR-14-0200.

la Rosa, Maurus de, Sascha Rutz, Heike Dorninger, and Alexander Scheffold. 2004. "Interleukin-2 Is Essential for CD4+CD25+ Regulatory T Cell Function." *European Journal of Immunology* 34 (9): 2480–88. https://doi.org/10.1002/eji.200425274.

Labrecque, Nathalie, Liam Simon Whitfield, Reinhard Obst, Caroline Waltzinger, Christophe Benoist, and Diane Mathis. 2001. "How Much TCR Does a T Cell Need?" *Immunity* 15 (1): 71–82. https://doi.org/10.1016/S1074-7613(01)00170-4.

Latchman, Yvette, Clive R. Wood, Tatyana Chernova, Divya Chaudhary, Madhuri Borde, Irene Chernova, Yoshiko Iwai, et al. 2001. "PD-L2 Is a Second Ligand for PD-1 and Inhibits T Cell Activation." *Nature Immunology* 2 (3): 261–68. https://doi.org/10.1038/85330.

Leach, Dana R., Matthew F. Krummel, and James P. Allison. 1996. "Enhancement of Antitumor Immunity by CTLA-4 Blockade." *Science* 271 (5256): 1734–36. https://doi.org/10.1126/science.271.5256.1734.

Lee, Daniel W., James N. Kochenderfer, Maryalice Stetler-Stevenson, Yongzhi K. Cui, Cindy Delbrook, Steven A. Feldman, Terry J. Fry, et al. 2015. "T Cells Expressing CD19 Chimeric Antigen Receptors for Acute Lymphoblastic Leukaemia in Children and Young Adults: A Phase 1 Dose-Escalation Trial." *The Lancet* 385 (9967): 517–28. https://doi.org/10.1016/S0140-6736(14)61403-3.

Lee, Hyang Mi, Jhoanne L. Bautista, James Scott-Browne, James F. Mohan, and Chyi Song Hsieh. 2012. "A Broad Range of Self-Reactivity Drives Thymic Regulatory T Cell Selection to Limit Responses to Self." *Immunity* 37 (3): 475–86. https://doi.org/10.1016/j.immuni.2012.07.009.

Lee, Kyung Mi, Ellen Chuang, Matthew Griffin, Roli Khattri, David K. Hong, Weiguo Zhang, David Straus, Lawrence E. Samelson, Craig B. Thompson, and Jeffrey A. Bluestone. 1998. "Molecular Basis of T Cell Inactivation by CTLA-4." *Science* 282 (5397): 2263–66. https://doi.org/10.1126/science.282.5397.2263.

Lenardo, Michael J. 1991. "Lnterleukin-2 Programs Mouse Aβ T Lymphocytes for Apoptosis." *Nature* 353 (6347): 858–61. https://doi.org/10.1038/353858a0.

Ley, Steven C., Mark Marsh, Chris R. Bebbington, Karen Proudfoot, and Peter Jordan. 1994. "Distinct Intracellular Localization of Lck and Fyn Protein Tyrosine Kinases in Human T Lymphocytes." *Journal of Cell Biology* 125 (3): 639–49. https://doi.org/10.1083/jcb.125.3.639.

Li, Jinzheng, Xing Lai, Wangyang Liao, Yong He, Yiming Liu, and Jianping Gong. 2011. "The Dynamic Changes of Th17/Treg Cytokines in Rat Liver Transplant Rejection and Tolerance." *International Immunopharmacology* 11 (8): 962–67. https://doi.org/10.1016/j.intimp.2011.02.010.

Li, Ming O., Yisong Y. Wan, and Richard A. Flavell. 2007. "T Cell-Produced Transforming Growth Factor-B1 Controls T Cell Tolerance and Regulates Th1- and Th17-Cell Differentiation." *Immunity* 26 (5): 579–91. https://doi.org/10.1016/j.immuni.2007.03.014.

Li, Ming O, Shomyseh Sanjabi, and Richard A Flavell. 2006. "Transforming Growth Factor-Beta Controls Development, Homeostasis, and Tolerance of T Cells by Regulatory T Cell-Dependent and -Independent Mechanisms." *Immunity* 25 (3): 455–71. https://doi.org/10.1016/j.immuni.2006.07.011.

Liao, Gongxian, Sushrusha Nayak, Jose R. Regueiro, Scott B. Berger, Cynthia Detre, Xavier Romero, Rene de Waal Malefyt, Talal A. Chatila, Roland W. Herzog, and Cox Terhorst. 2010. "GITR Engagement Preferentially Enhances Proliferation of Functionally Competent CD4+CD25+FoxP3+ Regulatory T Cells." *International Immunology* 22 (4): 259–70. https://doi.org/10.1093/intimm/dxq001.

Liao, Wei, Dustin E. Schones, Jangsuk Oh, Yongzhi Cui, Kairong Cui, Tae Young Roh, Keji Zhao, and Warren J. Leonard. 2008. "Priming for T Helper Type 2 Differentiation by Interleukin 2-Mediated Induction of Interleukin 4 Receptor α-Chain Expression." *Nature Immunology* 9 (11): 1288–96. https://doi.org/10.1038/ni.1656.

Liénart, Stéphanie, Romain Merceron, Christophe Vanderaa, Fanny Lambert, Didier Colau, Julie Stockis, Bas van der Woning, et al. 2018. "Structural Basis of Latent TGF-B1 Presentation and Activation by GARP on Human Regulatory T Cells." *Science*. https://doi.org/10.1126/science.aau2909.

Lin, Joseph, and Arthur Weiss. 2001. "Identification of the Minimal Tyrosine Residues Required for Linker for Activation of T Cell Function." *Journal of Biological Chemistry* 276 (31): 29588–95. https://doi.org/10.1074/jbc.M102221200.

Lin, Kui, Jennie B. Dorman, Aylin Rodan, and Cynthia Kenyon. 1997. "Daf-16: An HNF-3/Forkhead Family Member That Can Function to Double the Life-Span of Caenorhabditis Elegans." *Science* 278 (5341): 1319–22. https://doi.org/10.1126/science.278.5341.1319.

Linsley, Peter S., Jo Anne L. Greene, William Brady, Jürgen Bajorath, Jeffrey A. Ledbetter, and Robert Peach. 1994. "Human B7-1 (CD80) and B7-2 (CD86) Bind with Similar Avidities but Distinct Kinetics to CD28 and CTLA-4 Receptors." *Immunity* 1 (9): 793–801. https://doi.org/10.1016/S1074-7613(94)80021-9.

Lio, Chan Wang Joaquim, and Chyi Song Hsieh. 2008. "A Two-Step Process for Thymic Regulatory T Cell Development." *Immunity* 28 (1): 100–111. https://doi.org/10.1016/j.immuni.2007.11.021.

Liu, Yang, and Charles A. Janeway. 1990. "Interferon γ Plays a Critical Role in Induced Cell Death of Effector T Cell: A Possible Third Mechanism of Self-Tolerance." *Journal of Experimental Medicine* 172 (6): 1735–39. https://doi.org/10.1084/jem.172.6.1735.

Liu, Yongzhong, Pin Zhang, Jun Li, Ashok B. Kulkarni, Sylvain Perruche, and Wan Jun Chen. 2008. "A Critical Function for TGF-β Signaling in the Development of Natural CD4+CD25+Foxp3+ Regulatory T Cells." *Nature Immunology* 9 (6): 632–40. https://doi.org/10.1038/ni.1607.

Liu, Zhiduo, Michael Y. Gerner, Nicholas Van Panhuys, Andrew G. Levine, Alexander Y. Rudensky, and Ronald N. Germain. 2015. "Immune Homeostasis Enforced by Co-Localized Effector and Regulatory T Cells." *Nature* 528 (7581): 225–30. https://doi.org/10.1038/nature16169.

Llangumaran, Subburaj, Anne Briol, and Daniel C. Hoessli. 1998. "CD44 Selectively Associates with Active Src Family Protein Tyrosine Kinases Lck and Fyn in Glycosphingolipid-Rich Plasma Membrane Domains of Human Peripheral Blood Lymphocytes." *Blood* 91 (10): 3901–8. https://doi.org/10.1182/blood.v91.10.3901.

Lo, Roger S., Ye Guang Chen, Yigong Shi, Nikola P. Pavletich, and Joan Massagué. 1998. "The L3 Loop: A Structural Motif Determining Specific Interactions between SMAD Proteins and TGF-β Receptors." *EMBO Journal* 17 (4): 996–1005. https://doi.org/10.1093/emboj/17.4.996.

Long, Adrienne H., Waleed M. Haso, Jack F. Shern, Kelsey M. Wanhainen, Meera Murgai, Maria Ingaramo, Jillian P. Smith, et al. 2015. "4-1BB Costimulation Ameliorates T Cell Exhaustion Induced by Tonic Signaling of Chimeric Antigen Receptors." *Nature Medicine* 21 (6): 581–90. https://doi.org/10.1038/nm.3838.

Lorenzo, Patricia S., Maryam Beheshti, George R. Pettit, James C. Stone, and Peter M. Blumberg. 2000. "The Guanine Nucleotide Exchange Factor RasGRP Is a High-Affinity Target for Diacylglycerol and Phorbol Esters." *Molecular Pharmacology* 57 (5).

Louis, Chrystal U., Barbara Savoldo, Gianpietro Dotti, Martin Pule, Eric Yvon, G. Doug Myers, Claudia Rossig, et al. 2011. "Antitumor Activity and Long-Term Fate of Chimeric Antigen Receptor-Positive T Cells in Patients with Neuroblastoma." *Blood* 118 (23): 6050–56. https://doi.org/10.1182/blood-2011-05-354449.

Lu, Yong, Sungyoul Hong, Haiyan Li, Jungsun Park, Bangxing Hong, Lijuan Wang, Yuhuan Zheng, et al. 2012. "Th9 Cells Promote Antitumor Immune Responses in Vivo." *Journal of Clinical Investigation* 122 (11): 4160–71. https://doi.org/10.1172/JCI65459.

Luo, Kunxin, and Harvey F. Lodish. 1996. "Signaling by Chimeric Erythropoietin-TGF-β Receptors: Homodimerization of the Cytoplasmic Domain of the Type I TGF-β Receptor and Heterodimerization with the Type II Receptor Are Both Required for Intracellular Signal Transduction." *EMBO Journal* 15 (17): 4485–96. https://doi.org/10.1002/j.1460-2075.1996.tb00826.x.

Ma, Chaoyu, and Nu Zhang. 2015. "Transforming Growth Factor-β Signaling Is Constantly Shaping Memory T-Cell Population." *Proceedings of the National Academy of Sciences of the United States of America* 112 (35): 11013–17. https://doi.org/10.1073/pnas.1510119112.

MacKay, Laura K., Azad Rahimpour, Joel Z. Ma, Nicholas Collins, Angus T. Stock, Ming Li Hafon, Javier Vega-Ramos, et al. 2013. "The Developmental Pathway for CD103+ CD8+ Tissue-Resident Memory T Cells of Skin." *Nature Immunology* 14 (12): 1294–1301. https://doi.org/10.1038/ni.2744.

Maeda, H, and A Shiraishi. 1996. "TGF-Beta Contributes to the Shift toward Th2-Type Responses through Direct and IL-10-Mediated Pathways in Tumor-Bearing Mice." *Journal of Immunology (Baltimore, Md. : 1950)* 156 (1): 73–78. http://www.ncbi.nlm.nih.gov/pubmed/8598496.

Manel, Nicolas, Derya Unutmaz, and Dan R. Littman. 2008. "The Differentiation of Human TH-17 Cells Requires Transforming Growth Factor-β and Induction of the Nuclear Receptor RORγt." *Nature Immunology* 9 (6): 641–49. https://doi.org/10.1038/ni.1610.

Mangan, Paul R., Laurie E. Harrington, Darrell B. O'Quinn, Whitney S. Helms, Daniel C. Bullard, Charles O. Elson, Robin D. Hatton, Sharon M. Wahl, Trenton R. Schoeb, and Casey T. Weaver. 2006. "Transforming Growth Factor-β Induces Development of the T H17 Lineage." *Nature* 441 (7090): 231–34. https://doi.org/10.1038/nature04754.

Mao, Huawei, Wanling Yang, Sylvain Latour, Jing Yang, Sarah Winter, Jian Zheng, Ke Ni, et al. 2018. "RASGRP1 Mutation in Autoimmune Lymphoproliferative Syndrome-like Disease." *Journal of Allergy and Clinical Immunology* 142 (2): 595-604.e16. https://doi.org/10.1016/j.jaci.2017.10.026.

Marengère, Luc E.M., Paul Waterhouse, Gordon S. Duncan, Hans Willi Mittrücker, Gen Sheng Feng, and Tak W. Mak. 1996. "Regulation of T Cell Receptor Signaling by Tyrosine Phosphatase SYP Association with CTLA-4." *Science* 272 (5265): 1170–73. https://doi.org/10.1126/science.272.5265.1170.

Marie, Julien C, John J Letterio, Marc Gavin, and Alexander Y Rudensky. 2005. "TGF-Beta1 Maintains Suppressor Function and Foxp3 Expression in CD4+CD25+ Regulatory T Cells." *The Journal of Experimental Medicine* 201 (7): 1061–67. https://doi.org/10.1084/jem.20042276.

Market, Eleonora, and F. Nina Papavasiliou. 2003. "V(D)J Recombination and the Evolution of the Adaptive Immune System." *PLoS Biology*. Public Library of Science. https://doi.org/10.1371/journal.pbio.0000016.

MARSHALL, A. H., and R. G. WHITE. 1961. "The Immunological Reactivity of the Thymus." *British Journal of Experimental Pathology* 42 (August): 379–85. https://doi.org/10.1097/00006534-196204000-00081.

Marson, Alexander, Karsten Kretschmer, Garrett M. Frampton, Elizabeth S. Jacobsen, Julia K. Polansky, Kenzie D. MacIsaac, Stuart S. Levine, Ernest Fraenkel, Harald Von Boehmer, and Richard A. Young. 2007. "Foxp3 Occupancy and Regulation of Key Target Genes during T-Cell Stimulation." *Nature* 445 (7130): 931–35. https://doi.org/10.1038/nature05478.

Martin, Patrick, Marie Christine Poggi, Jean Claude Chambard, Kim E Boulukos, and Philippe Pognonec. 2006. "Low Dose Cadmium Poisoning Results in Sustained ERK Phosphorylation and Caspase Activation." *Biochemical and Biophysical Research Communications* 350 (3): 803–7. https://doi.org/10.1016/j.bbrc.2006.09.126.

Martinez, Gustavo J., Roza I. Nurieva, Xuexian O. Yang, and Chen Dong. 2008. "Regulation and Function of Proinflammatory TH17 Cells." *Annals of the New York Academy of Sciences*. Blackwell Publishing Inc. https://doi.org/10.1196/annals.1443.021.

Masopust, David, Daniel Choo, Vaiva Vezys, E. John Wherry, Jaikumar Duraiswamy, Rama Akondy, Jun Wang, et al. 2010. "Dynamic T Cell Migration Program Provides Resident Memory within Intestinal Epithelium." *Journal of Experimental Medicine* 207 (3): 553–64. https://doi.org/10.1084/jem.20090858.

Masopust, David, Vaiva Vezys, Amanda L. Marzo, and Leo Lefrançois. 2014. "Preferential Localization of Effector Memory Cells in Nonlymphoid Tissue." *Journal of Immunology* 192 (3): 845–49. https://doi.org/10.1126/science.1058867.

Masopust, David, Vaiva Vezys, E. John Wherry, Daniel L. Barber, and Rafi Ahmed. 2006. "Cutting Edge: Gut Microenvironment Promotes Differentiation of a Unique Memory CD8 T Cell Population." *The Journal of Immunology* 176 (4): 2079–83. https://doi.org/10.4049/jimmunol.176.4.2079.

Masuda, E S, Y Naito, H Tokumitsu, D Campbell, F Saito, C Hannum, K Arai, and N Arai. 1995. "NFATx, a Novel Member of the Nuclear Factor of Activated T Cells Family That Is Expressed Predominantly in the Thymus." *Molecular and Cellular Biology* 15 (5): 2697–2706. https://doi.org/10.1128/mcb.15.5.2697.

Matloubian, Mehrdad, Charles G. Lo, Guy Cinamon, Matthew J. Lesneski, Ying Xu, Volker Brinkmann, Maria L. Allende, Richard L. Proia, and Jason G. Cyster. 2004. "Lymphocyte Egress from Thymus and Peripheral Lymphoid Organs Is Dependent on S1P Receptor 1." *Nature* 427 (6972): 355–60. https://doi.org/10.1038/nature02284.

Matoba, Takuma, Masaki Imai, Naganari Ohkura, Daisuke Kawakita, Kei Ijichi, Tatsuya Toyama, Akimichi Morita, Shingo Murakami, Shimon Sakaguchi, and Sayuri Yamazaki. 2019. "Regulatory T Cells Expressing Abundant CTLA-4 on the Cell Surface with a Proliferative Gene Profile Are Key Features of Human Head and Neck Cancer." *International Journal of Cancer* 144 (11): 2811–22. https://doi.org/10.1002/ijc.32024.

Maude, Shannon L., Noelle Frey, Pamela A. Shaw, Richard Aplenc, David M. Barrett, Nancy J. Bunin, Anne Chew, et al. 2014. "Chimeric Antigen Receptor T Cells for Sustained Remissions in Leukemia." *New England Journal of Medicine* 371 (16): 1507–17. https://doi.org/10.1056/nejmoa1407222.

McHeyzer-Williams, Michael G., and Mark M. Davis. 1995. "Antigen-Specific Development of Primary and Memory T Cells in Vivo." *Science* 268 (5207): 106–11. https://doi.org/10.1126/science.7535476.

McKallip, Robert J., Yoon Do, Michael T. Fisher, John L. Robertson, Prakash S. Nagarkatti, and Mitzi Nagarkatti. 2002. "Role of CD44 in Activation-Induced Cell Death: CD44-Deficient Mice Exhibit Enhanced T Cell Response to Conventional and Superantigens." *International Immunology* 14 (9): 1015–26. https://doi.org/10.1093/intimm/dxf068.

McKarns, Susan C., Ronald H. Schwartz, and Norbert E. Kaminski. 2004. "Smad3 Is Essential for TGF-B1 to Suppress IL-2 Production and TCR-Induced Proliferation, but Not IL-2-Induced Proliferation." *The Journal of Immunology* 172 (7): 4275–84. https://doi.org/10.4049/jimmunol.172.7.4275.

Mercado, Roberto, Sujata Vijh, S. Elise Allen, Kristen Kerksiek, Ingrid M. Pilip, and Eric G. Pamer. 2000. "Early Programming of T Cell Populations Responding to Bacterial Infection." *The Journal of Immunology* 165 (12): 6833–39. https://doi.org/10.4049/jimmunol.165.12.6833.

Michalek, Ryan D, Valerie A Gerriets, Sarah R Jacobs, Andrew N Macintyre, Nancie J MacIver, Emily F Mason, Sarah A Sullivan, Amanda G Nichols, and Jeffrey C Rathmell. 2011. "Cutting Edge: Distinct Glycolytic and Lipid Oxidative Metabolic Programs Are Essential for Effector and Regulatory CDMichalek, R. D., Gerriets, V. A., Jacobs, S. R., Macintyre, A. N., MacIver, N. J., Mason, E. F., … Rathmell, J. C. (2011). Cutting Edge: D." *Journal of Immunology (Baltimore, Md. : 1950)* 186 (6): 3299–3303. https://doi.org/10.4049/jimmunol.1003613.

Miller, J. F.A.P. 1961. "IMMUNOLOGICAL FUNCTION OF THE THYMUS." *The Lancet* 278 (7205): 748–49. https://doi.org/10.1016/S0140-6736(61)90693-6.

Miyazono, K., A. Olofsson, P. Colosetti, and C. H. Heldin. 1991. "A Role of the Latent TGF-Beta 1-Binding Protein in the Assembly and Secretion of TGF-Beta 1." *The EMBO Journal* 10 (5): 1091–1101. https://doi.org/10.1002/j.1460-2075.1991.tb08049.x.

Miyazono, Kohei, Hidenori Ichijo, and Carl Henrik Heldin. 1993. "Transforming Growth Factor-β: Latent Forms, Binding Proteins and Receptors." *Growth Factors*. Informa Healthcare. https://doi.org/10.3109/08977199309029130.

Mombaerts, Peter, Alan R. Clarke, Michael A. Rudnicki, John Iacomini, Shigeyoshi Itohara, Juan J. Lafaille, Lili Wang, et al. 1992. "Mutations in T-Cell Antigen Receptor Genes α and β Block Thymocyte Development at Different Stages." *Nature* 360 (6401): 225–31. https://doi.org/10.1038/360225a0.

Mombaerts, Peter, John Iacomini, Randall S. Johnson, Karl Herrup, Susumu Tonegawa, and Virginia E. Papaioannou. 1992. "RAG-1-Deficient Mice Have No Mature B and T Lymphocytes." *Cell* 68 (5): 869–77. https://doi.org/10.1016/0092-8674(92)90030-G.

Morgan, Doris Anne, Francis W. Ruscetti, and Robert Gallo. 1976. "Selective in Vitro Growth of T Lymphocytes from Normal Human Bone Marrows." *Science* 193 (4257): 1007–8. https://doi.org/10.1126/science.181845.

Moskophidis, Demetrius, Ursula Assmann-Wischer, Markus M. Simon, and Fritz Lehmann-Grube. 1987. "The Immune Response of the Mouse to Lymphocytic Choriomeningitis Virus V. High Numbers of Cytolytic T Lymphocytes Are Generated in the Spleen during Acute Infection." *European Journal of Immunology* 17 (7): 937–42. https://doi.org/10.1002/eji.1830170707.

Mosmann, T. R., and R. L. Coffman. 1989. "TH1 and TH2 Cells: Different Patterns of Lymphokine Secretion Lead to Different Functional Properties." *Annual Review of Immunology*. Annu Rev Immunol. https://doi.org/10.1146/annurev.iy.07.040189.001045.

Mrass, Paulus, Ichiko Kinjyo, Lai Guan Ng, Steven L. Reiner, Ellen Puré, and Wolfgang Weninger. 2008. "CD44 Mediates Successful Interstitial Navigation by Killer T Cells and Enables Efficient Antitumor Immunity." *Immunity* 29 (6): 971–85. https://doi.org/10.1016/j.immuni.2008.10.015.

Mummert, Mark E., Diana Mummert, Dale Edelbaum, Francis Hui, Hiroyuki Matsue, and Akira Takashima. 2002. "Synthesis and Surface Expression of Hyaluronan by Dendritic Cells and Its Potential Role in Antigen Presentation." *The Journal of Immunology* 169 (8): 4322–31. https://doi.org/10.4049/jimmunol.169.8.4322.

Munger, John S., Xiaozhu Huang, Hisaaki Kawakatsu, Mark J.D. Griffiths, Stephen L. Dalton, Jianfeng Wu, Jean François Pittet, et al. 1999. "The Integrin Avβ6 Binds and Activates Latent TGFβ1: A Mechanism for Regulating Pulmonary Inflammation and Fibrosis." *Cell* 96 (3): 319–28. https://doi.org/10.1016/S0092-8674(00)80545-0.

Munn, David H., Ebrahim Shafizadeh, John T. Attwood, Igor Bondarev, Achal Pashine, and Andrew L. Mellor. 1999. "Inhibition of T Cell Proliferation by Macrophage Tryptophan Catabolism." *Journal of Experimental Medicine* 189 (9): 1363–72. https://doi.org/10.1084/jem.189.9.1363.

Munn, David H., Madhav D. Sharma, Deyan Hou, Babak Baban, Jeffrey R. Lee, Scott J. Antonia, Jane L. Messina, Phillip Chandler, Pandelakis A. Koni, and Andrew L. Mellor. 2004. "Expression of Indoleamine 2,3-Dioxygenase by Plasmacytoid Dendritic Cells in Tumor-Draining Lymph Nodes." *Journal of Clinical Investigation* 114 (2): 280–90. https://doi.org/10.1172/JCI200421583.

Mustelin, T., K. M. Coggeshall, and A. Altman. 1989. "Rapid Activation of the T-Cell Tyrosine Protein Kinase Pp56(Lck) by the CD45 Phosphotyrosine Phosphatase." *Proceedings of the National Academy of Sciences of the United States of America* 86 (16): 6302–6. https://doi.org/10.1073/pnas.86.16.6302.

Nakamura, Kazuhiko, Atsushi Kitani, Ivan Fuss, Aasta Pedersen, Naohiko Harada, Hajime Nawata, and Warren Strober. 2004. " TGF-B1 Plays an Important Role in the Mechanism of CD4 + CD25 + Regulatory T Cell Activity in Both Humans and Mice ." *The Journal of Immunology* 172 (2): 834–42. https://doi.org/10.4049/jimmunol.172.2.834.

Nakamura, Takafumi, Tomoko Shima, Ai Saeki, Takao Hidaka, Akitoshi Nakashima, Osamu Takikawa, and Shigeru Saito. 2007. "Expression of Indoleamine 2, 3-Dioxygenase and the Recruitment of Foxp3-Expressing Regulatory T Cells in the Development and Progression of Uterine Cervical Cancer." *Cancer Science* 98 (6): 874–81. https://doi.org/10.1111/j.1349-7006.2007.00470.x.

Nakano, Kazuhisa, Kazuyoshi Saito, Shinichiro Mine, Sho Matsushita, and Yoshiya Tanaka. 2007. "Engagement of CD44 Up-Regulates Fas Ligand Expression on T Cells Leading to Activation-Induced Cell Death." *Apoptosis* 12 (1): 45–54. https://doi.org/10.1007/s10495-006-0488-8.

Nakao, Atsuhito, Mozhgan Afrakhte, Anita Morén, Takuya Nakayama, Jan L. Christian, Rainer Heuchef, Susumu Itoh, et al. 1997. "Identification of Smad7, a TGFβ-Inducible Antagonist of TGF-β Signalling." *Nature* 389 (6651): 631–35. https://doi.org/10.1038/39369.

Nakayama, Toshinori, Alfred Singer, Eric D. Hsi, and Lawrence E. Samelson. 1989. "Intrathymic Signalling in Immature CD4+CD8+ Thymocytes Results in Tyrosine Phosphorylation of the T-Cell Receptor Zeta Chain." *Nature* 341 (6243): 651–54. https://doi.org/10.1038/341651a0.

Nelson, Alexander, Christina Cunha, Michael I. Nishimura, and Makio Iwashima. 2018. "Activated Human Foxp3+ Regulatory T Cells Produce Membrane-Bound TNF." *Cytokine* 111 (November): 454–59. https://doi.org/10.1016/j.cyto.2018.05.036.

Neumeister, E N, Y Zhu, S Richard, C Terhorst, A C Chan, and A S Shaw. 1995. "Binding of ZAP-70 to Phosphorylated T-Cell Receptor Zeta and Eta Enhances Its Autophosphorylation and Generates Specific Binding Sites for SH2 Domain-Containing Proteins." *Molecular and Cellular Biology* 15 (6): 3171–78. https://doi.org/10.1128/mcb.15.6.3171.

Nishibe, Shunzo, Matthew I. Wahl, S. M.Teresa Hernández-Sotomayor, Nicholas K. Tonks, Sue Goo Rhee, and Graham Carpenter. 1990. "Increase of the Catalytic Activity of Phospholipase C-Γ1 by Tyrosine Phosphorylation." *Science* 250 (4985): 1253–56. https://doi.org/10.1126/science.1700866.

Nishimura, Hiroyuki, Masato Nose, Hiroshi Hiai, Nagahiro Minato, and Tasuku Honjo. 1999. "Development of Lupus-like Autoimmune Diseases by Disruption of the PD-1 Gene Encoding an ITIM Motif-Carrying Immunoreceptor." *Immunity* 11 (2): 141–51. https://doi.org/10.1016/S1074-7613(00)80089-8.

Nishizuka, Yasuaki, and Teruyo Sakakura. 1969. "Thymus and Reproduction: Sex-Linked Dysgenesis of the Gonad after Neonatal Thymectomy in Mice." *Science* 166 (3906): 753–55. https://doi.org/10.1126/science.166.3906.753.

Norment, Anne M., Lisa Y. Bogatzki, Mark Klinger, Ethan W. Ojala, Michael J. Bevan, and Robert J. Kay. 2003. "Transgenic Expression of RasGRP1 Induces the Maturation of Double-Negative Thymocytes and Enhances the Production of CD8 Single-Positive Thymocytes." *The Journal of Immunology* 170 (3): 1141–49. https://doi.org/10.4049/jimmunol.170.3.1141.

Norment, Anne M., Russell D. Salter, Peter Parham, Victor H. Engelhard, and Dan R. Littman. 1988. "Cell-Cell Adhesion Mediated by CD8 and MHC Class I Molecules." *Nature* 336 (6194): 79–81. https://doi.org/10.1038/336079a0.

Nunes, I, R L Shapiro, and D B Rifkin. 1995. "Characterization of Latent TGF-Beta Activation by Murine Peritoneal Macrophages." *Journal of Immunology (Baltimore, Md. : 1950)* 155 (3): 1450–59. http://www.ncbi.nlm.nih.gov/pubmed/7636210.

Nunes, Irene, Pierre Emmanuel Gleizes, Christine N. Metz, and Daniel B. Rifkin. 1997. "Latent Transforming Growth Factor-β Binding Protein Domains Involved in Activation and Transglutaminase-Dependent Cross-Linking of Latent Transforming Growth Factor-β." *Journal of Cell Biology* 136 (5): 1151–63. https://doi.org/10.1083/jcb.136.5.1151.

O'Connor, Liam, Andreas Strasser, Lorraine A. O'Reilly, George Hausmann, Jerry M. Adams, Suzanne Cory, and David C.S. Huang. 1998. "Bim: A Novel Member of the Bcl-2 Family That Promotes Apoptosis." *EMBO Journal* 17 (2): 384–95. https://doi.org/10.1093/emboj/17.2.384.

Oderup, Cecilia, Lukas Cederbom, Anna Makowska, Corrado M. Cilio, and Fredrik Ivars. 2006. "Cytotoxic T Lymphocyte Antigen-4-Dependent down-Modulation of Costimulatory Molecules on Dendritic Cells in CD4+ CD25+ Regulatory T-Cell-Mediated Suppression." *Immunology* 118 (2): 240–49. https://doi.org/10.1111/j.1365-2567.2006.02362.x.

Oehm, A., I. Behrmann, W. Falk, M. Pawlita, G. Maier, C. Klas, M. Li-Weber, et al. 1992. "Purification and Molecular Cloning of the APO-1 Cell Surface Antigen, a Member of the Tumor Necrosis Factor/Nerve Growth Factor Receptor Superfamily. Sequence Identity with the Fas Antigen." *Journal of Biological Chemistry* 267 (15): 10709–15. https://doi.org/10.1016/s0021-9258(19)50076-x.

Oers, Nicolai S.C. van, Nigel Killeen, and Arthur Welss. 1994. "ZAP-70 Is Constitutively Associated with Tyrosine-Phosphorylated TCR ζ in Murine Thymocytes and Lymph Node T Cells." *Immunity* 1 (8): 675–85. https://doi.org/10.1016/1074-7613(94)90038-8.

Ogg, Scott, Suzanne Paradis, Shoshanna Gottlieb, Garth I. Patterson, Linda Lee, Heidi A. Tissenbaum, and Gary Ruvkun. 1997. "The Fork Head Transcription Factor DAF-16 Transduces Insulin-like Metabolic and Longevity Signals in C. Elegans." *Nature* 389 (6654): 994–99. https://doi.org/10.1038/40194.

Oh, Soyoung A., Dai-Chen Wu, Jeanne Cheung, Armando Navarro, Huizhong Xiong, Rafael Cubas, Klara Totpal, et al. 2020. "PD-L1 Expression by Dendritic Cells Is a Key Regulator of T-Cell Immunity in Cancer." *Nature Cancer* 1 (7): 681–91. https://doi.org/10.1038/s43018-020-0075-x.

Olofsson, Anders, Hidenori Ichijo, Anita Morén, Peter Ten Dijke, Kohei Miyazono, and Carl Henrik Heldin. 1995. "Efficient Association of an Amino-Terminally Extended Form of Human Latent Transforming Growth Factor-β Binding Protein with the Extracellular Matrix." *Journal of Biological Chemistry* 270 (52): 31294–97. https://doi.org/10.1074/jbc.270.52.31294.

Ono, Masahiro, Hiroko Yaguchi, Naganari Ohkura, Issay Kitabayashi, Yuko Nagamura, Takashi Nomura, Yoshiki Miyachi, Toshihiko Tsukada, and Shimon Sakaguchi. 2007. "Foxp3 Controls Regulatory T-Cell Function by Interacting with AML1/Runx1." *Nature* 446 (7136): 685–89. https://doi.org/10.1038/nature05673.

Ouyang, Weiming, Omar Beckett, Richard A. Flavell, and Ming O. Li. 2009. "An Essential Role of the Forkhead-Box Transcription Factor Foxo1 in Control of T Cell Homeostasis and Tolerance." *Immunity* 30 (3): 358–71. https://doi.org/10.1016/j.immuni.2009.02.003.

Ouyang, Weiming, Omar Beckett, Qian Ma, Ji Hye Paik, Ronald A. Depinho, and Ming O. Li. 2010. "Foxo Proteins Cooperatively Control the Differentiation of Foxp3 + Regulatory T Cells." *Nature Immunology* 11 (7): 618–27. https://doi.org/10.1038/ni.1884.

Ouyang, Weiming, Soyoung A. Oh, Qian Ma, Michael R. Bivona, Jinfang Zhu, and Ming O. Li. 2013. "TGF-β Cytokine Signaling Promotes CD8+ T Cell Development and Low-Affinity CD4+ T Cell Homeostasis by Regulation of Interleukin-7 Receptor α Expression." *Immunity* 39 (2): 335–46. https://doi.org/10.1016/j.immuni.2013.07.016.

Pacholczyk, Rafal, Hanna Ignatowicz, Piotr Kraj, and Leszek Ignatowicz. 2006. "Origin and T Cell Receptor Diversity of Foxp3+CD4+CD25+ T Cells." *Immunity* 25 (2): 249–59. https://doi.org/10.1016/j.immuni.2006.05.016.

Paik, Ji Hye, Ramya Kollipara, Gerald Chu, Hongkai Ji, Yonghong Xiao, Zhihu Ding, Lili Miao, et al. 2007. "FoxOs Are Lineage-Restricted Redundant Tumor Suppressors and Regulate Endothelial Cell Homeostasis." *Cell* 128 (2): 309–23. https://doi.org/10.1016/j.cell.2006.12.029.

Pandiyan, Pushpa, Lixin Zheng, Satoru Ishihara, Jennifer Reed, and Michael J. Lenardo. 2007. "CD4+CD25+Foxp3+ Regulatory T Cells Induce Cytokine Deprivation-Mediated Apoptosis of Effector CD4+ T Cells." *Nature Immunology* 8 (12): 1353–62. https://doi.org/10.1038/ni1536.

Park, Benjamin V., Zachary T. Freeman, Ali Ghasemzadeh, Michael A. Chattergoon, Alleluiah Rutebemberwa, Jordana Steigner, Matthew E. Winter, et al. 2016. "TGFβ1-Mediated SMAD3 Enhances PD-1 Expression on Antigen-Specific T Cells in Cancer." *Cancer Discovery* 6 (12): 1366–81. https://doi.org/10.1158/2159-8290.CD-15-1347.

Park, Heon, Zhaoxia Li, Xuexian O. Yang, Seon Hee Chang, Roza Nurieva, Yi Hong Wang, Ying Wang, et al. 2005. "A Distinct Lineage of CD4 T Cells Regulates Tissue Inflammation by Producing Interleukin 17." *Nature Immunology* 6 (11): 1133–41. https://doi.org/10.1038/ni1261.

Park, Il Kyoo, John J. Letterio, and James D. Gorham. 2007. "TGF-B1 Inhibition of IFN-γ-Induced Signaling and Th1 Gene Expression in CD4+ T Cells Is Smad3 Independent but MAP Kinase Dependent." *Molecular Immunology* 44 (13): 3283–90. https://doi.org/10.1016/j.molimm.2007.02.024.

Park, Jae Hong, Isabelle Riviere, Xiuyan Wang, Yvette Bernal, Terence Purdon, Elizabeth Halton, Kevin Joseph Curran, Craig Steven Sauter, Michel Sadelain, and Renier J. Brentjens. 2015. "Efficacy and Safety of CD19-Targeted 19-28z CAR Modified T Cells in Adult Patients with Relapsed or Refractory B-ALL." *Journal of Clinical Oncology* 33 (15_suppl): 7010–7010. https://doi.org/10.1200/jco.2015.33.15_suppl.7010.

Parrot, Tiphaine, Mathilde Allard, Romain Oger, Houssem Benlalam, Diane Raingeard de la Blétière, Anne Coutolleau, Laurence Preisser, et al. 2016. "IL-9 Promotes the Survival and Function of Human Melanoma-Infiltrating CD4+CD8+ Double-Positive T Cells." *European Journal of Immunology* 46 (7): 1770–82. https://doi.org/10.1002/eji.201546061.

Paul, William E. 2010. *What Determines Th2 Differentiation, in Vitro and in Vivo?* Vol. 88. https://doi.org/10.1038/icb.2010.2.

Pearce, Erika L., Alan C. Mullen, Gislâine A. Martins, Connie M. Krawczyk, Anne S. Hutchins, Valerie P. Zediak, Monica Banica, et al. 2003. "Control of Effector CD8+ T Cell Function by the Transcription Factor Eomesodermin." *Science* 302 (5647): 1041–43. https://doi.org/10.1126/science.1090148.

Pelak, Maciej J., Mirosław Śnietura, Dariusz Lange, Barbara Nikiel, and Katarzyna M. Pecka. 2015. "The Prognostic Significance of Indoleamine-2,3- Dioxygenase and the Recepto Rs for Transforming Growth Facto r β and Interferon γ in Metastatic Lymph Nodes in Malignant Melanoma." *Polish Journal of Pathology* 66 (4): 376–82. https://doi.org/10.5114/pjp.2015.57249.

Pelosi, Michele, Vincenzo Di Bartolo, Virginie Mounier, Dominique Mège, Jean Marc Pascussi, Evelyne Dufour, Arnaud Blondel, and Oreste Acuto. 1999. "Tyrosine 319 in the Interdomain B of ZAP-70 Is a Binding Site for the Src Homology 2 Domain of Lck." *Journal of Biological Chemistry* 274 (20): 14229–37. https://doi.org/10.1074/jbc.274.20.14229.

Philipson, Benjamin I., Roddy S. O'Connor, Michael J. May, Carl H. June, Steven M. Albelda, and Michael C. Milone. 2020. "4-1BB Costimulation Promotes CAR T Cell Survival through Noncanonical NF-KB Signaling." *Science Signaling* 13 (625). https://doi.org/10.1126/scisignal.aay8248.

Polic, Bojan, Desiree Kunkel, Alexander Scheffold, and Klaus Rajewsky. 2001. "How Aβ T Cells Deal with Induced TCRα Ablation." *Proceedings of the National Academy of Sciences of the United States of America* 98 (15): 8744–49. https://doi.org/10.1073/pnas.141218898.

Priatel, John J., Xiaoxi Chen, Salim Dhanji, Ninan Abraham, and Hung-Sia Teh. 2006. "RasGRP1 Transmits Prodifferentiation TCR Signaling That Is Crucial for CD4 T Cell Development." *The Journal of Immunology* 177 (3): 1470–80. https://doi.org/10.4049/jimmunol.177.3.1470.

Priatel, John J., Xiaoxi Chen, Lauren A. Zenewicz, Hao Shen, Kenneth W. Harder, Marc S. Horwitz, and Hung-Sia Teh. 2007. "Chronic Immunodeficiency in Mice Lacking RasGRP1 Results in CD4 T Cell Immune Activation and Exhaustion." *The Journal of Immunology* 179 (4): 2143–52. https://doi.org/10.4049/jimmunol.179.4.2143.

Priatel, John J., Soo Jeet Teh, Nancy A. Dower, James C. Stone, and Hung Sia Teh. 2002. "RasGRP1 Transduces Low-Grade TCR Signals Which Are Critical for T Cell Development, Homeostasis, and Differentiation." *Immunity* 17 (5): 617–27. https://doi.org/10.1016/S1074-7613(02)00451-X.

Prinz, Immo, Amandine Sansoni, Adrien Kissenpfennig, Laurence Ardouin, Marie Malissen, and Bernard Malissen. 2006. "Visualization of the Earliest Steps of Γδ T Cell Development in the Adult Thymus." *Nature Immunology* 7 (9): 995–1003. https://doi.org/10.1038/ni1371.

Qin, Bin Y., Suvana S. Lam, John J. Correia, and Kai Lin. 2002. "Smad3 Allostery Links TGF-β Receptor Kinase Activation to Transcriptional Control." *Genes and Development* 16 (15): 1950–63. https://doi.org/10.1101/gad.1002002.

Radtke, Freddy, Anne Wilson, Gerlinde Stark, Michelle Bauer, Joost Van Meerwijk, H. Robson MacDonald, and Michel Aguet. 1999. "Deficient T Cell Fate Specification in Mice with an Induced Inactivation of Notch1." *Immunity* 10 (5): 547–58. https://doi.org/10.1016/S1074-7613(00)80054-0.

Radvanyi, L. G., G. B. Mills, and R. G. Miller. 1993. "Religation of the T Cell Receptor after Primary Activation of Mature T Cells Inhibits Proliferation and Induces Apoptotic Cell Death." *The Journal of Immunology (1950)* 150 (12): 5704–15.

Rao, Rajesh R., Qingsheng Li, Melanie R.Gubbels Bupp, and Protul A. Shrikant. 2012. "Transcription Factor Foxo1 Represses T-Bet-Mediated Effector Functions and Promotes Memory CD8 + T Cell Differentiation." *Immunity* 36 (3): 374–87. https://doi.org/10.1016/j.immuni.2012.01.015.

Ravichandran, K S, U Lorenz, S E Shoelson, and S J Burakoff. 1995. "Interaction of Shc with Grb2 Regulates Association of Grb2 with MSOS." *Molecular and Cellular Biology* 15 (2): 593–600. https://doi.org/10.1128/mcb.15.2.593.

Read, Simon, Vivianne Malmström, and Fiona Powrie. 2000. "Cytotoxic T Lymphocyte-Associated Antigen 4 Plays an Essential Role in the Function of CD25+CD4+ Regulatory Cells That Control Intestinal Inflammation." *Journal of Experimental Medicine* 192 (2): 295–302. https://doi.org/10.1084/jem.192.2.295.

Refaeli, Y, L Van Parijs, C A London, J Tschopp, and A K Abbas. 1998. "Biochemical Mechanisms of IL-2-Regulated Fas-Mediated T Cell Apoptosis." *Immunity* 8 (5): 615–23. http://www.ncbi.nlm.nih.gov/pubmed/9620682.

Reinherz, Ellis L., Stefan Meuer, Kathleen A. Fitzgerald, Rebecca E. Hussey, Herbert Levine, and Stuart F. Schlossman. 1982. "Antigen Recognition by Human T Lymphocytes Is Linked to Surface Expression of the T3 Molecular Complex." *Cell* 30 (3): 735–43. https://doi.org/10.1016/0092-8674(82)90278-1.

Ren, X., F. Ye, Z. Jiang, Y. Chu, S. Xiong, and Y. Wang. 2007. "Involvement of Cellular Death in TRAIL/DR5-Dependent Suppression Induced by CD4+CD25+ Regulatory T Cells." *Cell Death and Differentiation* 14 (12): 2076–84. https://doi.org/10.1038/sj.cdd.4402220.

Rena, G., A. R. Prescott, S. Guo, P. Cohen, and T. G. Unterman. 2001. "Roles of the Forkhead in Rhabdomyosarcoma (FKHR) Phosphorylation Sites in Regulating 14-3-3 Binding, Transactivation and Nuclear Targetting." *Biochemical Journal* 354 (3): 605–12. https://doi.org/10.1042/0264-6021:3540605.

Richter, G. H.S., A. Mollweide, K. Hanewinkel, C. Zobywalski, and S. Burdach. 2009. "CD25 Blockade Protects T Cells from Activation-Induced Cell Death (AICD) via Maintenance of TOSO Expression." *Scandinavian Journal of Immunology* 70 (3): 206–15. https://doi.org/10.1111/j.1365-3083.2009.02281.x.

Riddle, Donald L., Margaret M. Swanson, and Patrice S. Albert. 1981. "Interacting Genes in Nematode Dauer Larva Formation." *Nature* 290 (5808): 668–71. https://doi.org/10.1038/290668a0.

Riese, Matthew J., Jashanpreet Grewal, Jayajit Das, Tao Zou, Vineet Patil, Arup K. Chakraborty, and Gary A. Koretzky. 2011. "Decreased Diacylglycerol Metabolism Enhances ERK Activation and Augments CD8+ T Cell Functional Responses." *Journal of Biological Chemistry* 286 (7): 5254–65. https://doi.org/10.1074/jbc.M110.171884.

Riou, Catherine, Bader Yassine-Diab, Julien Van Grevenynghe, Roland Somogyi, Larry D. Greller, Dominic Gagnon, Sylvain Gimmig, et al. 2007. "Convergence of TCR and Cytokine Signaling Leads to FOXO3a Phosphorylation and Drives the Survival of CD4+ Central Memory T Cells." *Journal of Experimental Medicine* 204 (1): 79–91. https://doi.org/10.1084/jem.20061681.

Robertson, H. 2001. "Relationship between TGFβ1, Intratubular CD103 Positive T Cells and Acute Renal Allograft Rejection." In *Transplantation Proceedings*, 33:1159. Transplant Proc. https://doi.org/10.1016/S0041-1345(00)02441-6.

Robinson, Douglas S., Qutayba Hamid, Sun Ying, Anne Tsicopoulos, Julia Barkans, Andrew M. Bentley, Christopher Corrigan, Stephen R. Durham, and A. Barry Kay. 1992. "Predominant TH2-like Bronchoalveolar T-Lymphocyte Population in Atopic Asthma." *New England Journal of Medicine* 326 (5): 298–304. https://doi.org/10.1056/NEJM199201303260504.

Rollings, Christina M., Linda V. Sinclair, Hugh J.M. Brady, Doreen A. Cantrell, and Sarah H. Ross. 2018. "Interleukin-2 Shapes the Cytotoxic T Cell Proteome and Immune Environment–Sensing Programs." *Science Signaling*. American Association for the Advancement of Science. https://doi.org/10.1126/scisignal.aap8112.

Ronchetti, Simona, Erika Ricci, Maria Grazia Petrillo, Luigi Cari, Graziella Migliorati, Giuseppe Nocentini, and Carlo Riccardi. 2015. "Glucocorticoid-Induced Tumour Necrosis Factor Receptor-Related Protein: A Key Marker of Functional Regulatory T Cells." *Journal of Immunology Research*. Hindawi Publishing Corporation. https://doi.org/10.1155/2015/171520.

Rooke, Ronald, Caroline Waltzinger, Christophe Benoist, and Diane Mathis. 1997. "Targeted Complementation of MHC Class II Deficiency by Intrathymic Delivery of Recombinant Adenoviruses." *Immunity* 7 (1): 123–34. https://doi.org/10.1016/S1074-7613(00)80515-4.

Roose, Jeroen P., Marianne Mollenauer, Vikas A. Gupta, James Stone, and Arthur Weiss. 2005. "A Diacylglycerol-Protein Kinase C-RasGRP1 Pathway Directs Ras Activation upon Antigen Receptor Stimulation of T Cells." *Molecular and Cellular Biology* 25 (11): 4426–41. https://doi.org/10.1128/mcb.25.11.4426-4441.2005.

Roose, Jeroen P, Marianne Mollenauer, Mary Ho, Tomohiro Kurosaki, and Arthur Weiss. 2007. "Unusual Interplay of Two Types of Ras Activators, RasGRP and SOS, Establishes Sensitive and Robust Ras Activation in Lymphocytes." *Molecular and Cellular Biology* 27 (7): 2732–45. https://doi.org/10.1128/MCB.01882-06.

Rosenberg, Steven A., Beverly S. Packard, Paul M. Aebersold, Diane Solomon, Suzanne L. Topalian, Stephen T. Toy, Paul Simon, et al. 1988. "Use of Tumor-Infiltrating Lymphocytes and Interleukin-2 in the Immunotherapy of Patients with Metastatic Melanoma." *New England Journal of Medicine* 319 (25): 1676–80. https://doi.org/10.1056/nejm198812223192527.

Rosenberg, Steven A., Paul Spiess, and Rene Lafreniere. 1986. "A New Approach to the Adoptive Immunotherapy of Cancer with Tumor-Infiltrating Lymphocytes." *Science* 233 (4770): 1318–21. https://doi.org/10.1126/science.3489291.

Ruffell, Brian, and Pauline Johnson. 2008. "Hyaluronan Induces Cell Death in Activated T Cells through CD44." *The Journal of Immunology* 181 (10): 7044–54. https://doi.org/10.4049/jimmunol.181.10.7044.

Sadlack, Benjamin, Hartmut Merz, Hubert Schorle, Anneliese Schimpl, Alfred C. Feller, and Ivan Horak. 1993. "Ulcerative Colitis-like Disease in Mice with a Disrupted Interleukin-2 Gene." *Cell* 75 (2): 253–61. https://doi.org/10.1016/0092-8674(93)80067-O.

Sadlon, Timothy J., Bridget G. Wilkinson, Stephen Pederson, Cheryl Y. Brown, Suzanne Bresatz, Tessa Gargett, Elizabeth L. Melville, et al. 2010. "Genome-Wide Identification of Human FOXP3 Target Genes in Natural Regulatory T Cells." *The Journal of Immunology* 185 (2): 1071–81. https://doi.org/10.4049/jimmunol.1000082.

Sakaguchi, S, N Sakaguchi, M Asano, M Itoh, and M Toda. 1995. "Immunologic Self-Tolerance Maintained by Activated T Cells Expressing IL-2 Receptor Alpha-Chains (CD25). Breakdown of a Single Mechanism of Self-Tolerance Causes Various Autoimmune Diseases." *Journal of Immunology (Baltimore, Md. : 1950)* 155 (3): 1151–64. http://www.ncbi.nlm.nih.gov/pubmed/7636184.

Sallusto, Federica, Danielle Lenig, Reinhold Förster, Martin Lipp, and Antonio Lanzavecchia. 1999. "Two Subsets of Memory T Lymphocytes with Distinct Homing Potentials and Effector Functions." *Nature* 401 (6754): 708–12. https://doi.org/10.1038/44385.

Salzer, Elisabeth, Deniz Cagdas, Miroslav Hons, Emily M. MacE, Wojciech Garncarz, Özlem Yüce Petronczki, René Platzer, et al. 2016. "RASGRP1 Deficiency Causes Immunodeficiency with Impaired Cytoskeletal Dynamics." *Nature Immunology* 17 (12): 1352–60. https://doi.org/10.1038/ni.3575.

Samelson, Lawrence E., Andrew F. Phillips, Elise T. Luong, and Richard D. Klausner. 1990. "Association of the Fyn Protein-Tyrosine Kinase with the T-Cell Antigen Receptor." *Proceedings of the National Academy of Sciences of the United States of America* 87 (11): 4358–62. https://doi.org/10.1073/pnas.87.11.4358.

Sandau, Michelle M., Jacob E. Kohlmeier, David L. Woodland, and Stephen C. Jameson. 2010. "IL-15 Regulates Both Quantitative and Qualitative Features of the Memory CD8 T Cell Pool." *The Journal of Immunology* 184 (1): 35–44. https://doi.org/10.4049/jimmunol.0803355.

Sanjabi, Shomyseh, Munir M. Mosaheb, and Richard A. Flavell. 2009. "Opposing Effects of TGF-β and IL-15 Cytokines Control the Number of Short-Lived Effector CD8 + T Cells." *Immunity* 31 (1): 131–44. https://doi.org/10.1016/j.immuni.2009.04.020.

Savoldo, Barbara, Carlos Almeida Ramos, Enli Liu, Martha P. Mims, Michael J. Keating, George Carrum, Rammurti T. Kamble, et al. 2011. "CD28 Costimulation Improves Expansion and Persistence of Chimeric Antigen Receptor-Modified T Cells in Lymphoma Patients." *Journal of Clinical Investigation* 121 (5): 1822–26. https://doi.org/10.1172/JCI46110.

Schluns, Kimberly S., William C. Kieper, Stephen C. Jameson, and Leo Lefrançois. 2000. "Interleukin-7 Mediates the Homeostasis of Naïve and Memory CD8 T Cells in Vivo." *Nature Immunology* 1 (5): 426–32. https://doi.org/10.1038/80868.

Schmitt, E, T Germann, S Goedert, P Hoehn, C Huels, S Koelsch, R Kühn, W Müller, N Palm, and E Rüde. 1994. "IL-9 Production of Naive CD4+ T Cells Depends on IL-2, Is Synergistically Enhanced by a Combination of TGF-Beta and IL-4, and Is Inhibited by IFN-Gamma." *Journal of Immunology (Baltimore, Md. : 1950)* 153 (9): 3989–96. http://www.ncbi.nlm.nih.gov/pubmed/7930607.

Schneider, Helga, Elke Valk, Rufina Leung, and Christopher E. Rudd. 2008. "CTLA-4 Activation of Phosphatidylinositol 3-Kinase (PI 3-K) and Protein Kinase B (PKB/AKT) Sustains T-Cell Anergy without Cell Death." *PLoS ONE* 3 (12). https://doi.org/10.1371/journal.pone.0003842.

Schorle, Hubert, Thomas Holtschke, Thomas Hünig, Anneliese Schimpi, and Ivan Horak. 1991. "Development and Function of T Cells in Mice Rendered Interleukin-2 Deficient by Gene Targeting." *Nature* 352 (6336): 621–24. https://doi.org/10.1038/352621a0.

Schumann, Julia, Katarina Stanko, Ulrike Schliesser, Christine Appelt, and Birgit Sawitzki. 2015. "Differences in CD44 Surface Expression Levels and Function Discriminates IL-17 and IFN-γ Producing Helper T Cells." *PLoS ONE* 10 (7). https://doi.org/10.1371/journal.pone.0132479.

Seddon, Benedict, Peter Tomlinson, and Rose Zamoyska. 2003. "Interleukin 7 and T Cell Receptor Signals Regulate Homeostasis of CD4 Memory Cells." *Nature Immunology* 4 (7): 680–86. https://doi.org/10.1038/ni946.

Seoane, Joan, Hong Van Le, Lijian Shen, Stewart A. Anderson, and Joan Massagué. 2004. "Integration of Smad and Forkhead Pathways in the Control of Neuroepithelial and Glioblastoma Cell Proliferation." *Cell* 117 (2): 211–23. https://doi.org/10.1016/S0092-8674(04)00298-3.

Serafini, Andrew T., Richard S. Lewis, Neil A. Clipstone, Richard J. Bram, Christopher Fanger, Steve Flering, Leonard A. Herzenberg, and Gerald R. Crabtree. 1995. "Isolation of Mutant T Lymphocytes with Defects in Capacitative Calcium Entry." *Immunity* 3 (2): 239–50. https://doi.org/10.1016/1074-7613(95)90093-4.

Setoguchi, Ruka, Shohei Hori, Takeshi Takahashi, and Shimon Sakaguchi. 2005. "Homeostatic Maintenance of Natural Foxp3+ CD25+ CD4+ Regulatory T Cells by Interleukin (IL)-2 and Induction of Autoimmune Disease by IL-2 Neutralization." *Journal of Experimental Medicine* 201 (5): 723–35. https://doi.org/10.1084/jem.20041982.

Shahinian, Arda, Klaus Pfefer, Kelvin P. Lee, Thomas M. Kündig, Kenji Kishihara, Andrew Wakeham, Kazuhiro Kawai, Pamela S. Ohashi, Craig B. Thompson, and Tak W. Mak. 1993. "Differential T Cell Costimulatory Requirements in CD28-Deficient Mice." *Science* 261 (5121): 609–12. https://doi.org/10.1126/science.7688139.

Shaw, A S, J Chalupny, J A Whitney, C Hammond, K E Amrein, P Kavathas, B M Sefton, and J K Rose. 1990. "Short Related Sequences in the Cytoplasmic Domains of CD4 and CD8 Mediate Binding to the Amino-Terminal Domain of the P56lck Tyrosine Protein Kinase." *Molecular and Cellular Biology* 10 (5): 1853–62. https://doi.org/10.1128/mcb.10.5.1853-1862.1990.

Shen, Shudan, Yong Chen, Balachandra K. Gorentla, Jianxin Lu, James C. Stone, and Xiao-Ping Zhong. 2011. "Critical Roles of RasGRP1 for Invariant NKT Cell Development." *The Journal of Immunology* 187 (9): 4467–73. https://doi.org/10.4049/jimmunol.1003798.

Shimizu, T., S. Nomiyama, F. Hirata, and O. Hayaishi. 1978. "Indoleamine 2,3 Dioxygenase. Purfication and Some Properties." *Journal of Biological Chemistry* 253 (13): 4700–4706. https://doi.org/10.1016/s0021-9258(17)30447-7.

Shinkai, Yoichi, Shigeo Koyasu, Kei Ichi Nakayama, Kenneth M. Murphy, Dennis Y. Loh, Ellis L. Reinherz, and Frederick W. Alt. 1993. "Restoration of T Cell Development in RAG-2-Deficient Mice by Functional TCR Transgenes." *Science* 259 (5096): 822–25. https://doi.org/10.1126/science.8430336.

Shull, Marcia M., Ilona Ormsby, Ann B. Kier, Sharon Pawlowski, Ronald J. Diebold, Moying Yin, Ruth Allen, et al. 1992. "Targeted Disruption of the Mouse Transforming Growth Factor-B1 Gene Results in Multifocal Inflammatory Disease [14]." *Nature* 359 (6397): 693–99. https://doi.org/10.1038/359693a0.

Singh, Nagendra, Mutsumi Yamamoto, Mariko Takami, Yoichi Seki, Mayuko Takezaki, Andrew L Mellor, and Makio Iwashima. 2010. "CD4(+)CD25(+) Regulatory T Cells Resist a Novel Form of CD28- and Fas-Dependent P53-Induced T Cell Apoptosis." *Journal of Immunology (Baltimore, Md. : 1950)* 184 (1): 94–104. https://doi.org/10.4049/jimmunol.0900753.

Sisirak, Vanja, Julien Faget, Michael Gobert, Nadège Goutagny, Nelly Vey, Isabelle Treilleux, Sarah Renaudineau, et al. 2012. "Impaired IFN-α Production by Plasmacytoid Dendritic Cells Favors Regulatory T-Cell Expansion That May Contribute to Breast Cancer Progression." *Cancer Research* 72 (20): 5188–97. https://doi.org/10.1158/0008-5472.CAN-11-3468.

Skelton, Timothy P., Chunxun Zeng, Aaron Nocks, and Ivan Stamenkovic. 1998. "Glycosylation Provides Both Stimulatory and Inhibitory Effects on Cell Surface and Soluble CD44 Binding to Hyaluronan." *Journal of Cell Biology* 140 (2): 431–46. https://doi.org/10.1083/jcb.140.2.431.

Skon, Cara N., June Yong Lee, Kristin G. Anderson, David Masopust, Kristin A. Hogquist, and Stephen C. Jameson. 2013. "Transcriptional Downregulation of S1pr1 Is Required for the Establishment of Resident Memory CD8+ T Cells." *Nature Immunology* 14 (12): 1285–93. https://doi.org/10.1038/ni.2745.

Smeltz, Ronald B., June Chen, Rolf Ehrhardt, and Ethan M. Shevach. 2002. "Role of IFN-γ in Th1 Differentiation: IFN-γ Regulates IL-18Rα Expression by Preventing the Negative Effects of IL-4 and by Inducing/Maintaining IL-12 Receptor B2 Expression." *The Journal of Immunology* 168 (12): 6165–72. https://doi.org/10.4049/jimmunol.168.12.6165.

Smith, C A, G T Williams, R Kingston, E J Jenkinson, and J J Owen. 1989. "Antibodies to CD3/T-Cell Receptor Complex Induce Death by Apoptosis in Immature T Cells in Thymic Cultures." *Nature* 337 (6203): 181–84. https://doi.org/10.1038/337181a0.

Soper, David M., Deborah J. Kasprowicz, and Steven F. Ziegler. 2007. "IL-2Rβ Links IL-2R Signaling with Foxp3 Expression." *European Journal of Immunology* 37 (7): 1817–26. https://doi.org/10.1002/eji.200737101.

Sprent, J., and A. Basten. 1973. "Circulating T and B Lymphocytes of the Mouse. II. Lifespan." *Cellular Immunology* 7 (1): 40–59. https://doi.org/10.1016/0008-8749(73)90181-0.

Stahl, Marie, Pascale F Dijkers, Geert J P L Kops, Susanne M A Lens, Paul J Coffer, Boudewijn M T Burgering, and René H Medema. 2002. "The Forkhead Transcription Factor FoxO Regulates Transcription of P27Kip1 and Bim in Response to IL-2." *Journal of Immunology (Baltimore, Md. : 1950)* 168 (10): 5024–31. https://doi.org/10.4049/jimmunol.168.10.5024.

Stang, Stacey L, Ana Lopez-Campistrous, Xiaohua Song, Nancy A Dower, Peter M Blumberg, Paul A Wender, and James C Stone. 2009. "A Proapoptotic Signaling Pathway Involving RasGRP, Erk, and Bim in B Cells." *Experimental Hematology* 37 (1): 122–34. https://doi.org/10.1016/j.exphem.2008.09.008.

Stankunas, K., I. A. Graef, J. R. Neilson, S. H. Park, and G. R. Crabtree. 1999. "Signaling through Calcium, Calcineurin, and NF-AT in Lymphocyte Activation and Development." In *Cold Spring Harbor Symposia on Quantitative Biology*, 64:505–16. Cold Spring Harbor Laboratory Press. https://doi.org/10.1101/sqb.1999.64.505.

Stienne, Caroline, Michaël F. F Michieletto, Mehdi Benamar, Nadège Carrié, Isabelle Bernard, Xuan-Hung Nguyen, Yannick Lippi, et al. 2016. "Foxo3 Transcription Factor Drives Pathogenic T Helper 1 Differentiation by Inducing the Expression of Eomes." *Immunity* 45 (4): 774–87. https://doi.org/10.1016/j.immuni.2016.09.010.

Stipdonk, Marianne J.B. Van, Edward E. Lemmens, and Stephen P. Schoenberger. 2001. "Naïve CTLs Require a Single Brief Period of Antigenic Stimulation for Clonal Expansion and Differentiation." *Nature Immunology* 2 (5): 423–29. https://doi.org/10.1038/87730.

Stirnweiss, Anja, Roland Hartig, Steffi Gieseler, Jonathan A. Lindquist, Peter Reichardt, Lars Philipsen, Luca Simeoni, et al. 2013. "T Cell Activation Results in Conformational Changes in the Src Family Kinase Lck to Induce Its Activation." *Science Signaling* 6 (263). https://doi.org/10.1126/scisignal.2003607.

Straus, David B., Andrew C. Chan, Barbara Patai, and Arthur Weiss. 1996. "SH2 Domain Function Is Essential for the Role of the Lck Tyrosine Kinase in T Cell Receptor Signal Transduction." *Journal of Biological Chemistry* 271 (17): 9976–81. https://doi.org/10.1074/jbc.271.17.9976.

Suarez, Eloah Rabello, De Kuan Chang, Jiusong Sun, Jianhua Sui, Gordon J. Freeman, Sabina Signoretti, Quan Zhu, and Wayne A. Marasco. 2016. "Chimeric Antigen Receptor T Cells Secreting Anti-PD-L1 Antibodies More Effectively Regress Renal Cell Carcinoma in a Humanized Mouse Model." *Oncotarget* 7 (23): 34341–55. https://doi.org/10.18632/oncotarget.9114.

Sullivan, Brandon M., Amy Juedes, Susanne J. Szabo, Matthias Von Herrath, and Laurie H. Glimcher. 2003. "Antigen-Driven Effector CD8 T Cell Function Regulated by T-Bet." *Proceedings of the National Academy of Sciences of the United States of America* 100 (26): 15818–23. https://doi.org/10.1073/pnas.2636938100.

Sullivan, Jeremy A., Eui Ho Kim, Erin H. Plisch, Stanford L. Peng, and M. Suresh. 2012. "FOXO3 Regulates CD8 T Cell Memory by T Cell-Intrinsic Mechanisms." Edited by Christopher M. Walker. *PLoS Pathogens* 8 (2): e1002533. https://doi.org/10.1371/journal.ppat.1002533.

Sung, James L., Jack T. Lin, and James D. Gorham. 2003. "CD28 Co-Stimulation Regulates the Effect of Transforming Growth Factor-B1 on the Proliferation of Naïve CD4+ T Cells." *International Immunopharmacology* 3 (2): 233–45. https://doi.org/10.1016/S1567-5769(02)00276-X.

Surh, Charles D., and Jonathan Sprent. 2008. "Homeostasis of Naive and Memory T Cells." *Immunity*. https://doi.org/10.1016/j.immuni.2008.11.002.

Szabo, Susanne J., Sean T. Kim, Gina L. Costa, Xiankui Zhang, C. Garrison Fathman, and Laurie H. Glimcher. 2000. "A Novel Transcription Factor, T-Bet, Directs Th1 Lineage Commitment." *Cell* 100 (6): 655–69. https://doi.org/10.1016/S0092-8674(00)80702-3.

Szostak, Bartosz, Filip Machaj, Jakub Rosik, and Andrzej Pawlik. 2019. "CTLA4 Antagonists in Phase I and Phase II Clinical Trials, Current Status and Future Perspectives for Cancer Therapy." *Expert Opinion on Investigational Drugs*. Taylor and Francis Ltd. https://doi.org/10.1080/13543784.2019.1559297.

Taghon, Tom, Mary A. Yui, Rashmi Pant, Rochelle A. Diamond, and Ellen V. Rothenberg. 2006. "Developmental and Molecular Characterization of Emerging β- and Γδ-Selected Pre-T Cells in the Adult Mouse Thymus." *Immunity* 24 (1): 53–64. https://doi.org/10.1016/j.immuni.2005.11.012.

Taher, Taher Elamin I., Linda Smit, Arjan W. Griffioen, Esther J.M. Schilder-Tol, Jannie Borst, and Steven T. Pals. 1996. "Signaling through CD44 Is Mediated by Tyrosine Kinases: Association with P56lck in T Lymphocytes." *Journal of Biological Chemistry* 271 (5): 2863–67. https://doi.org/10.1074/jbc.271.5.2863.

Takahashi, Takeshi, Yuhshi Kuniyasu, Masaaki Toda, Noriko Sakaguchi, Misako Itoh, Makoto Iwata, Jun Shimizu, and Shimon Sakaguchi. 1998. "Immunologic Self-Tolerance Maintained by CD25+CD4+ Naturally Anergic and Suppressive T Cells: Induction of Autoimmune Disease by Breaking Their Anergic/Suppressive State." *International Immunology* 10 (12): 1969–80. https://doi.org/10.1093/intimm/10.12.1969.

Takahashi, Takeshi, Tomoyuki Tagami, Sayuri Yamazaki, Toshimitsu Uede, Jun Shimizu, Noriko Sakaguchi, Tak W. Mak, and Shimon Sakaguchi. 2000. "Immunologic Self-Tolerance Maintained by CD25+CD4+ Regulatory T Cells Constitutively Expressing Cytotoxic T Lymphocyte-Associated Antigen 4." *Journal of Experimental Medicine* 192 (2): 303–9. https://doi.org/10.1084/jem.192.2.303.

Takami, Mariko, Robert B Love, and Makio Iwashima. 2012. "TGF-β Converts Apoptotic Stimuli into the Signal for Th9 Differentiation." *Journal of Immunology (Baltimore, Md. : 1950)* 188 (9): 4369–75. https://doi.org/10.4049/jimmunol.1102698.

Takami, Mariko, Christina Cunha, Shinichiro Motohashi, Toshinori Nakayama, and Makio Iwashima. 2018. "TGF-β Suppresses RasGRP1 Expression and Supports Regulatory T Cell Resistance against P53-Induced CD28-Dependent T-Cell Apoptosis" 48 (12): 1938–43. https://doi.org/10.1002/eji.201847587.

Takeda, Shunichi, Hans Reimer Rodewald, Hiroshi Arakawa, Horst Bluethmann, and Takeyuki Shimizu. 1996. "MHC Class II Molecules Are Not Required for Survival of Newly Generated CD4+ T Cells, but Affect Their Long-Term Life Span." *Immunity* 5 (3): 217–28. https://doi.org/10.1016/S1074-7613(00)80317-9.

Tamada, K, M Harada, K Abe, T Li, H Tada, Y Onoe, and K Nomoto. 1997. "Immunosuppressive Activity of Cloned Natural Killer (NK1.1+) T Cells Established from Murine Tumor-Infiltrating Lymphocytes." *Journal of Immunology (Baltimore, Md. : 1950)* 158 (10): 4846–54. http://www.ncbi.nlm.nih.gov/pubmed/9144500.

Tan, Joyce T., Eric Dudl, Eric LeRoy, Richard Murray, Jonathan Sprent, Kenneth I. Weinberg, and Charles D. Surh. 2001. "IL-7 Is Critical for Homeostatic Proliferation and Survival of Naïve T Cells." *Proceedings of the National Academy of Sciences of the United States of America* 98 (15): 8732–37. https://doi.org/10.1073/pnas.161126098.

Tanchot, Corinne, François A. Lemonnier, Beatrice Pérarnau, Antonio A. Freitas, and Benedita Rocha. 1997. "Differential Requirements for Survival and Proliferation of CD8 Naive or Memory T Cells." *Science* 276 (5321): 2057–62. https://doi.org/10.1126/science.276.5321.2057.

Tang, Qizhi, Elisa K. Boden, Kammi J. Henriksen, Helene Bour-Jordan, Mingying Bi, and Jeffrey A. Bluestone. 2004. "Distinct Roles of CTLA-4 and TGF-β in CD4+CD25+ Regulatory T Cell Function." *European Journal of Immunology* 34 (11): 2996–3005. https://doi.org/10.1002/eji.200425143.

Taniguchi, Tadatsugu, Hiroshi Matsui, Takashi Fujita, Chikako Takaoka, Nobukazu Kashima, Ryota Yoshimoto, and Junji Hamuro. 1983. "Structure and Expression of a Cloned CDNA for Human Interleukin-2." *Nature* 302 (5906): 305–10. https://doi.org/10.1038/302305a0.

Taylor, A. W. 2008. "Review of the Activation of TGF- in Immunity." *Journal of Leukocyte Biology* 85 (1): 29–33. https://doi.org/10.1189/jlb.0708415.

Teh, Hung Sia, Pawel Kisielow, Bernadette Scott, Hiroyuki Kishi, Yasushi Uematsu, Horst Blüthmann, and Harald Von Boehmer. 1988. "Thymic Major Histocompatibility Complex Antigens and the Aβ T-Cell Receptor Determine the CD4/CD8 Phenotype of T Cells." *Nature* 335 (6187): 229–33. https://doi.org/10.1038/335229a0.

Teijaro, John R., Damian Turner, Quynh Pham, E. John Wherry, Leo Lefrançois, and Donna L. Farber. 2011. "Cutting Edge: Tissue-Retentive Lung Memory CD4 T Cells Mediate Optimal Protection to Respiratory Virus Infection." *The Journal of Immunology* 187 (11): 5510–14. https://doi.org/10.4049/jimmunol.1102243.

Thomas, Dori A., and Joan Massagué. 2005. "TGF-β Directly Targets Cytotoxic T Cell Functions during Tumor Evasion of Immune Surveillance." *Cancer Cell* 8 (5): 369–80. https://doi.org/10.1016/j.ccr.2005.10.012.

Thornton, Angela M., and Ethan M. Shevach. 1998. "CD4+CD25+ Immunoregulatory T Cells Suppress Polyclonal T Cell Activation in Vitro by Inhibiting Interleukin 2 Production." *Journal of Experimental Medicine* 188 (2): 287–96. https://doi.org/10.1084/jem.188.2.287.

Tivol, Elizabeth A., Frank Borriello, A. Nicola Schweitzer, William P. Lynch, Jeffrey A. Bluestone, and Arlene H. Sharpe. 1995. "Loss of CTLA-4 Leads to Massive Lymphoproliferation and Fatal Multiorgan Tissue Destruction, Revealing a Critical Negative Regulatory Role of CTLA-4." *Immunity* 3 (5): 541–47. https://doi.org/10.1016/1074-7613(95)90125-6.

Togher, Susan, Alexandre Larange, Stephen P Schoenberger, and Sonia Feau. 2015. "FoxO3 Is a Negative Regulator of Primary CD8 [+] T-Cell Expansion but Not of Memory Formation." *Immunology and Cell Biology* 93 (2): 120–25. https://doi.org/10.1038/icb.2014.78.

Tognon, C E, H E Kirk, L A Passmore, I P Whitehead, C J Der, and R J Kay. 1998. "Regulation of RasGRP via a Phorbol Ester-Responsive C1 Domain." *Molecular and Cellular Biology* 18 (12): 6995–7008. https://doi.org/10.1128/MCB.18.12.6995.

Tone, Yukiko, Keiji Furuuchi, Yoshitsugu Kojima, Mark L. Tykocinski, Mark I. Greene, and Masahide Tone. 2008. "Smad3 and NFAT Cooperate to Induce Foxp3 Expression through Its Enhancer." *Nature Immunology* 9 (2): 194–202. https://doi.org/10.1038/ni1549.

Tough, David F., and Jonathan Sprent. 1994. "Turnover of Naive- and Memory-Phenotype T Cells." *Journal of Experimental Medicine* 179 (4): 1127–35. https://doi.org/10.1084/jem.179.4.1127.

Townsend, Sarah E., and James P. Allison. 1993. "Tumor Rejection after Direct Costimulation of CD8+ T Cells by B7-Transfected Melanoma Cells." *Science* 259 (5093): 368–70. https://doi.org/10.1126/science.7678351.

Trauth, Bernhard C., Christiane Klas, Anke M.J. Peters, Siegfried Matzku, Peter Möller, Werner Falk, Klaus Michael Debatin, and Peter H. Krammer. 1989. "Monoclonal Antibody-Mediated Tumor Regression by Induction of Apoptosis." *Science* 245 (4915): 301–5. https://doi.org/10.1126/science.2787530.

Travis, Mark A., Boris Reizis, Andrew C. Melton, Emma Masteller, Qizhi Tang, John M. Proctor, Yanli Wang, et al. 2007. "Loss of Integrin Avβ8 on Dendritic Cells Causes Autoimmunity and Colitis in Mice." *Nature* 449 (7160): 361–65. https://doi.org/10.1038/nature06110.

Tripp, R A, S Hou, A McMickle, J Houston, and P C Doherty. 1995. "Recruitment and Proliferation of CD8+ T Cells in Respiratory Virus Infections." *The Journal of Immunology* 154 (11).

Tschumi, Benjamin O, Nina Dumauthioz, Bastien Marti, Lianjun Zhang, Pascal Schneider, Jean-Pierre Mach, Pedro Romero, and Alena Donda. 2018. "CART Cells Are Prone to Fas- and DR5-Mediated Cell Death." *Journal for Immunotherapy of Cancer* 6 (1): 71. https://doi.org/10.1186/s40425-018-0385-z.

Tsukazaki, Tomoo, Theodore A. Chiang, Anne F. Davison, Liliana Attisano, and Jeffrey L. Wrana. 1998. "SARA, a FYVE Domain Protein That Recruits Smad2 to the TGFβ Receptor." *Cell* 95 (6): 779–91. https://doi.org/10.1016/S0092-8674(00)81701-8.

Tu, Eric, Cheryl P Z Chia, Weiwei Chen, Dunfang Zhang, Sang A Park, Wenwen Jin, Dandan Wang, et al. 2018. "T Cell Receptor-Regulated TGF-β Type I Receptor Expression Determines T Cell Quiescence and Activation." *Immunity* 48 (4): 745-759.e6. https://doi.org/10.1016/j.immuni.2018.03.025.

Tzachanis, Dimitrios, Gordon J. Freeman, Naoto Hirano, Andre A.F.L. Van Puijenbroek, Michael W. Delfs, Alla Berezovskaya, Lee M. Nadler, and Vassiliki A. Boussiotis. 2001. "Tob Is a Negative Regulator of Activation That Is Expressed in Anergic and Quiescent T Cells." *Nature Immunology* 2 (12): 1174–82. https://doi.org/10.1038/ni730.

Tzelepis, Fanny, Julie Joseph, Elias K Haddad, Susanne Maclean, Renu Dudani, Fabien Agenes, Stanford L Peng, Rafick-Pierre Sekaly, and Subash Sad. 2013. "Intrinsic Role of FoxO3a in the Development of CD8+ T Cell Memory." *Journal of Immunology (Baltimore, Md. : 1950)* 190 (3): 1066–75. https://doi.org/10.4049/jimmunol.1200639.

Ueno, Tomoo, Fumi Saito, Daniel H.D. Gray, Sachiyo Kuse, Kunio Hieshima, Hideki Nakano, Terutaka Kakiuchi, Martin Lipp, Richard L. Boyd, and Yousuke Takahama. 2004. "CCR7 Signals Are Essential for Cortex-Medulla Migration of Developing Thymocytes." *Journal of Experimental Medicine* 200 (4): 493–505. https://doi.org/10.1084/jem.20040643.

Veillette, André, Michael A. Bookman, Eva M. Horak, and Joseph B. Bolen. 1988. "The CD4 and CD8 T Cell Surface Antigens Are Associated with the Internal Membrane Tyrosine-Protein Kinase P56lck." *Cell* 55 (2): 301–8. https://doi.org/10.1016/0092-8674(88)90053-0.

Veldhoen, Marc, Richard J. Hocking, Christopher J. Atkins, Richard M. Locksley, and Brigitta Stockinger. 2006. "TGFβ in the Context of an Inflammatory Cytokine Milieu Supports de Novo Differentiation of IL-17-Producing T Cells." *Immunity* 24 (2): 179–89. https://doi.org/10.1016/j.immuni.2006.01.001.

Veldhoen, Marc, Catherine Uyttenhove, Jacques van Snick, Helena Helmby, Astrid Westendorf, Jan Buer, Bruno Martin, Christoph Wilhelm, and Brigitta Stockinger. 2008. "Transforming Growth Factor-β 'reprograms' the Differentiation of T Helper 2 Cells and Promotes an Interleukin 9-Producing Subset." *Nature Immunology* 9 (12): 1341–46. https://doi.org/10.1038/ni.1659.

Vella, Anthony, T. Kent Teague, James Ihle, John Kappler, and Philippa Marrack. 1997. "Interleukin 4 (IL-4) or IL-7 Prevents the Death of Resting T Cells: Stat6 Is Probably Not Required for the Effect of IL-4." *Journal of Experimental Medicine* 186 (2): 325–30. https://doi.org/10.1084/jem.186.2.325.

Vignaux, Franfoise, Eric Vivier, Bernard Malissen, V. Depraeter, S. Nagata, and Pierre Golstein. 1995. "Tcr/Cd3 Coupling to Fas-Based Cytotoxicity." *Journal of Experimental Medicine* 181 (2): 781–86. https://doi.org/10.1084/jem.181.2.781.

Vivien, Laëtitia, Christophe Benoist, and Diane Mathis. 2001. "T Lymphocytes Need IL-7 but Not IL-4 or IL-6 to Survive in Vivo." *International Immunology* 13 (6): 763–68. https://doi.org/10.1093/intimm/13.6.763.

Wakabayashi, Yu, Taiga Tamiya, Ichiro Takada, Tomohiro Fukaya, Yuki Sugiyama, Naoko Inoue, Akihiro Kimura, et al. 2011. "Histone 3 Lysine 9 (H3K9) Methyltransferase Recruitment to the Interleukin-2 (IL-2) Promoter Is a Mechanism of Suppression of IL-2 Transcription by the Transforming Growth Factor-β-Smad Pathway." *Journal of Biological Chemistry* 286 (41): 35456–65. https://doi.org/10.1074/jbc.M111.236794.

Wakefield, L. M., D. M. Smith, K. C. Flanders, and M. B. Sporn. 1988. "Latent Transforming Growth Factor-β from Human Platelets. A High Molecular Weight Complex Containing Precursor Sequences." *Journal of Biological Chemistry* 263 (16): 7646–54.

Walsh, C. M., M. Matloubian, C. C. Liu, R. Ueda, C. G. Kurahara, J. L. Christensen, M. T.F. Huang, J. D.E. Young, R. Ahmed, and W. R. Clark. 1994. "Immune Function in Mice Lacking the Perforin Gene." *Proceedings of the National Academy of Sciences of the United States of America* 91 (23): 10854–58. https://doi.org/10.1073/pnas.91.23.10854.

Walunas, Theresa L, Deborah J Lenschow, Christina Y Bakker, Peter S Linsley, Gordon J Freeman, Jonathan M Green, Craig B Thompson, and Jeffrey A Bluestone. 2011. "Pillars Article: CTLA-4 Can Function as a Negative Regulator of T Cell Activation. Immunity. 1994. 1: 405-413." *Journal of Immunology (Baltimore, Md. : 1950)* 187 (7): 3466–74. http://www.ncbi.nlm.nih.gov/pubmed/21934098.

Wang, Haiyong, Ping Han, Xinyue Qi, Fanlin Li, Min Li, Lilv Fan, Huihui Zhang, Xiaoqing Zhang, and Xuanming Yang. 2021. "Article Bcl-2 Enhances Chimeric Antigen Receptor T Cell Persistence by Reducing Activation-Induced Apoptosis." *Cancers* 13 (2): 1–16. https://doi.org/10.3390/cancers13020197.

Wang, Jun, Andreea Ioan-Facsinay, Ellen I.H. van der Voort, Tom W.J. Huizinga, and René E.M. Toes. 2007. "Transient Expression of FOXP3 in Human Activated Nonregulatory CD4+ T Cells." *European Journal of Immunology* 37 (1): 129–38. https://doi.org/10.1002/eji.200636435.

Wardenburg, Juliane Bubeck, Chong Fu, Janet K. Jackman, Horst Flotow, Sandra E. Wilkinson, David H. Williams, Robin Johnson, Guanghui Kong, Andrew C. Chan, and Paul R. Findel. 1996. "Phosphorylation of SLP-76 by the ZAP-70 Protein-Tyrosine Kinase Is Required for T-Cell Receptor Function." *Journal of Biological Chemistry* 271 (33): 19641–44. https://doi.org/10.1074/jbc.271.33.19641.

Warnecke, Nicole, Mateusz Poltorak, Bhavani S. Kowtharapu, Boerge Arndt, James C. Stone, Burkhart Schraven, and Luca Simeoni. 2012. "TCR-Mediated Erk Activation Does Not Depend on Sos and Grb2 in Peripheral Human T Cells." *EMBO Reports* 13 (4): 386–91. https://doi.org/10.1038/embor.2012.17.

Waterhouse, Paul, Josef M. Penninger, Emma Timms, Andrew Wakeham, Arda Shahinian, Kelvin P. Lee, Craig B. Thompson, Henrik Griesser, and Tak W. Mak. 1995. "Lymphoproliferative Disorders with Early Lethality in Mice Deficient in Ctla-4." *Science* 270 (5238): 985–88. https://doi.org/10.1126/science.270.5238.985.

Watts, Julian D., Michael Affolter, Danielle L. Krebs, Ronald L. Wange, Lawrence E. Samelson, and Ruedi Aebersold. 1994. "Identification by Electrospray Ionization Mass Spectrometry of the Sites of Tyrosine Phosphorylation Induced in Activated Jurkat T Cells on the Protein Tyrosine Kinase ZAP-70." *Journal of Biological Chemistry* 269 (47): 29520–29. https://doi.org/10.1016/s0021-9258(18)43911-7.

Webb, John R., Katy Milne, Peter Watson, Ronald J. DeLeeuw, and Brad H. Nelson. 2014. "Tumor-Infiltrating Lymphocytes Expressing the Tissue Resident Memory Marker Cd103 Are Associated with Increased Survival in High-Grade Serous Ovarian Cancer." *Clinical Cancer Research* 20 (2): 434–44. https://doi.org/10.1158/1078-0432.CCR-13-1877.

Webb, Susan, Claudia Morris, and Jonathan Sprent. 1990. "Extrathymic Tolerance of Mature T Cells: Clonal Elimination as a Consequence of Immunity." *Cell* 63 (6): 1249–56. https://doi.org/10.1016/0092-8674(90)90420-J.

Weiss, S., and B. Bogen. 1989. "B-Lymphoma Cells Process and Present Their Endogenous Immunoglobulin to Major Histocompatibility Complex-Restricted T Cells." *Proceedings of the National Academy of Sciences of the United States of America* 86 (1): 282–86. https://doi.org/10.1073/pnas.86.1.282.

Whitmire, Jason K., Richard A. Flavell, Iqbal S. Grewal, Christian P. Larsen, Thomas C. Pearson, and Rafi Ahmed. 1999. "CD40-CD40 Ligand Costimulation Is Required for Generating Antiviral CD4 T Cell Responses but Is Dispensable for CD8 T Cell Responses." *The Journal of Immunology* 163 (6): 3194–3201.

Williams, Brandi L, Brenda J Irvin, Shari L Sutor, Claudia C S Chini, Elaine Yacyshyn, Juliane Bubeck Wardenburg, Mark Dalton, et al. 1999. "Phosphorylation of Tyr319 in ZAP-70 Is Required for T-Cell Antigen Receptor-Dependent Phospholipase C-Γ1 and Ras Activation." *The EMBO Journal*. Vol. 18.

Wing, Kajsa, Yasushi Onishi, Paz Prieto-Martin, Tomoyuki Yamaguchi, Makoto Miyara, Zoltan Fehervari, Takashi Nomura, and Shimon Sakaguchi. 2008. "CTLA-4 Control over Foxp3+ Regulatory T Cell Function." *Science* 322 (5899): 271–75. https://doi.org/10.1126/science.1160062.

Winter, Sarah, Emmanuel Martin, David Boutboul, Christelle Lenoir, Sabah Boudjemaa, Arnaud Petit, Capucine Picard, Alain Fischer, Guy Leverger, and Sylvain Latour. 2018. " Loss of RASGRP 1 in Humans Impairs T-cell Expansion Leading to Epstein-Barr Virus Susceptibility ." *EMBO Molecular Medicine* 10 (2): 188–99. https://doi.org/10.15252/emmm.201708292.

Wolchok, Jedd D., Harriet Kluger, Margaret K. Callahan, Michael A. Postow, Naiyer A. Rizvi, Alexander M. Lesokhin, Neil H. Segal, et al. 2013. "Nivolumab plus Ipilimumab in Advanced Melanoma." *New England Journal of Medicine* 369 (2): 122–33. https://doi.org/10.1056/nejmoa1302369.

Wood, Joanne E., Helga Schneider, and Christopher E. Rudd. 2006. "TcR and TcR-CD28 Engagement of Protein Kinase B (PKB/AKT) and Glycogen Synthase Kinase-3 (GSK-3) Operates Independently of Guanine Nucleotide Exchange Factor VAV-1." *Journal of Biological Chemistry* 281 (43): 32385–94. https://doi.org/10.1074/jbc.M604878200.

Wrana, Jeffrey L., Liliana Attisano, Juan Cárcamo, Alejandro Zentella, Jacqueline Doody, Marikki Laiho, Xiao Fan Wang, and Joan Massague. 1992. "TGFβ Signals through a Heteromeric Protein Kinase Receptor Complex." *Cell* 71 (6): 1003–14. https://doi.org/10.1016/0092-8674(92)90395-S.

Wrana, Jeffrey L., Liliana Attisano, Rotraud Wieser, Francesc Ventura, and Joan Massagué. 1994. "Mechanism of Activation of the TGF-β Receptor." *Nature* 370 (6488): 341–47. https://doi.org/10.1038/370341a0.

Wu, Yongqing, Madhuri Borde, Vigo Heissmeyer, Markus Feuerer, Ariya D. Lapan, James C. Stroud, Darren L. Bates, et al. 2006. "FOXP3 Controls Regulatory T Cell Function through Cooperation with NFAT." *Cell* 126 (2): 375–87. https://doi.org/10.1016/j.cell.2006.05.042.

Xavier, Ramnik, Todd Brennan, Qingqin Li, Christine McCormack, and Brian Seed. 1998. "Membrane Compartmentation Is Required for Efficient T Cell Activation." *Immunity* 8 (6): 723–32. https://doi.org/10.1016/S1074-7613(00)80577-4.

Xiong, J. P., T. Stehle, B. Diefenbach, R. Zhang, R. Dunker, D. L. Scott, A. Joachimiak, S. L. Goodman, and M. A. Arnaout. 2001. "Crystal Structure of the Extracettutar Segment of Integrin AVβ3." *Science* 294 (5541): 339–45. https://doi.org/10.1126/science.1064535.

Xu, Yang, Ming Zhang, Carlos A. Ramos, April Durett, Enli Liu, Olga Dakhova, Hao Liu, et al. 2014. "Closely Related T-Memory Stem Cells Correlate with in Vivo Expansion of CAR.CD19-T Cells and Are Preserved by IL-7 and IL-15." *Blood* 123 (24): 3750–59. https://doi.org/10.1182/blood-2014-01-552174.

Yablonski, Deborah, Theresa Kadlecek, and Arthur Weiss. 2001. "Identification of a Phospholipase C-Γ1 (PLC-Γ1) SH3 Domain-Binding Site in SLP-76 Required for T-Cell Receptor-Mediated Activation of PLC-Γ1 and NFAT." *Molecular and Cellular Biology* 21 (13): 4208–18. https://doi.org/10.1128/mcb.21.13.4208-4218.2001.

Yamamoto-Taguchi, Nanae, Yorifumi Satou, Paola Miyazato, Koichi Ohshima, Masanori Nakagawa, Koko Katagiri, Tatsuo Kinashi, and Masao Matsuoka. 2013. "HTLV-1 BZIP Factor Induces Inflammation through Labile Foxp3 Expression." Edited by Jeremy Luban. *PLoS Pathogens* 9 (9): e1003630. https://doi.org/10.1371/journal.ppat.1003630.

Yamamoto, Mutsumi, Yoichi Seki, Kazuyuki Iwai, Iei Ko, Alicia Martin, Noriko Tsuji, Shuji Miyagawa, Robert B. Love, and Makio Iwashima. 2013. "Ontogeny and Localization of the Cells Produce IL-2 in Healthy Animals." *Cytokine* 61 (3): 831–41. https://doi.org/10.1016/j.cyto.2012.11.026.

Yamane, Hidehiro, Jinfang Zhu, and William E. Paul. 2005. "Independent Roles for IL-2 and GATA-3 in Stimulating Naive CD4 + T Cells to Generate a Th2-Inducing Cytokine Environment." *Journal of Experimental Medicine* 202 (6): 793–804. https://doi.org/10.1084/jem.20051304.

Yan, Qingrong, Tiago Barros, Patrick R. Visperas, Sebastian Deindl, Theresa A. Kadlecek, Arthur Weiss, and John Kuriyan. 2013. "Structural Basis for Activation of ZAP-70 by Phosphorylation of the SH2-Kinase Linker." *Molecular and Cellular Biology* 33 (11): 2188–2201. https://doi.org/10.1128/mcb.01637-12.

Yan, Yaping, Guang-Xian Zhang, Bruno Gran, Francesca Fallarino, Shuo Yu, Hongmei Li, Melissa L. Cullimore, Abdolmohamad Rostami, and Hui Xu. 2010. "IDO Upregulates Regulatory T Cells via Tryptophan Catabolite and Suppresses Encephalitogenic T Cell Responses in Experimental Autoimmune Encephalomyelitis." *The Journal of Immunology* 185 (10): 5953–61. https://doi.org/10.4049/jimmunol.1001628.

Yang, Tsung Ying, Gee Chen Chang, Kun Chieh Chen, Hsiao Wen Hung, Kuo Hsuan Hsu, Gwo Tarng Sheu, and Shih Lan Hsu. 2011. "Sustained Activation of ERK and Cdk2/Cyclin-A Signaling Pathway by Pemetrexed Leading to S-Phase Arrest and Apoptosis in Human Non-Small Cell Lung Cancer A549 Cells." *European Journal of Pharmacology* 663 (1–3): 17–26. https://doi.org/10.1016/j.ejphar.2011.04.057.

Yang, Zhiwei, Zhenyu Mu, Branka Dabovic, Vladimir Jurukovski, Dawen Yu, Joanne Sung, Xiaozhong Xiong, and John S. Munger. 2007. "Absence of Integrin-Mediated TGFβ1 Activation in Vivo Recapitulates the Phenotype of TGFβ1-Null Mice." *Journal of Cell Biology* 176 (6): 787–93. https://doi.org/10.1083/jcb.200611044.

Yasuda, Shinsuke, Richard L. Stevens, Tomoko Terada, Masumi Takeda, Toko Hashimoto, Jun Fukae, Tetsuya Horita, Hiroshi Kataoka, Tatsuya Atsumi, and Takao Koike. 2007. "Defective Expression of Ras Guanyl Nucleotide-Releasing Protein 1 in a Subset of Patients with Systemic Lupus Erythematosus." *The Journal of Immunology* 179 (7): 4890–4900. https://doi.org/10.4049/jimmunol.179.7.4890.

Yonehara, S., A. Ishii, and M. Yonehara. 1989. "A Cell-Killing Monoclonal Antibody (Anti-Fas) to a Cell Surface Antigen Co-Downregulated with the Receptor of Tumor Necrosis Factor." *Journal of Experimental Medicine* 169 (5): 1747–56. https://doi.org/10.1084/jem.169.5.1747.

Yoon, Jeong Hwan, Su Myung Jung, Seok Hee Park, Mitsuyasu Kato, Tadashi Yamashita, In Kyu Lee, Katsuko Sudo, et al. 2013. "Activin Receptor-like Kinase5 Inhibition Suppresses Mouse Melanoma by Ubiquitin Degradation of Smad4, Thereby Derepressing Eomesodermin in Cytotoxic T Lymphocytes." *EMBO Molecular Medicine* 5 (11): 1720–39. https://doi.org/10.1002/emmm.201302524.

You, Han, Marc Pellegrini, Katsuya Tsuchihara, Kazuo Yamamoto, Georg Hacker, Miriam Erlacher, Andreas Villunger, and Tak W. Mak. 2006. "FOXO3a-Dependent Regulation of Puma in Response to Cytokine/Growth Factor Withdrawal" 203 (7): 1657–63. https://doi.org/10.1084/jem.20060353.

Zhang, Weiguo, Joanne Sloan-Lancaster, Jason Kitchen, Ronald P. Trible, and Lawrence E. Samelson. 1998. "LAT: The ZAP-70 Tyrosine Kinase Substrate That Links T Cell Receptor to Cellular Activation." *Cell* 92 (1): 83–92. https://doi.org/10.1016/S0092-8674(00)80901-0.

Zhang, Xiaohong, Lizzie Giangreco, H. Elizabeth Broome, Catherine M. Dargan, and Susan L. Swain. 1995. "Control of CD4 Effector Fate: Transforming Growth Factor B1 and Interleukin 2 Synergize to Prevent Apoptosis and Promote Effector Expansion." *Journal of Experimental Medicine* 182 (3): 699–709. https://doi.org/10.1084/jem.182.3.699.

Zhang, Y., X. H. Feng, R. Y. Wu, and R. Derynck. 1996. "Receptor-Associated Mad Homologues Synergize as Effectors of the TGF-β Response." *Nature* 383 (6596): 168–72. https://doi.org/10.1038/383168a0.

Zheng, Shi-Jun, and Youhai H Chen. 2003. "TRAIL, Bim, and Thymic-Negative Selection." *Immunologic Research* 28 (3): 295–301. https://doi.org/10.1385/IR:28:3:295.

Zheng, Wei Ping, and Richard A. Flavell. 1997. "The Transcription Factor GATA-3 Is Necessary and Sufficient for Th2 Cytokine Gene Expression in CD4 T Cells." *Cell* 89 (4): 587–96. https://doi.org/10.1016/S0092-8674(00)80240-8.

Zheng, Xincheng, Lijie Yin, Yang Liu, and Pan Zheng. 2004. "Expression of Tissue-Specific Autoantigens in the Hematopoietic Cells Leads to Activation-Induced Cell Death of Autoreactive T Cells in the Secondary Lymphoid Organs." *European Journal of Immunology* 34 (11): 3126–34. https://doi.org/10.1002/eji.200425177.

Zheng, Ye, Steven Z. Josefowicz, Arnold Kas, Tin Tin Chu, Marc A. Gavin, and Alexander Y. Rudensky. 2007. "Genome-Wide Analysis of Foxp3 Target Genes in Developing and Mature Regulatory T Cells." *Nature* 445 (7130): 936–40. https://doi.org/10.1038/nature05563.

Zhong, Xiao Song, Maiko Matsushita, Jason Plotkin, Isabelle Riviere, and Michel Sadelain. 2010. "Chimeric Antigen Receptors Combining 4-1BB and CD28 Signaling Domains Augment PI 3 Kinase/AKT/Bcl-X L Activation and CD8 T Cell-Mediated Tumor Eradication." *Molecular Therapy* 18 (2): 413–20. https://doi.org/10.1038/mt.2009.210.

Zhou, Jing-Tao, Jiang-Hao Liu, Ting-Ting Song, Bo Ma, Nuermaimait Amidula, and Chao Bai. 2020. "EGLIF-CAR-T Cells Secreting PD-1 Blocking Antibodies Significantly Mediate the Elimination of Gastric Cancer." *Cancer Management and Research* 12: 8893–8902. https://doi.org/10.2147/CMAR.S260915.

Zhu, L., X. Yu, Y. Akatsuka, J. A. Cooper, and C. Anasetti. 1999. "Role of Mitogen-Activated Protein Kinases in Activation-Induced Apoptosis of T Cells." *Immunology* 97 (1): 26–35. https://doi.org/10.1046/j.1365-2567.1999.00756.x.

Zinkernagel, Rolf F., and Peter C. Doherty. 1974. "Immunological Surveillance against Altered Self Components by Sensitised T Lymphocytes in Lymphocytes Choriomeningitis." *Nature* 251 (5475): 547–48. https://doi.org/10.1038/251547a0.

Zorn, Emmanuel, Erik A. Nelson, Mehrdad Mohseni, Fabrice Porcheray, Haesook Kim, Despina Litsa, Roberto Bellucci, et al. 2006. "IL-2 Regulates FOXP3 Expression in Human CD4+CD25+ Regulatory T Cells through a STAT-Dependent Mechanism and Induces the Expansion of These Cells in Vivo." *Blood* 108 (5): 1571–79. https://doi.org/10.1182/blood-2006-02-004747.

Zuckerberg, A. L., L. I. Goldberg, and H. M. Lederman. 1994. "Effects of Hypoxia on Interleukin-2 MRNA Expression by T Lymphocytes." *Critical Care Medicine* 22 (2): 197–203. https://doi.org/10.1097/00003246-199402000-00008.

VITA

The author, Christina Rose Cunha, was born in Sacramento, CA on January 31st, 1992 to Julia Bashaw and Allyn Cunha. She attended Loyola Marymount University where she earned a Bachelor of Science in Biochemistry and a Minor in Music in 2014. During her undergraduate studies, Christina worked in the laboratory of Dr. David Moffet studying the formation of IAAP amyloid fibers in diabetes. After graduation, Christina worked as a high school teacher and AmeriCorps volunteer at Perspectives Leadership Academy in Chicago, IL.

In 2015, Christina matriculated into the Loyola University Chicago Interdisciplinary Program in Biomedical Sciences. She joined the Department of Microbiology and Immunology and the laboratory of Dr. Makio Iwashima, studying the effects of TGF-β signaling on T cell survival and function. This work was supported by the T32 Immunology Training Grant awarded to Dr. Katherine Knight and the Predoctoral Ruth L. Kirchstein National Research Service F31 Award from the National Cancer Institute of the National Institute of Health.

After completion of her graduate studies, Christina will continue to pursue her interest in scientific research and begin a postdoctoral position at Loyola in the laboratory of Dr. Bryan Mounce. She will investigate the role of polyamines in viral infection and host anti-viral responses.