The role of SHP-1 in regulating the T cell response to suppression by regulatory T cells

Emily Rose Mercadante
Succasunna, NJ

M.S., University of Virginia, 2012
B.S., Ursinus College, 2010

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ABSTRACT

The balance between activation of T cells and their suppression by regulatory T cells (Treg) is dysregulated in autoimmune diseases and cancer. In the past decade, evidence has accumulated to suggest that autoimmune diseases not only feature defective Tregs, but also feature T cells that are resistant to suppression by Tregs. On the other hand, in cancer, T cells are unable to mount anti-tumor responses due to the Treg-enriched suppressive microenvironment. Much remains unknown about what regulates this interplay between T cells and Tregs. However, it has become clear that maintaining the tenuous balance between T cell activation and suppression by Tregs is essential for immune homeostasis and prevention of disease.

In this work, we investigated the role of the protein tyrosine phosphatase SHP-1, in regulating T cell responses to T cell receptor (TCR) stimulation. SHP-1 has been defined as a negative regulator of TCR signaling based on studies in global SHP-1 knockout mice and immortalized cell lines. We expanded in-depth upon these findings by utilizing two mouse models in which SHP-1 is deleted specifically in T cells and a pharmacological inhibitor of SHP-1. With this approach, we show that SHP-1 limits the responsiveness of T cells to TCR stimulation in a cell-intrinsic manner. Furthermore, we identified a novel function of SHP-1 in regulating the susceptibility of T cells to Treg-mediated suppression. Thus, SHP-1 deficiency rendered naïve CD4+ and CD8+ T cells resistant to Treg-mediated suppression *in vitro,* and regulated *in vivo* CD4+ T cell susceptibility to Treg suppression under conditions of homeostatic expansion.

Mechanistically, SHP-1-deficient T cells did not mediate resistance to Treg suppression via soluble factors *in vitro*. We specifically ruled out an influence of IL-4

signaling on Treg-resistance in SHP-1-deficient T cells, as previous work had shown that SHP-1-deficient T cells are hyper-sensitive to IL-4 and that IL-4 can induce wildtype T cells to resist suppression. Rather, SHP-1 controlled the activation of the PI3K/Akt pathway, the enhancement of which has been previously linked to the induction of T cell resistance to Treg suppression. Collectively, these data establish SHP-1 as a critical player in setting the threshold downstream of TCR signaling and identify a novel function of SHP-1 as a regulator of T cell susceptibility to Treg-mediated suppression *in vitro* and *in vivo*. Thus, SHP-1 and the PI3K/Akt pathway could represent potential immunotherapeutic targets to modulate susceptibility of T cells to Treg suppression.

In addition to investigating the role of SHP-1 in T cells, we also preliminarily assessed the regulatory function of SHP-1 in Tregs. Previous work from our lab established that Tregs from global knockout SHP-1 mice were more suppressive than wildtype Tregs. Thus, we generated a mouse model in which SHP-1 is deleted specifically in Tregs to assess the Treg cell-intrinsic function of SHP-1. Preliminary results herein recapitulate the inhibitory role of SHP-1 on Treg function. Thus, SHP-1-deficient Tregs were more potently suppressive than wildtype Tregs. The dual role of SHP-1 in T cells and Tregs provides the possibility of inhibiting SHP-1 in Tregs for treatment of autoimmune disease, and inhibiting SHP-1 in T cells to boost anti-tumor responses. Overall, this work better informs immunotherapeutic strategies for autoimmune disease and cancer, and highlights the importance of targeted approaches to avoid counterproductive systemic effects.

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List of Abbreviations

4-1BB	Tumor necrosis factor receptor superfamily member 9
αCD3	anti-CD3
Ab	Antibody(s)
ACT	Adoptive cell transfer
Ag	Antigen(s)
Akt	Protein kinase B
ANOVA	Analysis of variance
AP-1	Activator protein 1
APC	Antigen-presenting cell(s)
BL6	C57BL/6
cAMP	cyclic adenosine monophosphate
CAR-T	Chimeric antigen receptor T cell
Casp-3	Caspase-3
Cbl-b/c-Cbl	Casitas-B-lineage-lymphoma proteins
CFSE	Carboxyfluorescein succinimidyl ester
CTL(s)	Cytotoxic T lymphocyte(s)
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4

DC(s)	Dendritic cell(s)
dLck	Distal promoter of Lck
DN	Double negative (CD4-CD8-) thymocyte
DP	Double positive (CD4+CD8+) thymocyte
EAE	Experimentally-induced autoimmune encephalomyelitis
ELISA	Enzyme-linked immunosorbent assay
Erk	Extracellular-regulated kinases
FMO	Fluorescence minus one
Foxp3	Forkhead box P3
GITR	Glucocorticoid-induced TNFR-related protein
GVHD	Graft-versus-host disease
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HSV	Herpes simplex virus
IBD	Inflammatory bowel disease
ICAM-1	Intracellular adhesion molecule 1
IDO	Indoleamine 2,3-dioxygenase

IFNγ	Interferon gamma
IL	Interleukin
i.p.	Intraperitoneal
i.v.	Intravenous
IPEX syndrome	Immunodysregulation polyendocrinopathy enteropathy X-linked
ITAM(s)	Immune tyrosine-based activation motif(s)
Jak	Janus kinase proteins
JIA	Juvenile idiopathic arthritis
KD	Knockdown
KO	Knockout
LAG3	Lymphocyte-activation gene 3
LAT	Linker for activation of T cells
Lck	Lymphocyte-specific protein tyrosine kinase
LFA-1	Lymphocyte function-associated antigen 1
LN	Lymph node(s)
MACS	Magnetic activated cell sorting
me/me	*motheaten* mice

MFI	Mean fluorescence intensity
MHC I/II	Major histocompatibility complex class I or class II
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
mTOR	Mechanistic (formerly mammalian) target of rapamycin
mut	Mutant
Nedd4	Neural precursor cell expressed developmentally downregulated protein 4
NFAT	Nuclear factor of activated T-cells
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
OVA	Ovalbumin
OX40	Tumor necrosis factor receptor superfamily member 4
p85	Regulatory subunit of PI3K
pAkt	phosphorylated Akt
PBS	Phosphate-buffered saline
pDC(s)	Plasmacytoid dendritic cell(s)
PI3K	Phosphoinositide 3-kinase
PKCθ	Protein kinase C theta
PMA	Phorbol 12-myristate 13-acetate

pMHC	peptide: Major histocompatibility complex
PTEN	Phosphatase and tensin homolog
PTP	Protein tyrosine phosphatase
pTregs	Peripherally-induced Tregs
pY	Phospho-tyrosine(s)
RA	Rheumatoid arthritis
Rag1$^{-/-}$	Recombination activating gene 1 knockout
RNA-seq	Ribonucleic acid sequencing
scFv	single-chain variable fragment
SEM	Standard error of the mean
SH2	Src homology region 2
SHP-1	Src homology region 2 domain-containing phosphatase 1
shRNA	Short hairpin ribonucleic acid
siRNA	Small interfering ribonucleic acid
SLE	Systemic lupus erythematosus
SLP-76	Lymphocyte cytosolic protein 2
SP	Single positive thymocyte
SSG	Sodium stibogluconate

STAT	Signal transducer and activator of transcription proteins
T1D	Type 1 Diabetes
T308	Threonine 308 (in Akt)
Tcon	Conventional T cell(s)
TCR	T cell receptor
Tg	Transgenic
TGF-β	Transforming growth factor beta
T_H	T helper
TLR(s)	Toll-like receptor(s)
TNFα	Tumor necrosis factor alpha
TNFR(s)	Tumor necrosis factor receptor(s)
TRAF6	TNF receptor associated factor 6
tTregs	Thymic-derived Tregs
Treg(s)	Regulatory T cell(s)
Trx	Treated
Vav1	Vav guanine nucleotide exchange factor 1
wt	Wildtype
Zap-70	Zeta-chain-associated protein kinase 70

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CHAPTER 1

INTRODUCTION

1.1 Adaptive Immune System

The immune system encounters a myriad of pathogens on a regular basis and therefore must engage an array of defenses against infection. The first line of defense is the innate immune response, which is composed mostly of myeloid cells that are equipped to recognize and immediately respond to pathogens. The innate response also serves to activate the adaptive immune response, comprised of lymphocytes that recognize specific antigens (Ag). As part of the adaptive response, T cells become activated upon encountering Ag presented in complex with a major histocompatibility complex (MHC) molecule by an antigen-presenting cell (APC). The environment during Ag presentation informs the quality and type of T cell response. Effector functions of T cells include production of cytokines, direct killing of infected cells, as well as recruitment of other cell types to clear infected and dead cells. The humoral arm of the adaptive immune response (B cell production of antibodies) works in tandem with T cells to clear infections. To protect against re-exposure to the same pathogen, some lymphocytes develop into long-term memory cells capable of rapidly responding to repeat infection[1].

Every phase of the immune response is exquisitely regulated to prevent inappropriate responses against self, to ensure that the correct type of response is elicited to clear a specific pathogen, and to limit the magnitude of a response to avoid tissue damage. T cells are restricted in their ability to respond by having to possess a T cell receptor (TCR) that is specific for the Ag being presented. This specificity helps ensure that T cells do not “see” self, because those that do are eliminated in the thymus during the process of negative selection (discussed in Section 1.2). Furthermore, T cells must

receive several activation signals to clonally expand and acquire effector function (discussed in Sections 1.2, 1.3). Regulatory T cells (Tregs, defined as CD4+CD25+Foxp3+ cells) can intervene at various stages of this process, from suppressing the activation of a naïve T cell to suppressing the effector function of a mature T cell, to directly killing effector T cells (discussed in Section 1.4). This counterbalance by Tregs is necessary to prevent autoreactivity and development of autoimmunity and to help resolve immune responses against pathogens[2].

1.2 T cell receptor signaling and thymocyte development

Signaling through the pre-T cell receptor (pre-TCR) and the TCR complexes determines the developmental path of a T cell. The pre-TCR and TCR are multi-subunit complexes associated with the plasma membrane, composed of the pre-Tα/β and αβ subunits, respectively, as well as CD3 γδε and TCR ζ chains[3]. The TCR binds peptide ligands presented on the surface of MHC molecules, and signals through immune tyrosine based activation motifs (ITAMs) present in the cytoplasmic tails of CD3 and TCR ζ chains (Fig. 1.1). Signals transduced through ITAMs are propagated by protein tyrosine kinases. Ag engagement results in the upregulation of genes involved in proliferation, differentiation, and effector function[3].

TCR signaling is necessary at the earliest stages of T cell development in order to direct many of the complex processes that occur throughout T cell maturation. Pre-thymocytes enter the thymus from the bone marrow and do not express a TCR. These cells transition to pre-T cells, and upon successful gene rearrangement, express a TCR β chain paired with a pre-T α chain. Signals through the pre-TCR induce rearrangement of

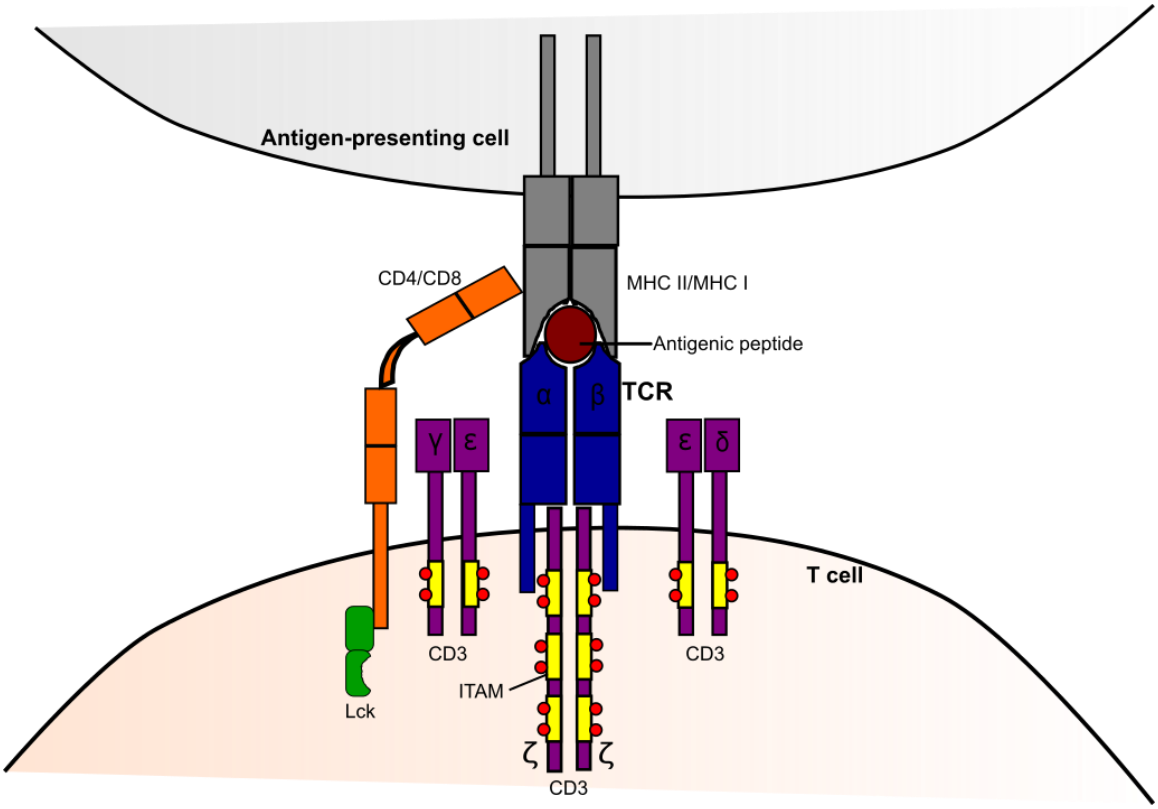

Figure 1.1. T cell receptor complex. The TCR engages with antigenic peptide presented on an MHC molecule by an APC, and the CD4 or CD8 co-receptor stabilizes the complex and brings Lck into proximity of the TCR-CD3 complex. Lck then phosphorylates the ITAMs in the cytoplasmic tails of CD3 and the TCR, initiating the TCR signaling cascade.

the TCR α chain and these thymocytes subsequently express both CD4 and CD8 co-receptors (double positive, DP stage). Expression of a complete αβ TCR leads to assembly of CD3 and ζ proteins, leading to a complete TCR complex (Fig. 1.1)[4]. These cells can now respond to antigen and undergo positive and negative selection, in addition to transitioning to CD4+ or CD8+ (single positive, SP) thymocytes (Fig. 1.2). Thymocytes possessing a TCR that recognizes self-antigen with low avidity will be stimulated to survive in the process of positive selection. This process ensures that T cells are self-MHC restricted, enforcing class I restriction for CD8+ T cells and class II restriction for CD4+ T cells. T cells whose TCR recognizes self-antigen with a high-affinity interaction undergo apoptosis (negative selection) to prevent maturation of autoreactive T cells[5]. The process of negative selection establishes central tolerance; however, not all autoreactive T cells are deleted[5]. Some that recognize self-antigen with an intermediate- or high-affinity differentiate into Foxp3+ Treg cells[6]. Still, others are able to escape negative selection and have the potential to mount an autoreactive response. Therefore, peripheral tolerance mechanisms, including the induction of anergy in autoreactive T cells[7], and active suppression by Tregs[5] (Section 1.4) are in place to prevent autoreactive T cells from becoming activated in the periphery.

After positive selection, naïve T cells leave the thymus and home to peripheral lymphoid organs where they may experience a productive encounter with antigen[1]. The naïve T cell becomes activated when it interacts with an APC displaying antigenic peptide on an MHC molecule of the specificity recognized by that particular TCR. The TCR:pMHC interaction provides the first activation signal to the T cell; the second comes from the interaction of costimulatory molecules on the APC (CD80/CD86)

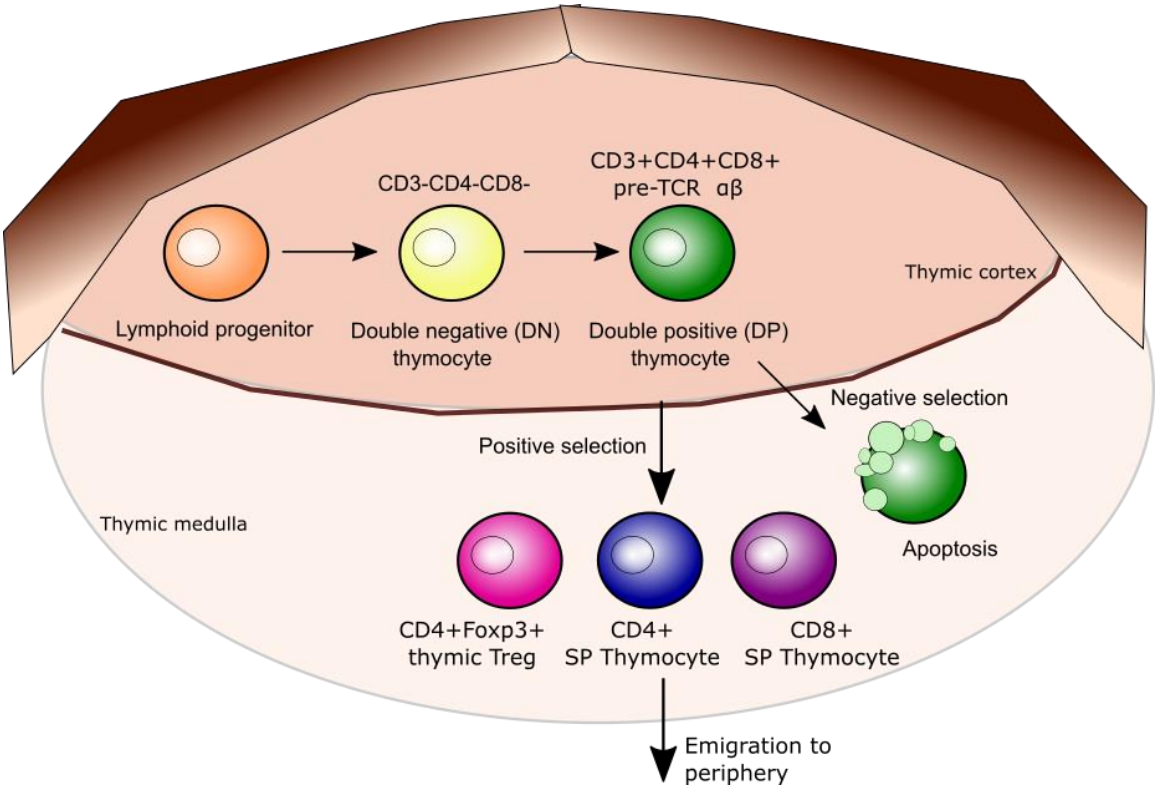

Figure 1.2. Thymocyte development. Lymphoid progenitors arise in the bone marrow and migrate to the thymus. In the thymic cortex, lymphoid progenitors become early committed T cells, which lack expression of CD3, CD4, or CD8 and are termed DN. DN thymocytes can also be subdivided into further stages, DN1-DN4. DN2-DN4 thymocytes express the pre-TCR, composed of a non-rearranged pre-Tα chain and rearranged TCR β chain. During the DN4 to DP thymocyte transition, cells express a rearranged TCRα chain, resulting in a complete αβ TCR. Cortical epithelial cells express MHC I/II molecules with self-peptides, beginning the process of negative and positive selection. At this stage, DP cells receive signals to become committed to develop into either CD4+ or CD8+ SP thymocytes. DP cells that interact strongly with self-peptides undergo negative selection, which typically occurs in the thymic medulla. Cells that receive intermediate-

affinity signals are positively selected and develop into SP CD4+ or CD8+ thymocytes. Cells that receive intermediate to high-affinity signals give rise to CD4+Foxp3+ thymic Treg cells.

interacting with CD28 on the T cell[8]. This interaction induces IL-2 production by the T cell, promoting its clonal expansion[9]. Without co-stimulatory signals, T cells become anergic[10]. Depending on the context and cytokine milieu in which T cell activation occurs (considered the "third" signal)[11], CD4+ T cells will differentiate into a specific lineage, any of a number of T helper (T_H) lineages or the peripheral regulatory T cell (pTreg) lineage[12] (Fig. 1.3). Unlike the variety of T_H lineages a CD4+ T cell can become, activation of naïve CD8+ T cells leads to their maturation into cytotoxic T lymphocytes[13] (CTLs; discussed in Section 1.3).

The extracellular environment triggers intracellular signaling responses that govern a panoply of fate decisions, from early development to terminal differentiation and acquisition of effector function. Upon engagement of the TCR with the appropriate peptide ligand, the CD4 or CD8 co-receptor binds to MHCII or MHCI, respectively, and an immunological synapse begins to form. The engagement leads to tyrosine phosphorylation of ITAMs primarily by Lck and somewhat by Fyn, creating Src homology 2 (SH2) docking sites for Zap-70. Zap-70 is then phosphorylated by Lck, activating it. Once activated, Zap-70 phosphorylates linker molecules LAT and SLP-76. These molecules form docking sites for activation of other substrates, providing a platform on which signal propagation events can occur. Ultimately, multiple downstream transcription factors become activated, including NFAT, NF-κB, and AP-1, which bind to the IL-2 promoter, inducing IL-2 production[14] (Fig. 1.4). Early TCR signaling events are enhanced by CD28 costimulation[14,15]. Upon CD28 costimulation, tyrosine residues in its cytoplasmic tail are phosphorylated, providing a docking site for phosphatidylinositide-3 kinase (PI3K). PI3K then phosphorylates membrane phospholipids, yielding PI-3,4,5-P_3

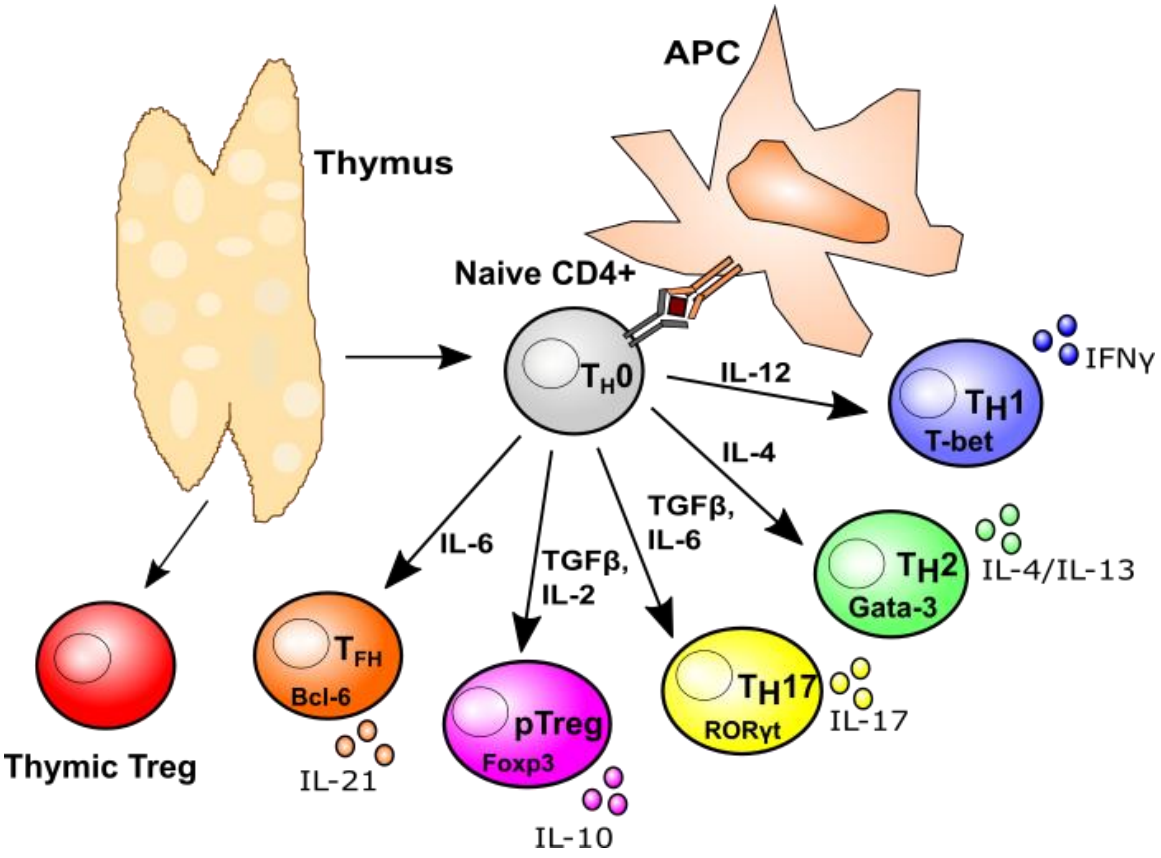

Figure 1.3. CD4+ Helper T cell differentiation. Naïve T cells exit the thymus and encounter Ag presented on an APC in secondary lymphoid organs. The cytokine milieu present during activation instructs the T cell to differentiate into a specific lineage. Cytokines that induce specific T_H lineages are depicted, and the transcription factors that regulate lineage differentiation are shown. Each T_H lineage is defined based on the cytokines which they produce. In addition to naïve, "T_H0" CD4+ T cells, Tregs also arise in the thymus. These tTregs are distinct from peripherally-induced Tregs (pTregs), which arise in response to TGF-β and IL-2 signals in the periphery.

and PI-3,4-P_2, which anchor PLCγ1 and Vav1 to the plasma membrane. Activation of Vav1 leads to downstream activation of Jnk and NF-κB, further increasing IL-2 production[14]. Costimulation through CD28 also lowers the threshold for naïve T cell activation and prevents anergy[14,15]. PI3K not only functions in response to CD28 engagement; it is recruited to LAT and other adapters, through its p85 subunit, during the TCR signaling cascade. Lipid mediators produced by PI3K recruit and activate Akt, which results in upregulation of transcription factors involved in cellular survival, proliferation, differentiation, and metabolic changes[15,16]. It is extremely important that T cells respond appropriately to a given TCR stimulus. Since the TCR is capable of rapidly binding or "sampling" potential ligands, it must discriminate between agonist foreign peptides and self-antigen. One way it distinguishes agonist from antagonist is by differences in the time the antigen remains bound to the TCR[17]. Besides temporal regulation, many proteins act as negative regulators of TCR signaling molecules to fine-tune signals and set activation thresholds. As early TCR signaling occurs mainly via tyrosine phosphorylation, phosphatases act to negatively regulate T cell activation[18]. The protein tyrosine phosphatase SHP-1 is one such negative regulator of TCR signaling[19] (discussed in Section 1.6). TCR signaling can result in T cell differentiation, proliferation, effector function, development into memory cells, apoptosis, or anergy[14]. The outcome is dictated by the integration and quality of signals through the TCR, costimulatory and cytokine receptors, as well as many layers of regulation.

1.3 Effector CD4+ and CD8+ T cells

Upon leaving the thymus, naïve CD4+ and CD8+ single positive thymocytes enter secondary lymphoid organs through high endothelial venules, ending up in the T

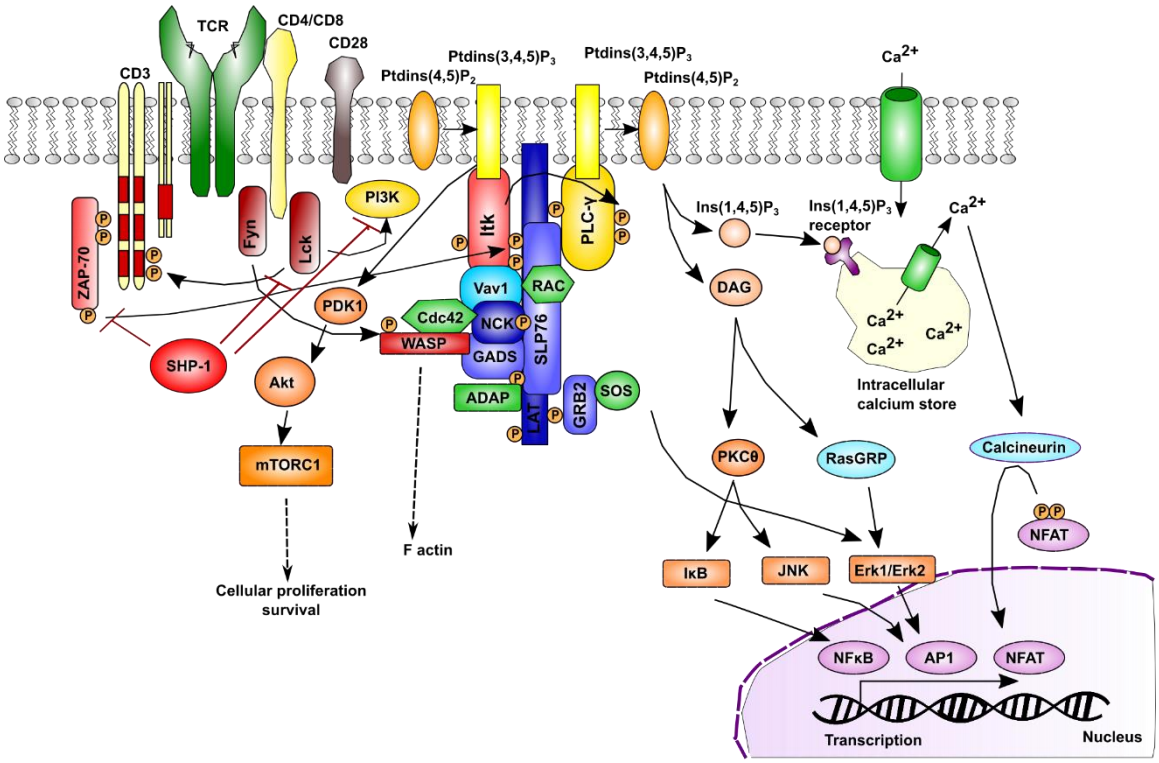

Figure 1.4 T cell receptor signaling cascade. Engagement of antigen with the TCR/CD3 complex initiates a cascade of signaling events. ITAMs in CD3 cytoplasmic tails are phosphorylated by Lck and Zap-70. A complex with several molecules forms, which is propagated by the adapter molecules LAT and SLP-76. PLCɣ and PI3K are activated and generate lipid second messengers, which mediate calcium influx. Elevated Ca^{2+} activates calcineurin, which dephosphorylates NFAT, allowing it to translocate to the nucleus and bind to the IL-2 promoter. DAG activates members of the PKC family and Ras, leading to activation of Jnk and Erk1/Erk2, which regulate gene transcription. PKCθ is activated by a Vav1/Rac complex, and it then activates NF-κB, also a transcriptional regulator. PI3K activation also results in the activation of Akt, which activates mTORC1, involved in cellular proliferation and survival. As depicted, SHP-1 is a negative regulator of early TCR signaling events, dephosphorylating molecules such as Zap-70, Lck, and Vav1.

cell zone[1]. In this area, naïve T cells encounter dendritic cells (DCs) presenting Ag, and if the T cell encounters a peptide/MHC (pMHC) complex recognized by its TCR, then the T cell will undergo activation. However, if the naïve T cell does not encounter its cognate Ag in a particular lymph node, it can recirculate through efferent lymphatic vessels and migrate to other lymph nodes. The ability of naïve T cells to enter or exit a lymph node is mediated by a complex array of chemokine receptors and adhesion molecules. Each lymph node drains local tissues, concentrating Ag from pathogens present in those tissues and increasing the likelihood that a cognate T cell will recognize the Ag and become activated[1,20].

As discussed above, T cell activation requires engagement of the TCR with its cognate pMHC as well as costimulation via CD80/CD86 molecules on the APC. A downstream consequence of costimulatory signals is the rapid transcription of IL-2, which acts in an autocrine manner to induce the T cell to express the high-affinity IL-2Rα, or CD25. Engagement of CD25 by IL-2, along with other activation signals, induces T cells to proliferate[21]. Once naïve CD4+ T cells undergo proliferation, additional signals from cytokines produced by APCs as well as other signals from the innate immune response instruct CD4+ T cells to differentiate into specific T_H lineages[11] (Fig. 1.3). T_H lineages are relatively stable, and are defined by the cytokine which they produce, the transcription factor that helps determine their differentiation, and the type of response in which they play a role[11]. T_H1 cells differentiate in response to IL-12 and/or IFNγ, turn on the transcription factor T-bet, and produce IL-12 and IFNγ themselves. T_H1 cells specialize in activating macrophages and controlling intracellular bacterial infections[11]. In response to IL-4 signals, naïve CD4+ T cells turn on GATA3 and develop into T_H2 cells,

which produce IL-4, IL-5, and IL-13. T_H2 cells recruit eosinophils, basophils, and mast cells to clear infection by helminth parasites[11]. However, T_H2 cells are also culprits in allergic responses[22]. TGF-β and IL-6 signals together induce RORɣt expression, leading to T_H17 cells, which produce IL-17. T_H17 cells recruit neutrophils to help clear fungi and extracellular bacteria[11]. Another important lineage is T_{FH} cells, which are induced by the expression of Bcl6 in response to IL-6 signaling. T_{FH} cells produce IL-21 and express CXCR5, allowing them into the B cell follicle. Once there, T_{FH} cells produce cytokines and provide signals that promote B cell class switching[11]. Finally, signals from TGF-β promote the expression of Foxp3 in the pTreg lineage[11] (discussed in Section 1.4). Each of these T_H subsets also upregulates expression of surface molecules during differentiation that allow them to migrate to sites of infection, where they must find APCs displaying cognate Ag. Upon engagement with a cognate pMHC, these effector T cells can become activated and release cytokines without needing costimulation[20].

For naïve CD8+ T cells, activation can occur in response to peptide presented by a DC, or may require simultaneous "help" from CD4+ T_H cells via the production of IL-2. CD8+ T cells then undergo proliferation and differentiation into CTLs, which produce cytotoxins such as granzymes and perforins. CTLs secrete these cytotoxins to induce virally-infected cells to undergo apoptosis, thereby clearing the viral infection[23]. The majority of effector CD4+ T cells and CTLs undergo apoptosis after an infection is cleared[24]. However, some of these cells persist as effector or central memory T cells. Effector memory T cells patrol tissues and can respond immediately to an infection, whereas central memory T cells reside in lymphoid organs and become activated more slowly, but can then generate more effector T cells[24]. For each of these subsets of T cells

(naïve, effector, and memory), Tregs can inhibit the activation, proliferation, differentiation, and effector function through a variety of mechanisms (discussed in Section 1.4). However, dysregulation of T_H1, T_H17[25], and CD8+ T cells[26] can result in autoimmune disease.

1.4 Regulatory T cells and immunological tolerance

Originally identified as "suppressor T cells" in 1970[27], Tregs were further defined in the 1990s upon the discovery that a portion of the CD4+ T cell population highly expressed CD25 (IL-2Rα) and that depletion of these cells caused autoimmunity in mice[28]. Neonatal thymectomy at day 3 of life resulted in mice that developed systemic autoimmunity, which could be rescued by transfer of thymocytes[29] (later discovered to be CD4+CD25+ Tregs). Therefore, these "suppressor" T cells were thymic in origin but migrated to the periphery to maintain self-tolerance. Subsequently, these same CD4+CD25+ cells were found in humans, and like their mouse counterparts, could suppress the *in vitro* proliferation of and production of IL-2 by CD4+CD25- T cells[30,31]. In the early 2000s, Foxp3 was identified as the master transcriptional regulator of Tregs in mice and humans, and later shown to be necessary for Treg development and function[32]. Mice with loss-of-function mutations in *Foxp3* develop systemic autoimmunity (*scurfy* mice)[33], similar to IPEX syndrome in humans which also results from a mutation in *Foxp3*[34]. It is now clear that Tregs are a crucial component for a healthy immune system due to their critical role in mediating immune homeostasis, preventing autoimmunity, and influencing the composition of the gut microbiome[35–37].

Following the identification of Foxp3 as the master transcriptional regulator of Tregs, it was shown that forced expression of Foxp3 in Tcon cells induced a Treg-like

suppressive phenotype, but without activating all Treg signature genes[32]. Foxp3 upregulates the expression of CD25, CTLA-4, and GITR, while repressing transcription of IL-2, IL-4, and IFNγ[38]. More recent studies have found that in addition to Foxp3 expression, Tregs also acquire epigenetic changes during development wherein chromatin becomes accessible at loci encoding genes important to Treg function (e.g. Foxp3, CTLA-4, Eos)[6]. Foxp3+ Tregs represent a heterogenous population of cells, distributed throughout lymphoid and non-lymphoid tissues[35]. The main distinction in Treg types is between thymic-derived Tregs (tTregs, formerly known as natural Tregs) and peripheral Tregs (pTregs, formerly induced Tregs). Thymic Tregs, as their name suggests, develop in the thymus, acquiring Foxp3 expression in response to a relatively high-affinity TCR interaction with self-peptide/MHC as well as other cytokine signals[2]. The duration of TCR stimulation seems to be important for inducing the Treg epigenetic pattern, but much is unknown about how this occurs[6]. In contrast to tTregs, pTregs develop in the periphery from naïve CD4+ T cells in response to TGF-β signaling, which promotes Smad3 binding to the CNS1 enhancer of *Foxp3*, inducing *Foxp3* transcription[39]. In the gut, retinoic acid and TGF-β signaling induce pTregs with a gut homing phenotype, and other studies have shown that gut microbiota influence pTreg development[39]. pTregs have been further divided into Tr1 cells which are induced by IL-10 and secrete large amounts of IL-10, and T_H3 Treg cells, which produce TGF-β and IL-10[40]. TCR usage only partially overlaps between tTregs and pTregs; whereas tTregs are self-Ag specific, pTregs are non-self-Ag specific and develop in response to allergens, food, and commensals[2]. It remains unclear if tTregs and pTregs have separate “divisions of labor”

within the immune system with regards to their suppressive function[41]. In this body of work, we focus on thymic CD4+CD25+Foxp3+ Tregs.

Tregs maintain a constitutive presence in secondary lymphoid organs and recirculate in the blood. Tregs can also be found in most tissues even in the absence of inflammation, especially in mucosal sites[35]. Tregs express a variety of chemokine receptors and integrins to home to different tissue locations, the expression of which is influenced by cytokines produced in the local environment. Depending on location, Tregs acquire specialized phenotypes. For example, a subset of Tregs acquire an effector/memory-like phenotype in peripheral lymphoid organs and have different homeostatic requirements than those that remain "naïve"[35]. Tregs must become activated through the TCR in order to exert their suppressive functions *in vitro* and *in vivo*. Once activated, Tregs can suppress a wide range of immune cells, in both an Ag-specific and non-specific manner[42]. Depending on cytokine signals received during their initial activation, Tregs not only express specific chemokine receptors to home to sites of inflammation, but have also been found to upregulate transcription factors associated with the specific type of T_H response occurring[35]. For example, during a T_H1 response, Tregs expressing T-bet accumulate at the site of inflammation and control the T_H1 response better than Tregs lacking T-bet[35]. It is unclear whether these functionally specialized Tregs represent tTregs that migrated from lymphoid tissues or pTregs generated at the site of inflammation[41]. It is clear, however, that Tregs are heavily influenced by the environment in which they are activated and the site to where they migrate, in a manner that tailors their suppressive function[35]. It is well known that Tregs

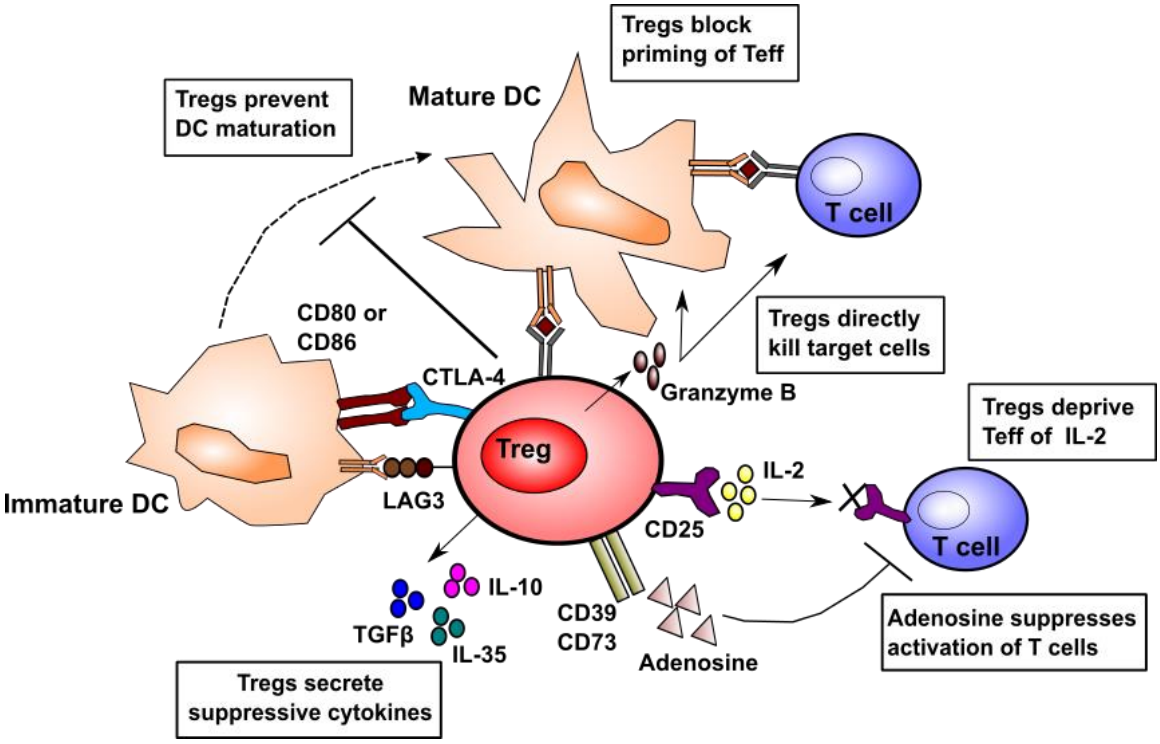

Figure 1.5. Treg suppressive mechanisms. Tregs employ many suppressive mechanisms, which can suppress both T cells and APCs. Expression of CTLA-4 on the surface of Tregs prevents the maturation of DCs by binding to costimulatory molecules CD80/CD86, and prevents adequate priming of T cells by DCs. Tregs also express LAG3, which binds to MHCII on DCs, inhibiting their maturation. Tregs secrete suppressive cytokines IL-10, IL-35, and TGF-β, and also secrete granzyme B, which mediates direct killing of target cells in a perforin-dependent manner. The constitutive expression of CD25 allows Tregs to bind up IL-2, depriving T cells of IL-2 needed to become activated and proliferate. Tregs also express ectonucleases CD39 and CD73 which synthesize adenosine, dampening T cell activation and proliferation.

can employ a diverse repertoire of suppressive mechanisms (Fig. 1.5), through secretion of soluble factors or through cell-cell contact[43]. Not only can Tregs suppress the activation of naïve T cells, but they also inhibit activated effector and memory CD4+ and CD8+ T cells[44]. Tregs can secrete suppressive cytokines IL-10, IL-35, TGF-β, which can act directly on target cells or on APCs[43]. Since Tregs express CD25 at high levels, they also bind up available IL-2, depriving effector T cells of IL-2 necessary for survival[44]. IL-2 deprivation does not appear to be a prominent suppressive mechanism *in vitro,* and it remains controversial whether it occurs *in vivo*[44]. Furthermore, Tregs can secrete granzyme B, mediating target cell lysis in a perforin-dependent manner[45]. Tregs express galectin-9, which can bind to TIM-3 on activated effector T cells and induce apoptosis[46]. Tregs reduce the ability of APCs to costimulate effector T cells by expressing CTLA-4, which binds to CD80/CD86 on APCs and downregulates their expression[46]. The molecule LAG-3 is expressed on Tregs and binds to MHCII molecules on DCs, sending signals that inhibit DC maturation[46]. DCs express indoleamine 2,3-dioxygenase (IDO) which can lead to cell cycle arrest in effector T cells and promote pTreg generation. Some Tregs can induce IDO expression by DCs via CTLA-4[44].

In addition to suppressive cytokines, cytolysis, and inhibition of APC costimulatory capacity, Tregs also employ metabolic disruption as a method of suppression. Tregs express the ectonucleases CD39 and CD73 on their surface, which synthesize extracellular adenosine. Adenosine dampens the proliferation of effector T cells and production of pro-inflammatory cytokines by T cells and APCs[45,46]. Tregs produce high levels of cAMP and can induce DCs to produce cAMP, which then acts through ICER to repress IL-2 and IL-4 in target T cells[44]. Much work has been devoted to delineating how

Treg suppressive mechanisms differ *in vitro* versus *in vivo*[43] and how these mechanisms function within specific tissues to shape immune responses[35,47]. The suppressive mechanisms and response of Tregs *in vitro* may be different from what occurs *in vivo*[43], especially since *in vivo* the microenvironment and physiological location inform Treg function. For example, IL-2 is needed for Treg survival and homeostasis *in vivo*, but IL-2 signaling is not only dispensable, but counteracts Treg suppressive function *in vitro*[48]. Furthermore, Tregs are anergic and generally non-proliferative *in vitro*, but can expand *in vivo* after antigen encounter[43]. *In vitro* Treg suppression relies on cell-cell contact with APCs and/or Tcon cells, at least to initiate suppression, which is then mediated by effects on APCs and possibly cytokine production[49]. Much remains unknown about what happens at the molecular level in a Tcon cell that is suppressed by a Treg cell, and *in vitro* assays are useful to investigate this. Interestingly, Tregs can suppress effector CD4+ or CD8+ T cell function independently of suppressing their proliferation, suggesting the inhibition of distinct intracellular pathways[44].

1.5 Tcon cell resistance to Treg suppression as a pathophysiological mechanism of autoimmune disease (adapted from[50], Appendix A)

As discussed above, central and peripheral tolerance mechanisms exist to ensure that the immune system can adequately respond to infectious threats while not responding to self-Ag. However, individuals with genetic susceptibility and/or other etiological factors can develop autoimmune disease if the fine balance of these regulatory mechanisms is perturbed. When there is such a break in tolerance, autoreactive T cells (and B cells) become activated in response to self-Ag and lead to autoimmune

pathologies. It remains unclear what initiates a break in tolerance, and whether environmental triggers such as microbial infections influence this[51]. However, it has been clearly established that reduced frequency of Tregs or functionally impaired Tregs lead to autoimmune disease[36]. This conclusion arose from the overwhelming evidence that systemic autoimmunity ensued in the absence of Tregs (discussed above Section 1.4). Furthermore, genetic models where key components of Treg function are impaired, such as CTLA-4 KO[52] or IL-10 KO[53] mice, supported the idea that Tregs were necessary for immune tolerance, and are the likely culprits in autoimmune disease.

More recently, there have been conflicting reports on whether Treg frequency and/or function is actually reduced in all autoimmune diseases[54]. Despite these discrepancies, both reduced Treg number and/or function remain as possible pathological mechanisms[46,54] (Fig. 1.6). However, compelling evidence acquired over the past decade now suggests that Tcon cells that are refractory to Treg suppression also act as mediators of autoimmune disease in mice[55–63] and humans (Table 1). It has been clearly demonstrated that Tcon cells - including naïve (also called "T_H0") T cells, differentiated effector T cells, and memory T cells - can become refractory to Treg-mediated suppression both *in vitro* and *in vivo*[55–61,63–77]. Tcon cells can become insensitive to Treg-mediated suppression when the ratio of Tcon cells to Tregs is skewed in favor of Tcon cells, when intracellular signaling pathways have been modified by mutations, or through extracellular signals, such as strong activation or a specific cytokine milieu, that induce Tcon cell-intrinsic changes[36]. The latter mechanism refers to potentially pathogenic Tcon cells that have become *resistant* to Treg suppression, a phenomenon which has been observed in several autoimmune diseases.

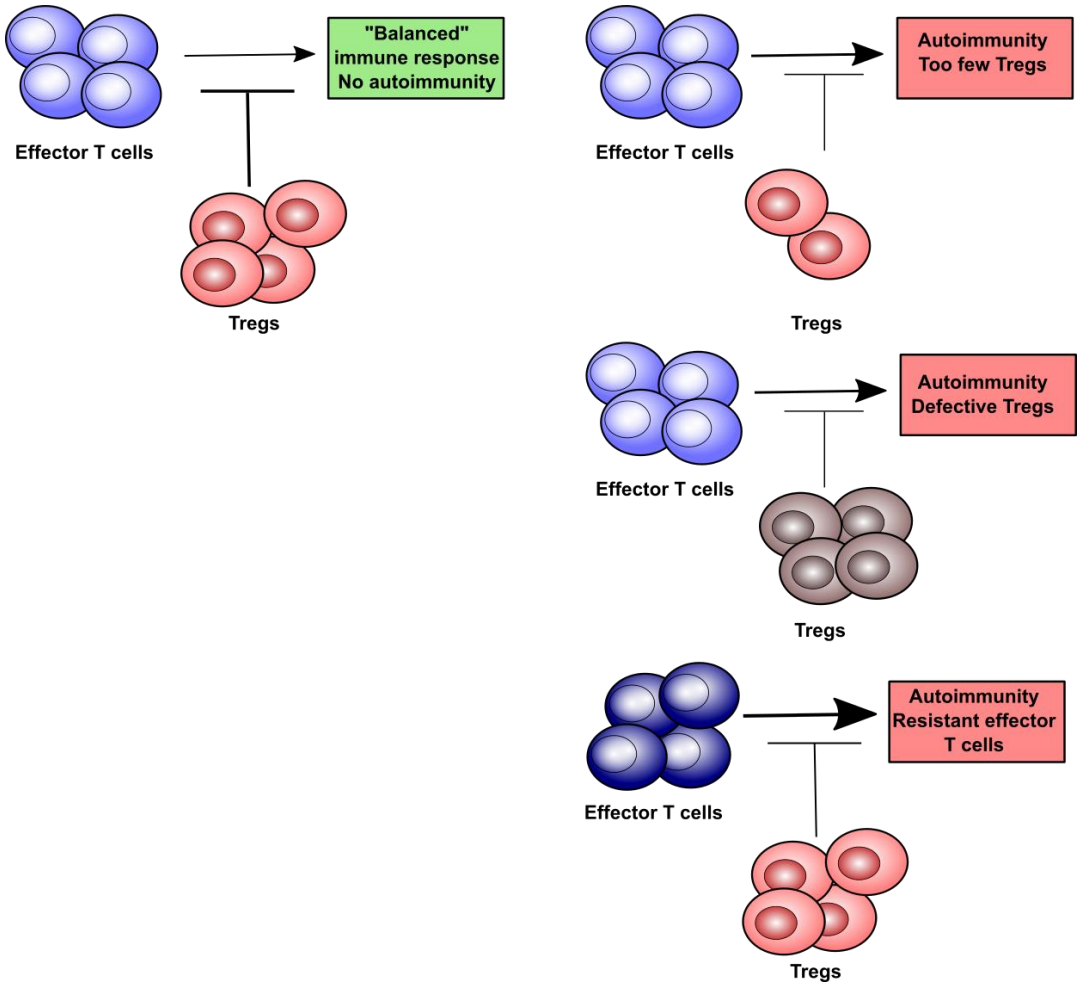

Figure 1.6. Dysregulation of the Treg/T cell balance. The balance between Treg suppression and activation of T effector cells maintains immune homeostasis. Autoimmunity can occur when there are inadequate numbers of Tregs to suppress autoreactive T cell activation, or when Treg function is impaired (gray Treg cells represent functionally defective Tregs). Autoimmunity can also occur when effector T cells become resistant to Treg suppression (dark blue effector T cells).

Table 1. Diseases in which Tcon cells resist Treg-mediated suppression[50].

Abbreviation: ND – not determined
CNS – central nervous system
[a]Teff – total synovial fluid or peripheral blood mononuclear cells (as indicated) isolated as CD4+ or CD8+
[b] Teff – contains both CD4+ and CD8+ Teff cells, isolated as CD3+

Disease	Subject	Type of effector cell	Suggested mechanism
Juvenile idiopathic arthritis (JIA)	Human	Synovial fluid CD4+CD25-	Enhanced activation[69]
		Synovial fluid CD4+ and CD8+ Teff[a]	Akt hyperactivation in response to IL-6/TNFα[70,73]
Rheumatoid arthritis (RA)	Human	Peripheral blood CD4+CD25-	Increased TRAIL expression on Teff leading to Treg apoptosis[77]
Type 1 diabetes (T1D)	NOD mice	Splenic CD4+CD25-	ND[56]
	DO11.10 RIP-mOVA mice	Lymph node CD4+CD25-	Increased IL-21[59]
	NOD mice	Splenic CD4+CD25-	ND[61]
	NOD mice	Splenic CD4+ and CD8+ Teff	Reduced ganglioside M1 expression on Teff[62]
	Human	Peripheral blood CD4+CD25-	ND[66]
		Peripheral blood CD4+CD25-	ND[65]
Systemic lupus erythematosus (SLE)	MRL/lpr and NZB/WF1 mice	Splenic and lymph node CD4+CD25-	ND[55]

	MRL/lpr mice	Lymph node CD4+CD25-	ND[60]
	Human	Peripheral blood CD4+CD25-	ND[68]
		Peripheral blood CD4+CD25-	ND[67]
		Peripheral blood CD4+CD45RA- FoxP3-	Akt hyperactivation, upregulation of OX40 and impaired TRAF6 in Teff [78]
	FoxP3.gfp KI mice	CNS CD4+GFP-	High IL-6 and TNFα[57]
	C57BL/6 mice	CNS CD4+CD25-	ND[58]
	B6.SLE mice	Splenic CD4+CD25-	ND[63]
	Human	Peripheral blood CD3+ Teff[b]	Accelerated production of IL-6 and higher expression of IL-6R on Teff leads to Akt hyperactivation[74]
		Peripheral blood CD4+CD25-	Increased IL-6 induction of pSTAT3[75]
		Peripheral blood CD4+CD25-	Increased Granzyme B production by Teff w/ TCR activation/IL-6 stimulation, inactivating Tregs[72]
Inflammatory bowel disease (IBD)	Human	Lamina propria CD4+CD25-	Higher expression of Smad7 interfering with TGF-β signaling[64]
		Lamina propria CD4+CD25-	Increased IL-15 in lamina propria[76]

1.6 Mechanisms of resistance (adapted from Appendix A[50])

1.6.1 Extracellular factors

1.6.1.1 Cytokine milieu

Autoimmune diseases are organ- or tissue-specific and characterized by overproduction of inflammatory cytokines. This is in line with the observation that numerous cytokines associated with autoimmune disease have been found to induce Tcon resistance to Treg suppression: IL-6[57,71,74,75,79–82], TNFα [57,73,83], IL-15[84–86], IL-21[59,81,87,88], IL-1β[89,90], and IL-4[91,92] (Fig. 1.7). Beyond pro-inflammatory cytokines, IL-2 has also long been known to overrule Treg suppression *in vitro*[86,93,94]. One of the difficulties with experiments assessing the effect of cytokines on Treg suppression is that simply adding a cytokine to an *in vitro* co-culture system simultaneously affects Tregs and Tcon cells, making it difficult to distinguish whether there is impaired Treg function, Tcon cell resistance to suppression, or both. Many studies have therefore focused on downstream signaling pathways, or used genetic deletion of cytokine receptors, to delineate effects on Tcon cells independent of Tregs. While many of these factors induce Tcon cells to resist suppression, they may also affect Treg function (for more detailed discussion on this topic, see Appendix A).

1.6.1.2 Toll-like receptors

Toll-like receptors (TLRs) are an essential line of defense against microbial and viral pathogens. Various pathogen-derived ligands signal through TLRs, which recruit adaptor molecules such as MyD88 to trigger the production of pro-inflammatory mediators[95]. The goal of TLR signaling is to sense a pathogenic threat and mount innate

and adaptive immune responses. TLR ligands can influence T cell responses via direct receptor activation or indirectly, by inducing APCs to produce cytokines that affect T cells[96,97]. For example, stimulation of DCs with LPS or CpG (TLR4 and 9 agonists, respectively) induced their production of IL-6, contributing to Tcon cell resistance to Treg suppression[79]. Both human and murine T cells express mRNA for TLRs 1-9, but protein expression levels vary and depend on the genetic background (in mice) and activation status of the T cell[97–99]. In general, TLR engagement acts as a costimulatory signal to T cells and subsequently activates the PI3K/Akt pathway, consistent with a role in inducing Tcon cells to resist Treg suppression[98,99]. Signals through TLR9[100,101] and TLR2[102,103104,105] thus far have been shown to induce Tcon resistance to Treg suppression.

IL-1β is a potent pro-inflammatory cytokine associated with a wide array of inflammatory states, including some autoimmune diseases[106]. Monocytes release IL-1β in response to pathogen or "danger" signals[106]. Like TLRs, the IL-1R also contains a Toll/interleukin-1 receptor domain and utilizes MyD88 in signaling[107]. Tcon cells and Tregs both express the IL-1R, and IL-1β has been found to enhance the expansion and survival of T cells by activating NF-κB and PI3K pathways[107,108]. IL-1β was found to inhibit Treg suppression of Tcon cells *in vitro*[89] by acting directly on Tcon cells rather than by impairing Treg function[90]. These data suggest that IL-1β may be another factor that, during pathogenic infection, allows Tcon cells to mount a response despite the presence of Tregs. It is possible that IL-1β also induces Tcon cell resistance to suppression in autoimmune disease settings, but this remains to be investigated.

1.6.1.3 TNF receptors

Engagement of certain tumor necrosis factor receptors (TNFRs) on T cells provides costimulatory signals that lead to activation, proliferation, differentiation, and survival[109]. In particular, signaling through GITR, 4-1BB, OX40, and TNFR2 has been found to render Tcon cells resistant to Treg suppression[110–117]. These TNFRs are constitutively expressed on Tregs and become upregulated on activated Tcon cells[115,118–120]. The ligands for these TNFRs are generally expressed on APCs, but can also be induced on other cell types during infection[110,111,121]. TNFRs, like TLRs, play an important role during an infectious threat by allowing Tcon cells to become efficiently activated in order to mount a response, unrestrained by Tregs. It has therefore been proposed that TNFR ligand expression becomes upregulated during inflammatory conditions and provides costimulatory signals to both Tregs and Tcon cells, with Tcon cells becoming activated, producing IL-2, and resisting Treg suppression. As TNFR ligand levels wane and Tcon cells are no longer able to resist suppression, Tregs can assume control of the immune response[110].

1.6.2 Intracellular signaling molecules linked to Tcon resistance

1.6.2.1 Cbl-b

Cbl-b is an E3 ubiquitin ligase that catalyzes the ubiquitylation of target proteins, which can result in their degradation by the proteasome, translocation inside the cell, or alteration in function[122]. In T cells, Cbl-b sets the threshold for weak antigen stimulation[123] and enforces the need for costimulation, or "signal 2", by regulating CD28 signaling[124]. Cbl-b negatively regulates the recruitment of the p85 subunit of PI3K to

CD28, thereby enforcing T cell anergy and tolerance when signal 2 is lacking[125]. Upon CD28 signaling, Cbl-b itself becomes ubiquitylated and degraded, allowing PI3K recruitment and other downstream signaling required for full T cell activation[126]. Consistent with its negative regulatory functions, Cbl-b knockout (KO) mice develop systemic autoimmunity due to hyper-proliferation and increased activation of lymphocytes, with T cells that can be activated in the absence of CD28 costimulation[127]. Cbl-b KO Tregs were found to be normal, whereas Tcon cells were found to resist suppression by both wild type and Cbl-b KO Tregs, *in vitro*[128] and *in vivo* in a graft-versus-host disease (GVHD) model[129]. In addition to CD4+ T cells, Cbl-b KO CD8+ T cells also resisted Treg-mediated suppression, providing a mechanism by which Cbl-b KO mice were able to spontaneously reject different types of xenograft tumors as well as ultraviolet-B light-induced skin cancer[130,131]. While the exact downstream mechanism of resistance in Cbl-b KO Tcon cells remains unclear, it is notable that Cbl-b KO T cells showed enhanced PI3K/Akt activation[125].

1.6.2.2 TRAF6

TRAF6 belongs to the E3 ubiquitin ligase family and transduces signals downstream of members of the TNFR superfamily, including IL-1R/TLRs[132], thereby activating NF-κB, NFAT, MAP kinases, and Akt signaling pathways[132]. A role for TRAF6 in the negative regulation of T cell signaling was discovered by Choi and colleagues in 2006[133]. Their study demonstrated that TRAF6 KO mice developed multi-organ inflammatory disease characterized by hyper-activated T cells. Using mice in which TRAF6 was specifically deleted in T cells, the group showed that while TRAF6 KO Tregs were normal, the Tcon cells resisted Treg suppression both *in vitro* and *in*

vivo[133]. Re-expression of TRAF6 via retroviral transduction restored susceptibility of Tcon cells to Treg-mediated suppression[133]. Like Cbl-b KO T cells, TRAF6 KO T cells could also be activated independently of CD28 costimulation, and showed enhanced Akt activation upon TCR signaling. Importantly, sensitivity to Tregs could be restored by overexpression of PTEN, an inhibitor of PI3K/Akt[133]. These findings were also supported by human studies indicating that T cells from SLE patients had reduced induction of TRAF6 mRNA upon TCR stimulation, which correlated with increased levels of phospho-Akt and resistance to Treg suppression[78].

1.6.3 PI3K/Akt: Node of convergence

Many of the studies discussed above directly demonstrated hyper-activation of the PI3K/Akt pathway in Tcon cells that resist Treg suppression. Evidence is accumulating to suggest that increased PI3K/Akt signaling may be at the heart of Tcon resistance. Wohlfert[134] was the first to propose that the PI3K/Akt pathway was central in allowing Tcon cells to resist suppression. Furthermore, murine models with genetic deficiencies in molecules that negatively regulate the PI3K pathway exhibit Tcon cells resistant to suppression[128,133]. Most compelling is the finding that inhibitors of PI3K and/or Akt can reverse Tcon cell resistance to Treg suppression, making Tcon cells once again susceptible to suppression[70,73,74,78,85,133].

It is unknown how increased activation of the PI3K/Akt pathway allows Tcon cells to overcome suppression, especially because the specific mechanisms of suppression employed by Tregs in a given setting vary. In T cells, signaling through the TCR and CD28 rapidly recruits and activates PI3K, but cytokines and other costimulatory

receptors can similarly activate PI3K[135]. Lipid second messengers produced by activated PI3K bind to Akt and relocate it to the plasma membrane, where it becomes primed for activation[136]. Upon activation, Akt promotes proliferation by increasing cell size, inactivating cell cycle inhibitors, and increasing glucose metabolism, as well as enhancing cell survival and allowing cytokine production[137]. Mice in which T cells overexpress constitutively active PI3K or Akt develop lymphadenopathy and autoimmunity, underscoring the importance of regulated PI3K/Akt signaling in T cells[137,138].

It is important to note that resistance to suppression occurs in both naïve and memory Tcon cells[70,85,129] and that hyper-activation of PI3K/Akt induces resistance in both subsets[85]. Interestingly, Tcon cells rendered hyper-responsive by NFATc2/NFATc3 double KO were also able to resist Treg suppression and become activated independently of CD28 costimulation[139]. NFAT proteins are regulators of T cell activation, inducing transcription of genes necessary for T cell responses[139]. However, the findings of this study suggest that NFATc2/NFATc3 also play a regulatory role in T cell activation, representing a signaling pathway aside from PI3K/Akt that can render Tcon cells resistant to suppression. This finding warrants further investigation into the signaling events that allow Tcon cells to become Treg-resistant, and whether there is a common molecular mediator downstream of both the PI3K/Akt and NFAT pathways.

1.7 SHP-1 Function in T cells

Src homology 2 domain-containing protein tyrosine phosphatase 1, or SHP-1 (also known as PTPN6, previously called PTP1C, SH-PTP1, or Hcph) is a non-receptor

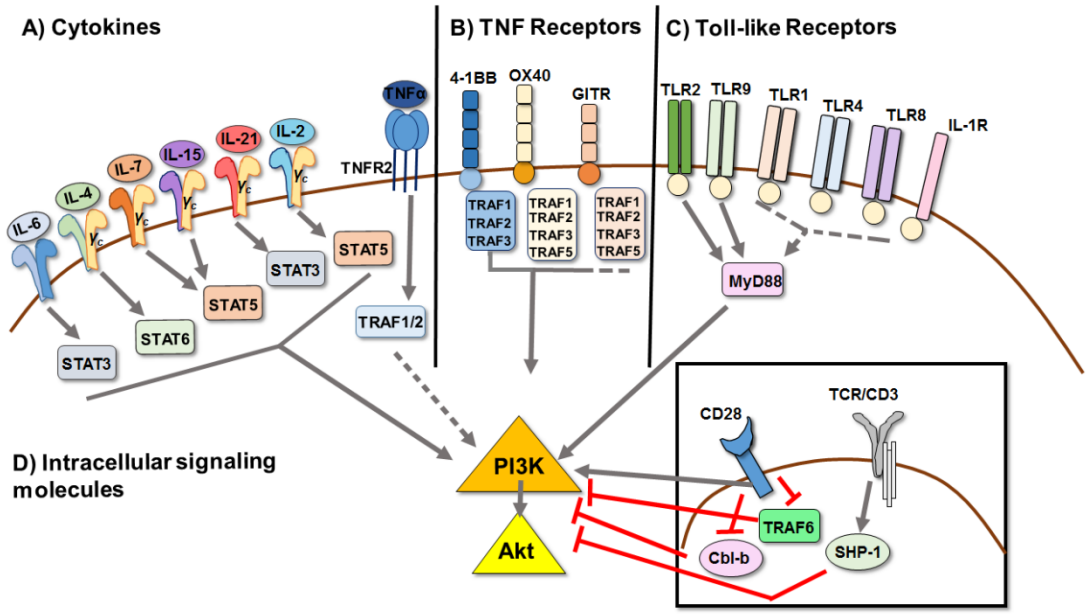

Figure 1.7. Signal transduction pathways that mediate Treg resistance converge on the PI3K/Akt pathway. **(A)** Cytokines IL-6, IL-4, IL-7, IL-15, IL-21, IL-2, and TNFα [ligand for TNFR2, see **(B)** as part of the TNFR superfamily] have been shown to induce Tcon cells to resist Treg suppression. The respective STAT molecule through which each predominantly signals is depicted. **(B)** Signaling through TNF receptors 4-1BB, OX40, GITR, and TNFR2 can induce Tcon cell resistance to Treg suppression, as they provide costimulatory signals similar to CD28 ligation. 4-1BB, OX40, and TNFR2 signaling has been shown to induce PI3K/ Akt activation *via* TRAF adaptor proteins, while GITR ligation has not been directly demonstrated to activate the PI3K/Akt pathway. **(C)** Toll-like receptors 1, 2, 4, 8, and 9, as well as IL-1R, also a member of the TLR family, have been shown to induce Treg resistance. Of these, only signaling through TLR2 and TLR9 has been shown to activate the PI3K/Akt pathway *via* recruitment of adaptor protein MyD88, which in turn recruits and activates PI3K *via* its Toll/interleukin-1 receptor domain. **(D)** Intracellular signaling molecules Cbl-b and SHP-1 act as negative regulators

downstream of TCR signaling, and genetic deficiency in either induces Treg resistance. Cbl-b enforces the requirement for CD28 costimulatory signaling by inhibiting the recruitment of PI3K to CD28. TRAF6 also negatively regulates activation of PI3K downstream of CD28 costimulation by an as yet undefined mechanism. Dashed lines indicate proposed, but unconfirmed, links between receptors and/or signaling molecules and the PI3K/Akt pathway.

protein tyrosine phosphatase expressed in all hematopoietic cells and at low levels in epithelial, endothelial, and brain cells[140]. The importance of SHP-1 in many signaling pathways in hematopoietic cells was discovered upon the characterization of the *motheaten* (*me/me*) mouse model in 1975. A random splicing mutation occurred on chromosome 6 in a C57BL/6 mouse, resulting in no expression of the of Hcph protein (later known as SHP-1) and leading to the "motheaten" phenotype[141,142]. *Motheaten* mice develop systemic autoimmunity and inflammation, with immune complex deposition and autoantibody production, and ultimately die by 2-4 weeks of age from pneumonia caused by macrophage and neutrophil accumulation in the lungs[141,142]. These mice get their name from their patchy fur and skin lesions, which have been shown to be caused by enhanced neutrophil infiltration of the skin[143]. A second random mutation led to in-frame insertion or deletion of a few amino acids in the catalytic site of SHP-1, such that SHP-1 protein is still expressed but is catalytically inactive. Mice with catalytically inactive SHP-1 were termed *motheaten*^{viable} since they live for 9-12 weeks and display a less severe phenotype, likely because there is still some phosphatase activity of SHP-1[141]. From these mouse models it was evident that SHP-1 regulated many signaling pathways in various immune cell lineages.

As a member of the family of "classical" protein tyrosine phosphatase (PTPs), SHP-1 has two SH2 domains (N- and C-terminal) as well as a catalytic PTP domain and a C-terminal tail with two tyrosyl phosphorylation sites (Fig. 1.8A). In resting cells, SHP-1 is auto-inhibited (Fig. 1.8B**)**; an interaction between the N-terminal SH2 domain and the PTP domain keeps the molecule allosterically inhibited. It is thought that the C-terminal SH2 domain is able to survey for target phospho-tyrosines (pY) on adapter molecules or

receptors, and upon binding to a pY, SHP-1 undergoes a conformational change that frees the PTP domain and allows for catalytic activity[144] (Fig. 1.8C). Other studies have suggested another regulatory mechanism for activation of SHP-1 that involves tyrosine phosphorylation of Y536 and Y564 in the C-terminal tail region by Lck[145]. Once phosphorylation of the tail tyrosine residues occurs, the PTP domain is freed from interaction with the N-SH2 domain, and SHP-1 is activated (Fig. 1.8D). These pY residues in the C-terminal tail of SHP-1 are thought to act as docking sites to recruit target proteins with SH2 domains directly or to bind adapter molecules that can in turn recruit SHP-1 targets[144]. In T cells, the C-terminus also contains an important lipid raft localization sequence, allowing SHP-1 to constitutively localize to lipid rafts where it mediates its effects on T cell receptor signaling molecules[146,147].

SHP-1 has been found to be involved in many signaling pathways, including signaling downstream of receptor tyrosine kinases (e.g. T and B cell receptors), cytokine and chemokine receptors, toll-like receptors, and integrin signaling[148]. The SH2 domains of SHP-1 interacts with phosphorylated tyrosine residues found in ITAMs (in the TCR and BCR) and immunoreceptor tyrosine-based inhibitory motifs (ITIMs) such as those found in NK cells (KIRs, Ly49 receptors) and B cells (CD22, PIR-B)[140]. Much work has gone into identifying substrates of SHP-1 in T cells, and while there are many putative targets, it has been difficult to reproducibly demonstrate direct interactions between SHP-1 and binding partners in T cells[19].

Based on several different model systems as well as predictive substrate mapping, SHP-1 is thought to negatively regulate Zap70, Lck, Fyn, TCRζ, Vav1, LAT, and SLP-76, all of which are involved in early TCR signaling events[19] (Fig. 1.4). Further

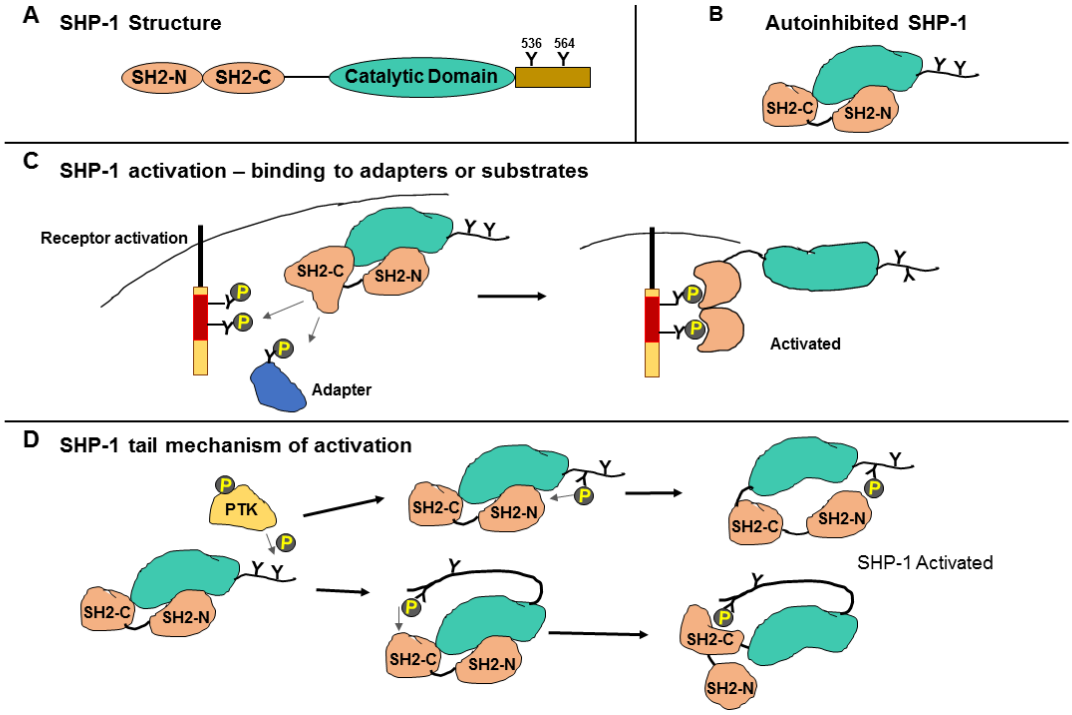

Figure 1.8. SHP-1 Structure and Regulation of SHP-1 Activation. (A) SHP-1 contains two SH2 domains, a catalytic domain, and a C-terminal tail with two tyrosine residues important for its activation. **(B)** In resting cells, SHP-1 is autoinhibited by the interaction between its N-SH2 domain with its catalytic domain. **(C)** SHP-1 can become activated when the C-SH2 domain binds to pY on ITAMs or adapter molecules, leading to the N-SH2 domain binding to pY and releasing the inhibition of the catalytic domain. **(D)** SHP-1 can also be activated when a protein tyrosine kinase (PTK) phosphorylates the tyrosine residues in the C-terminal tail. Upon phosphorylation, the pY residue in the tail is thought to interact with either the C-SH2 or N-SH2 domain, resulting in a conformational change that activates SHP-1.

downstream of TCR signaling, SHP-1 also negatively regulates PI3K by dephosphorylating its p85 regulatory subunit[149,150]. SHP-1 also negatively regulates the tyrosine phosphorylation of Jak/Stat molecules downstream of cytokine receptors. Specifically, SHP-1 has been shown to negatively regulate Stat3 downstream of the IL-6R[151], Stat6 downstream of the IL-4R[152], and Jak1/Stat1 downstream of the IFNγR[153].

Given its role in dephosphorylating proximal TCR signaling molecules, SHP-1 is considered a negative regulator of TCR signaling and has been shown to set the activation threshold for the TCR[19]. As explained above (Section 1.2), thymocytes undergo positive and negative selection based on the strength of interaction with pMHC complexes. SHP-1 was found to regulate the strength of TCR signaling in thymocytes during development, thereby regulating positive and negative selection[154–157]. The role for SHP-1 in regulating thymocyte selection was established using *me/me* and dominant negative mutant SHP-1 mouse models, in which functional SHP-1 protein is never expressed. A study using a CD4-Cre SHP-1$^{f/f}$ mouse model, in which SHP-1 is deleted during the DP thymocyte stage (pre-selection), challenged the notion that SHP-1 regulates thymocyte development[152] (for further discussion see Chapters 3-4). However, a subsequent study using the same model showed a higher occurrence of negative selection in the absence of SHP-1 because TCR signals were amplified, and therefore reduced the frequency of post-selection thymocytes[158]. Recently, the protein Themis was identified as a key player regulating positive thymocyte selection. Themis was found to bind to SHP-1 via Grb2 and block the catalytic activity of SHP-1 in order to enhance signaling in response to low affinity peptides, allowing positive selection[159,160]. These newer studies

along with previous work in SHP-1 null mice clearly establish its role in regulating TCR signaling in thymocytes and therefore regulating positive and negative selection.

In mature T cells, SHP-1 has also been found to play a role in distinguishing agonist versus antagonist peptides[161–163]. Low-affinity antagonist peptides fail to induce a T cell response and render T cells refractory to subsequent stimulation by agonists. Upon engagement with an antagonist peptide, Lck phosphorylates Y564 on SHP-1, leading to its rapid recruitment to the TCR complex and binding to Lck, where it can then dephosphorylate Lck and terminate the signal. However, when a strong agonist binds the TCR, Erk phosphorylates Lck, inactivates its SH2 domain and prevents SHP-1 recruitment, allowing signal propagation[161]. SHP-1 also regulates the differentiation of naïve CD4+ T cells into Th1, Th2, and Th17 subsets, likely through its negative regulation downstream of cytokine receptors[151–153,164–166]. While many studies demonstrated that SHP-1 negatively regulates the cytokine-driven skewing of naïve T cells into T_H1, T_H2, or T_H17 *in vitro*, several have also shown that lack of SHP-1 *in vivo* generates a bias toward T_H2 cells[152,167] or toward T_H17 cells[151]. It is likely that use of different mouse models may account for the differences in bias towards one T_H lineage versus another *in vivo*.

Aside from T_H cell differentiation, SHP-1 was found to negatively regulate the proliferative response to TCR stimulation of naïve and effector CD4+ and CD8+ T cells from *me/me* mice[157,168,169]. Deletion of SHP-1 mediated by dLck-Cre resulted in CD8+ T cells with enhanced accumulation of short-term effector cells in response to either tumor Ag or virus, and enhanced lytic function, without any effect on the formation of long-

term memory cells[170,171]. SHP-1 also regulates the effector function of Tregs, with SHP-1-deficient Tregs exhibiting more potent suppression[172].

1.8 Thesis rationale

Many studies using global knockout SHP-1 mice or immortalized cell lines have demonstrated the ability of SHP-1 to regulate the threshold for TCR signaling (reviewed in[19]) and influence peripheral T cell activation and differentiation[151,152,173,174]. However, global SHP-1 knockout mice have systemic inflammation and hyper-activation of myeloid cells, and immortalized cell lines typically have altered intracellular signaling. These factors have made it difficult to determine if SHP-1-deficient T cell phenotypes observed are cell-intrinsic. Therefore, we utilized two T cell-specific SHP-1-deletion mouse models (Fig. 1.9), wherein SHP-1 was deleted at the DP thymocyte stage under the control of CD4-Cre[175] (Chapter 4), or deleted post-thymocyte selection, under the control of distal Lck-Cre[176] (dLck-Cre) (Chapter 3). These mouse models allowed us to address the cell-intrinsic role of SHP-1 in regulating the response of peripheral T cells to TCR stimulation, and to identify effects on downstream signaling pathways.

Hyper-proliferation of SHP-1-deficient T cells in response to TCR stimulation had previously been reported, but the underlying mechanism remained unclear. Here, utilizing our dLck-Cre SHP-1$^{f/f}$ mouse model, we investigated three possible ways in which SHP-1 could regulate the proliferative response of T cells to TCR stimulation in a cell-intrinsic manner. It is possible that SHP-1 deficiency increases T cell survival, shortens cellular division time, and/or increases the proportion of cells responding to

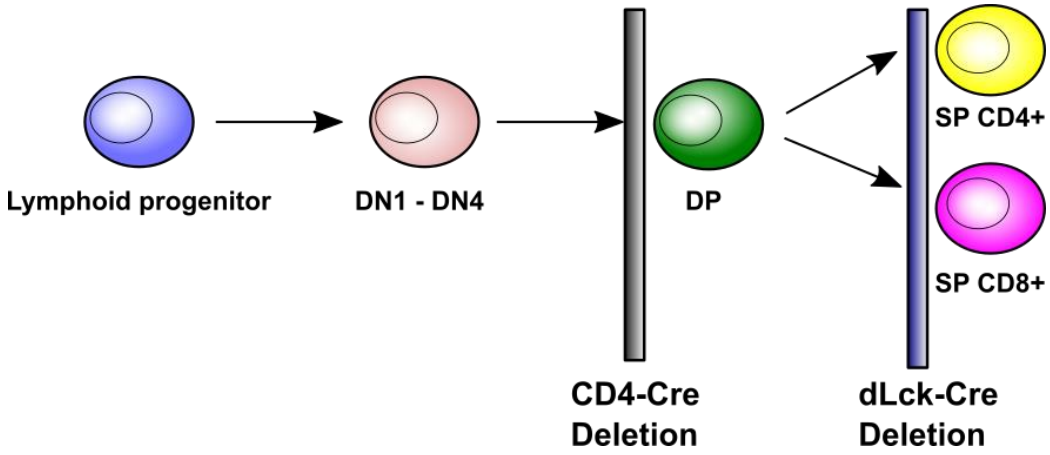

Figure 1.9. Timing of CD4-Cre- versus dLck-Cre-mediated deletion. CD4-Cre initiates recombination between the DN4 and DP thymocyte stage, before positive and negative selection. dLck-Cre mediates recombination at the SP thymocyte stage, post-selection.

stimulation. These studies provide new insight into how SHP-1 influences peripheral T cell activation and proliferation.

In addition to elucidating the regulatory function of SHP-1 in T cell responses to TCR stimulation, we sought to determine if SHP-1 also regulates the ability of a T cell to be suppressed by a Treg. As discussed above (Section 1.5), there is accumulating evidence to suggest that T cells are resistant to suppression by Tregs in many autoimmune diseases[50]. The molecular mechanisms underpinning T cell resistance to Treg suppression remain poorly understood, but T cells that receive strong activation can overcome Treg suppression[30,93,177]. Along these lines, deficiencies in two negative regulators of TCR signaling, Cbl-b[128,129] and TRAF6[133], induced T cells to resist Treg suppression. Thus, we hypothesized that SHP-1 deficiency would result in T cells that could potentially resist Treg suppression *in vitro* and *in vivo*. Furthermore, resistance to suppression has been documented in CD4+ and CD8+ T cells, in naïve and in memory-like T cells, but whether resistance occurs by the same mechanism in each of these subsets is unknown[50]. Therefore, we characterized whether SHP-1 regulated resistance to Treg suppression of naïve CD4+ and CD8+ T cells as well as CD4+CD25- Tcon cells and total CD8+ T cells.

Furthermore, we hypothesized that SHP-1 deficiency might induce T cells to resist Treg suppression by enhancing activation of the PI3K/Akt pathway (Fig. 1.10). Several studies have found that T cells resistant to Treg suppression have enhanced activation of the PI3K/Akt pathway (Section 1.6.3). SHP-1 has been described as a negative regulator of PI3K/Akt signaling in *me/me* thymocytes[150], providing a possible mechanism for SHP-1-deficient T cells to resist Treg suppression. Thus, the overall goal of this work was to

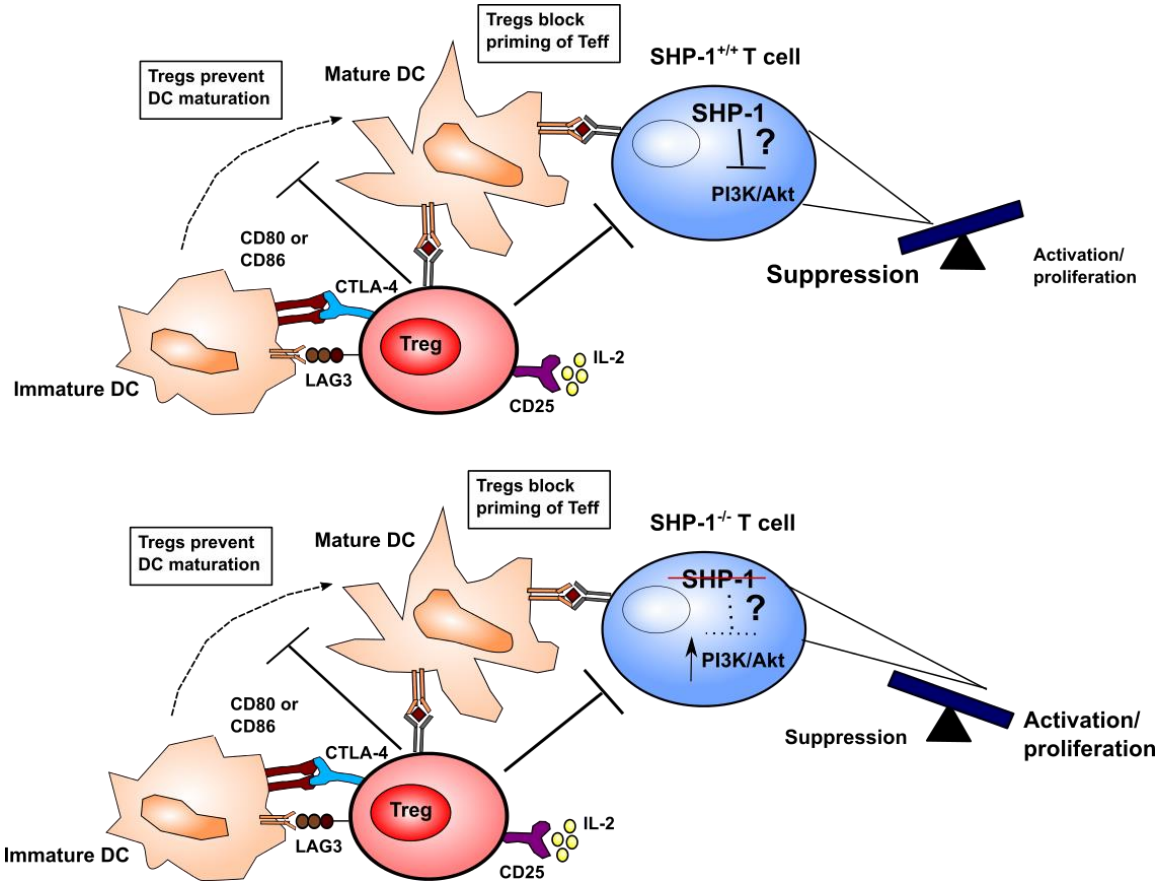

Figure 1.10. Questions addressed regarding molecular mechanism of SHP-1 in regulating T cell resistance to Treg suppression. This diagram represents the questions addressed in this work regarding how SHP-1 regulates T cell susceptibility to Treg suppression. We address whether SHP-1 negatively regulates the PI3K/Akt pathway in T cells, and whether this plays a role in determining if a T cell is suppressed by a Treg or is instead resistant to suppression and becomes activated and proliferates despite the presence of a Treg.

investigate a novel functional role for SHP-1 in regulating T cell resistance to Treg suppression, thereby identifying SHP-1 as a potential immunotherapeutic target to module susceptibility of T cells to Treg suppression.

CHAPTER 2

Materials & Methods

2.1 Mice

SHP-1$^{flox/flox}$ (SHP-1$^{f/f}$) mice[178] (generously provided by B. Neel) were crossed to distal Lck-Cre (*dLck*-Cre) mice[176] purchased from The Jackson Laboratory (Bar Harbor, ME). TCR-Tg DO11.10 mice (Balb/C) were purchased from The Jackson Laboratory and bred to *me/+* mice[142] to generate wt, *me/+*, and *me/me* mice on the TCR-Tg background. wt, *me/+*, and *me/me* mice were also bred on the C57BL/6 background. CD4-Cre mice[175] were purchased from Taconic Farms (Derwood, MD) and crossed to SHP-1$^{flox/flox}$ mice. DO11.10 mice were also bred to CD4-Cre SHP-1$^{f/f}$ mice and backcrossed for 8 generations to obtain a clean Balb/C background. *Foxp3*-Cre^{YFP} mice were obtained from The Jackson Laboratory and crossed to SHP-1$^{f/f}$ mice. CD45.1 wild type C57BL/6 mice were purchased from Charles River. Genotyping of all mice was done by PCR as described previously for the *me* allele[154], DO11.10 TCR[154], SHP-1$^{flox/flox}$ allele[178], *dLck*-Cre alelle[176], and the endogenous *Foxp3* allele and mutant (Cre) *Foxp3* allele (according to the Jackson Laboratory protocol). For all experiments using *me/me* mice, 17- to 21-day old mice were used. For all other experiments, 6- to 10-week old female and male mice were used. Control mice were either Cre- SHP-1$^{f/f}$ or Cre+ SHP-1$^{+/+}$ littermates of Cre+ SHP-1$^{f/f}$ mice. All mice were bred and maintained in accordance with the policies of the Institutional Animal Care and Use Committee at the University of Virginia. All experiments involving mice were conducted with the approval of Institutional Animal Care and Use Committee.

2.2 Isolation and Purification of Primary Cells

CD4+ T cells were isolated from peripheral lymph nodes (combined inguinal, axillary, brachial, cervical, sacral, and renal nodes) or spleens by negative selection using the CD4+ T cell isolation kit (Miltenyi Biotec, Auburn, CA) according to manufacturer's protocol. CD8+ T cells were isolated from spleen by negative selection using the CD8α+ T cell isolation kit (Miltenyi Biotec). For naïve CD4+ or CD8+ T cell experiments, $CD4+CD44^{lo}$ or $CD8+CD44^{lo}$ T cells were isolated from spleens by negative selection using the naïve CD4+ T cell isolation kit or the CD8+ T cell isolation kit, respectively (Miltenyi Biotec). For splenic T cell isolation, red blood cells were lysed using BD Pharm lyse buffer (BD Biosciences, San Jose, CA) before T cell isolation. For Treg isolation, CD4+ T cells were subsequently labeled with CD25-PE in order to separate conventional T cells (Tcon defined as $CD4^{+}CD25^{-}$) and Tregs ($CD4^{+}CD25^{+}$). Labeled CD4+ cells were run on an AutoMACS Pro separator (Miltenyi Biotec) using the posseld2 program in order to obtain Treg cells with >85% purity as assessed by Foxp3+CD25+ staining. CD4+ T cell-depleted splenocytes were irradiated (2000rad) and used as APCs in culture where indicated.

2.3 Flow cytometry

Cells were stained directly after isolation or harvested after 24, 72, or 96 hours of culture as indicated. Cells were surface stained with anti-CD4, anti-CD25 (eBiosciences, San Diego, CA), anti-CD8, anti-CD44, anti-CD62L, anti-CD69, anti-CD45.1, anti-CD45.2, anti-ICAM-1 (BD Biosciences) in PBS supplemented with 1% BSA and 0.1% sodium azide. Staining for live cells was done following surface staining and washing,

using Live/Dead Fixable Dye (Life Technologies, Carlsbad, CA). Cells were then fixed with BD Fix/Lyse (BD Biosciences) and washed. For intracellular Foxp3 staining, cells were fixed and permeabilized using the Foxp3 staining buffer set (eBiosciences) according to the manufacturer's protocol and stained with anti-Foxp3 (eBiosciences). For IFNγ and phospo-Akt intracellular staining, cells were fixed and permeabilized using BD Cytofix/Cytoperm (BD Biosciences) according to manufacturer's protocol and stained with anti-pAkt T308 (Cell Signaling Technology, Danvers, MA) or stained with anti-IFNγ (BD Pharmingen). For caspase-3 staining, cells were stained with the CaspGlow kit (eBiosciences) according to manufacturer's protocol for the last 60 minutes of *in vitro* culture. Stained cells were collected on a BD FacsCanto I or II, using FACSDiva version 8 software (BD Biosciences), or using a Beckman Coulter CytoFlex and CytExpert Software (Beckman Coulter, Brea, CA) and subsequent analyses were done using FlowJo Software version 9.9 or version 10.1 (FlowJo, LLC, Ashland, OR). Analyses were performed on singlet-gated cells as defined by FSC-W vs. FSC-A, and live cells as defined by Live/Dead dye negative. Gates were set based on FMO controls.

2.4 Proliferation and suppression assays

Assessment via CellTrace Violet and CFSE dilution. To assess proliferation, isolated T cells [CD4+CD25- (Tcon cells), CD4+CD44^{lo} (naïve CD4+ T cells), CD8+, or CD8+CD44^{lo} (naïve CD8+ T cells)] were stained with 5μM CellTrace Violet (Life Technologies) for 20min at 37°C followed by quenching with pre-warmed complete RPMI for 5min at 37°C. Where indicated, cells were stained with 10μM CFSE (carboxyfluorescein succinimidyl ester, Life Technologies) for 15 min at 37°C. Stained

cells were washed, and $2.5x10^4$ T cells were plated (in quadruplicate, pooled at time of harvest) in a total volume of 200µL RPMI 1640 complete medium (supplemented with 10% FBS, 50µM 2-ME, 2mM L-glutamine, 10mM HEPES, MEM non-essential amino acids, 1mM sodium pyruvate, and 100U/mL pen/strep) in round-bottom 96-well plates. Irradiated (2000rad), CD4+ T cell-depleted splenocytes were added at $5x10^4$ cells/well along with anti-CD3 Ab (2C11; CedarLane Laboratories, Burlington, NC) at 10-1000ng/mL as indicated. Alternatively, flat-bottom 96-well plates were coated with 10µg/mL IgG (goat anti-Armenian hamster, H+L; Jackson Laboratories) overnight followed by coating with 0, 5, 50, 100, or 1000ng/mL anti-CD3 ± 50ng/mL anti-CD28 (BD Biosciences) for 2 hours at 37°C. Plates were washed and $2.5x10^4$ T cells were plated in a total of 200uL complete RPMI for 72 hours. For suppression assays, CD4+CD25+ Treg cells were plated with responder T cells at indicated ratios. For proliferation assays, cells were cultured for 72 or 96 hours, and for suppression assays cells were cultured for 96hrs followed by flow cytometric analyses. Where indicated, cells were stimulated with 125ng/mL OVA peptide (OVA 323-339 peptide ISQAVHAAHAEINEAGR; AnaSpec, Inc., Fremont, CA.) instead of soluble anti-CD3.

Analysis of Proliferation Assay. CellTrace Violet dilution was assessed by flow cytometry, and subsequently analyzed using FlowJo v 9.9 Software Proliferation Wizard Platform (FlowJo, LLC.) Briefly, after sequentially gating on Singlets, Live cells, CD4-positive cells, and CellTrace Violet-positive cells, the percent of responding (dividing) cells relative to the input was obtained using the provided software algorithm.

Assessment via [^{3}H]thymidine incorporation. Proliferation and suppression assays were set up as described. Cells were cultured for 72 h before being pulsed with 1μCi [^{3}H]thymidine for 18 h. [^{3}H]thymidine incorporation was measured using a Tomec cell harvester and Betaplate counter (PerkinElmer, Waltham, MA).

Suppression assay with IL-4 blockade. Suppression assay was set up as described above, but cells were cultured in the presence of 10μg/mL anti-IL4 antibody (eBiosciences), or in the presence of 1μg/mL anti-IL-4R (CD124 mAb) antibody (BD Biosciences). After 4 days, cells were harvested and assessed by flow cytometry as described.

In vivo treatment with SSG. Wildtype Balb/C DO11.10 mice (2mo old) were injected i.p. with SSG (Merck Millipore, Billerica, MA) at a concentration of 10mg/mouse or sterile PBS as a control. 48hours post-injection, CD4+CD25- Tcon cells were isolated as described above, labeled with CellTrace Violet, and plated at $2.5x10^4$ cells per well in a round-bottom 96-well plate. CD4+CD25+ Treg cells were isolated from PBS-treated mice only (as described above) and cultured with Tcon cells at indicated ratios. CD4 T cell-depleted splenocytes were isolated from PBS-treated mice only, irradiated (2000rad) and cultured at a 2:1 ratio to Tcon cells. Soluble anti-CD3 was used to stimulate Tcon cells at indicated concentrations. Cells were cultured for 96 hours and subsequently harvested and stained for flow cytometric analysis.

Analysis of Suppression Assay. To compensate for the increased baseline responsiveness of SHP-$1^{-/-}$ T cells, the percentage of responding cells in the no Treg condition was set to

100% (maximum responsiveness) for each genotype. The percentage of responding cells was calculated as described above for all Treg:T cell ratios and normalized to the maximum responsiveness for their own genotype (no Treg condition). Percent suppression equals 100 minus percent responding cells.

2.5 24-hour T cell activation

CD4+CD25- Tcon cells or naïve ($CD44^{lo}$) CD8+ T cells were isolated from spleens of indicated mice and $2.5x10^4$ cells were cultured per well in a 96-well round bottom plate with $5x10^4$ irradiated (2000rad) CD4+ T cell-depleted splenocytes and indicated doses of anti-CD3 Ab (2C11, CedarLane Laboratories). After 24 hours, cells were harvested and stained for flow cytometric analysis of CD25 and pAkt T308 expression.

2.6 Immunoblotting

SHP-1 protein level in CD4+ T cells was assessed by lysing $5x10^5$ cells in NP40 lysis buffer (1% Nonidet P-40, 150mM sodium chloride, 50mM Tris, 4mM sodium pyrophosphate, 5mM sodium fluoride, 10μg/mL sodium vanadate, 50μg/mL antipain, 40μg/mL PMSF, 1X Protease Inhibitor Cocktail [Sigma Aldrich, St. Louis, MO]) and resolving lysates on an Any KD TGX Tris-glycine-SDS gel (BioRad, Hercules, CA). Blots were probed with monoclonal anti-SHP-1 (clone 1SH01, Neomarkers/ThermoFisher Scientific, Fremont, CA) and re-probed for β-actin as a loading control (anti-β actin-HRP, clone AC-15, Sigma-Aldrich). Blots were imaged using the ChemiDoc Touch gel imaging system (BioRad). Bands densities were quantified using ImageLab (BioRad) software after normalization to loading control. $2x10^6$ CD4+ T cells and non-CD4+ cells were lysed in NP40 and run on an 8% SDS-gel,

and immunoblotted using monoclonal anti-c-Cbl (clone A-9) and anti-Cbl-b (clone G-1, Santa Cruz Biotech, Dallas, TX). Blots were developed using film and band densities were quantified using ImageJ software after normalization to loading control.

2.7 Quantitative RT-PCR

Total lymph node cells or lymph node CD4+ T cells were isolated and purified as described, and total RNA was extracted using RNeasy mini-kit followed by DNase digestion (RNase Free DNase Set; Qiagen). cDNA was generated using the Superscript III First-strand Kit (Invitrogen). Quantitative RT-PCRs for c-Cbl, Cbl-b, and HPRT1 were performed using TaqMan Fast Universal PCR Master Mix and commercially available TaqMan Gene expression assays for c-Cbl, Cbl-b, and HPRT1 (Applied Biosystems). Using HPRT1 expression for normalization, relative c-Cbl and Cbl-b mRNA expression was calculated with $2^{-\Delta Ct}$ equation.

2.7 *In vivo* T cell transfer

Tcon (CD4+CD25-) cells were isolated by MACS as described above from spleens of CD45.2 SHP-$1^{+/+}$ or SHP-$1^{-/-}$ mice and CD45.1 wild type mice. Tcon cells were labeled with 5μM CellTrace Violet, and Treg (CD4+CD25+) cells were isolated from SHP-$1^{+/+}$ mice and pooled. Tcon cells were resuspended at a 1:1 ratio of either CD45.2 SHP-$1^{+/+}$: CD45.1 wild type Tcon cells or CD45.2 SHP-$1^{-/-}$: CD45.1 wild type Tcon cells, and a total of $3x10^6$ Tcon cells from either mix were injected i.v. in 200μL sterile PBS via the tail vein into *Rag1*$^{-/-}$ recipients. Additionally, half the recipient mice also received $7.5x10^5$ SHP-$1^{+/+}$ Tregs along with Tcon cells (1:4 Treg:Tcon ratio). After 10 days,

spleens were harvested from recipient mice and stained for flow cytometric analysis. Donor and recipient mice were age-matched.

2.8 IFNγ ELISA

IFNγ ELISA was performed using the Mouse IFNγ Femto HS Ready-Set-Go! Kit from eBiosciences (Affymetrix) according to manufacturer's protocol. Briefly, 96-well plates (Corning Costar 9018) were coated with purified anti-mouse IFNγ capture Ab (Affymetrix) in sodium carbonate buffer (pH 9.6). Culture supernatants were diluted in PBS containing 10% FBS and were added to the wells and incubated for 2 h at room temperature. Plates were washed five times with PBS containing 0.5% Tween 20 and incubated for 1 h at room temperature with IFNγ Detection Ab (Affymetrix). Plates were washed three times and bound Ab was detected with Avidin-HRP (Affymetrix) incubation for 30min at room temperature. Plates were developed with tetramethylbenzidine substrate and read at 450nm. All culture supernatants were assayed in duplicate, and concentrations of IFNγ were determined based on a standard curve using purified mouse IFNγ (Affymetrix).

2.9 T_H1 skewing and IFNγ Detection

CD4+CD25- Tcon cells were isolated from spleens of dLck-Cre- or dLck Cre+ SHP-$1^{f/f}$ mice as described above. Tcon cells were cultured in a 24-well plate ($1x10^6$ cells/mL) with $2x10^6$ APCs/mL (irradiated, CD4-depleted splenocytes) with 1μg/mL anti-CD3 (Cedarlane), 10ng/mL IL-12 (eBiosciences) and 10μg/mL anti-IL4 (eBiosciences). As a control, some wells had no IL-12 or anti-IL-4 so that skewing would not occur. Cells were cultured for 3 days, after which media was removed and

replenished with complete RPMI for 2 days. To confirm T_H1 skewing, cells were then washed and stimulated with 50ng/mL PMA (Sigma Aldrich) and 1μg/mL ionomycin (Sigma Aldrich) for 5h in the presence of Golgi Stop (BD Biosciences). Cells were then washed and stained for flow cytometric detection of IFNγ as described above. About 60% of cells produced IFNγ after the skewing process. For suppression of IFNγ production, cells were washed after skewing and counted, and $2.5x10^4$ T_H1 cells were cultured in a 96-well round bottom plate with Tregs at indicated ratios, 30ng/mL anti-CD3, and $5x10^4$ irradiated (2000rad) CD4-depleted splenocytes as APCs in quadruplicate wells. After 24h, Golgi Stop (BD Biosciences) was added to culture for 5h, then cells were collected and stained for flow cytometry as described above.

2.10 PI3K/Akt Inhibition Assays

Suppression assays were set up as described above, but with the addition of 0.1, 0.2, 0.5 or 1μM Akt Inhibitor VIII (Calbiochem) in the culture. Where indicated, Tcon cells were pre-treated for 60-90min with Akt Inhibitor VIII, washed, then plated with APCs, Tregs, and anti-CD3. The same set up was used but with the addition of the PI3K inhibitor, wortmannin (Sigma Aldrich) to culture. 0.25, 0.5, or 1μM of wortmannin was added to culture for the entire assay or Tcon cells were pre-treated for 60-90min then washed before plating. Another Akt inhibitor, MK-2206 (Santa Cruz Biotechnology), was added to culture or used to pre-treat cells at 0.25, 0.5, or 1μM.

2.11 Statistical analysis

Chapter 3. T cell proliferation, CD25 upregulation, proliferation index, and suppression assays using CD4+CD25- or total CD8+ T cells were analyzed using a three-way ANOVA with a 95% confidence interval. A Student's *t* test was used to analyze the comparison of percentage and absolute number of CD44+ T cells from SHP-$1^{+/+}$ or SHP-$1^{-/-}$ mice. A Student's *t* test was used to analyze naive CD4+ and naive CD8+ T cell suppression assay data for each Treg:T cell ratio. A two-way ANOVA with a Sidak's multiple comparison post-test was used to analyze cell death and apoptosis data. A one-way ANOVA with a Tukey's multiple comparison post-test was performed to analyze the absolute number of LN and splenic T cells in *dLck*-Cre SHP-$1^{f/f}$ mice, the percentage of responding cells in co-culture experiments, and the percent suppression of *in vivo* T cell transfer experiments. For pAkt T308 flow cytometric data, a one-column *t* test was applied to the fold change pAkt MFI values of SHP-$1^{-/-}$ T cells compared to the pAkt MFI of SHP-$1^{+/+}$ cells for each anti-CD3 dose, with a null hypothesis of 1 (if no change from control, fold change = 1). *p* values ≤ 0.05 were considered significant.

Chapter 4. For ^{3}H thymidine incorporation data, a one-way ANOVA with a Tukey's multiple comparison post-test was applied to each Treg:Tcon ratio. A three-way ANOVA was performed on SSG suppression assay data. For band densities of Cbl immunoblots, an independent one-group *t* test was applied to the ratio of *me/me* T cell densities normalized to wt T cell densities. For the comparison between *me/+* and *me/me* T cell band densities, a Student's *t* test was used. For qRT-PCR data, a Kruskal-Wallis analysis with a Dunn's multiple comparison post-test was applied. A Student's *t* test was performed on total cellularity, percentage CD44^{hi} cells, and absolute number of CD44^{hi} cells data.

Chapter 3

T cells deficient in the tyrosine phosphatase SHP-1 resist suppression by regulatory T cells

This chapter contains unpublished data and data adapted from the published manuscript: Mercadante E., and U. Lorenz. 2017. T cells deficient in the tyrosine phosphatase SHP-1 resist suppression by regulatory T cells. *Journal of Immunology*. (Issue/volume TBD).

We would like to thank Dr. Mark Conaway for lending us his expertise in statistical analyses, Ms. Amber Woods for help with the *in vivo* suppression experiments, and Dr. Kodi Ravichandran for critical reading of the manuscript.

3.1 INTRODUCTION

Regulatory T cells (Treg) play an essential role in shaping T cell responses and maintaining immune homeostasis[35]. Deficits in Treg function or number allow T cell responses to go unchecked, leading to the development of autoimmunity and chronic inflammatory diseases[36]. Dysregulation of the balance between activation and suppression of T cells can also occur when T cells become resistant to Treg-mediated suppression[36]. Many autoimmune diseases, including type 1 diabetes, multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus, and inflammatory bowel disease, feature not only impaired Tregs but also T cells that are resistant to suppression[50]. However, the potential mechanism(s) by which T cells might acquire resistance to Treg-mediated suppression remain unclear. While several extracellular factors have been linked to inducing resistance in T cells[50], the intracellular signaling mechanisms that can render T cells resistant to Treg suppression are poorly defined. Further, strong activation through the T cell receptor (TCR) and/or costimulatory receptors can cause T cells to become refractory to Treg suppression[93,94,177,179,180], but the specific pathways allowing this resistance remain elusive. Similarly, while resistance to suppression occurs in both CD4+ and CD8+ T cells[50], whether resistance is induced by the same mechanism in both subsets is not known.

SHP-1 is a cytoplasmic protein tyrosine phosphatase expressed in all hematopoietic cells, which has been implicated in the regulation of TCR-mediated signaling in T cells[19], including the PI3K/Akt pathway[149]. We[154] and others[155,181] have previously shown that SHP-1-deficient T cells are hyper-responsive to TCR stimulation. This was done using the *motheaten* (*me/me*) mouse model, in which all hematopoietic cells lack SHP-1 due to

a splicing mutation[142], as well as cell lines expressing dominant negative mutant forms of SHP-1[155]. However, one recent study[152], using conditional T cell deletion of SHP-1 via CD4-Cre, challenged the role of SHP-1 in regulating T cell development, while another report using the same mouse model confirmed the role of SHP-1 during T cell development[158]. Here, we generated a conditional knockout mouse model wherein SHP-1 deletion is driven by the distal Lck promoter[176], resulting in abrogation of SHP-1 expression in post-selection thymocytes. This model allows largely normal T cell development such that any phenotypic and/or functional alterations observed due to SHP-1 deficiency can be directly ascribed to its role in mature T cells[154–156]. Using this approach, we show that SHP-1 negatively regulates the activation and proliferation of CD4+ and CD8+ T cells in response to TCR stimulation, and that in the absence of SHP-1, T cells become resistant to Treg-mediated suppression. Such resistance is T cell-intrinsic, as SHP-1$^{-/-}$ T cells could not induce "bystander resistance" when co-cultured with wild type T cells. Our data also suggest a role for the PI3K/Akt pathway in allowing both CD4+ and CD8+ T cells to resist suppression. This resistance of CD4+ SHP-1$^{-/-}$ T cells to Treg-mediated suppression was also observed during homeostatic expansion *in vivo.* Collectively, these data identify a novel function of SHP-1 in regulating the susceptibility of T cells to Treg-mediated suppression *in vitro* and *in vivo*, through controlling the strength of signal received via the TCR and attenuating subsequent activation of the downstream PI3K/Akt pathway.

3.2 RESULTS

SHP-1 sets threshold for activation and proliferation of T cells in response to TCR stimulation

While several previous studies have suggested that SHP-1 is a negative regulator of signaling downstream of the TCR, based on *in vitro* cell culture studies and primary T cells from total body knockout of SHP-1 (reviewed in [19]), a recent study performed using conditional deletion of SHP-1 in T cells has disagreed with this notion[152]. One potential reason for this discrepancy might have been the type of Cre line that was used to delete SHP-1 by Johnson et al., as the CD4-Cre used gets expressed from earlier stages of T cell development. To test this possibility, we crossed mice carrying floxed alleles of *Ptpn6 (Shp1)*[178] with mice that express the Cre recombinase under the control of the distal promoter of *Lck*[176]. The distal Lck promoter drives Cre expression at late stages of T cell development, allowing TCR-dependent selection to occur under conditions of SHP-1 sufficiency[155,156,158,182]. We confirmed that SHP-1 was deleted in peripheral CD4+ (Fig. 3.1A) and CD8+ T cells (Fig. 3.1B) from the lymph nodes and spleen of *dLck*-Cre+ SHP-$1^{f/f}$ (referred to here as SHP-$1^{-/-}$) mice. Importantly, we observed no changes in the composition of the thymic or peripheral T cell compartments with respect to absolute numbers (Fig. 3.1C), or percentages of CD4+ or CD8+ T cells or Treg cells in the lymph nodes or spleens (Fig. 3.1 D and E). Expression of *dLck*-Cre alone did not affect the peripheral T cell compartment, consistent with previous reports[183].

When we assessed the role of SHP-1 during T cell activation, a greater percentage of SHP-$1^{-/-}$ CD4+CD25- T cells, referred to as conventional T (Tcon) cells, proliferated compared to Tcon cells from SHP-$1^{+/+}$ mice (Fig. 3.2A). Enhanced proliferation in SHP-

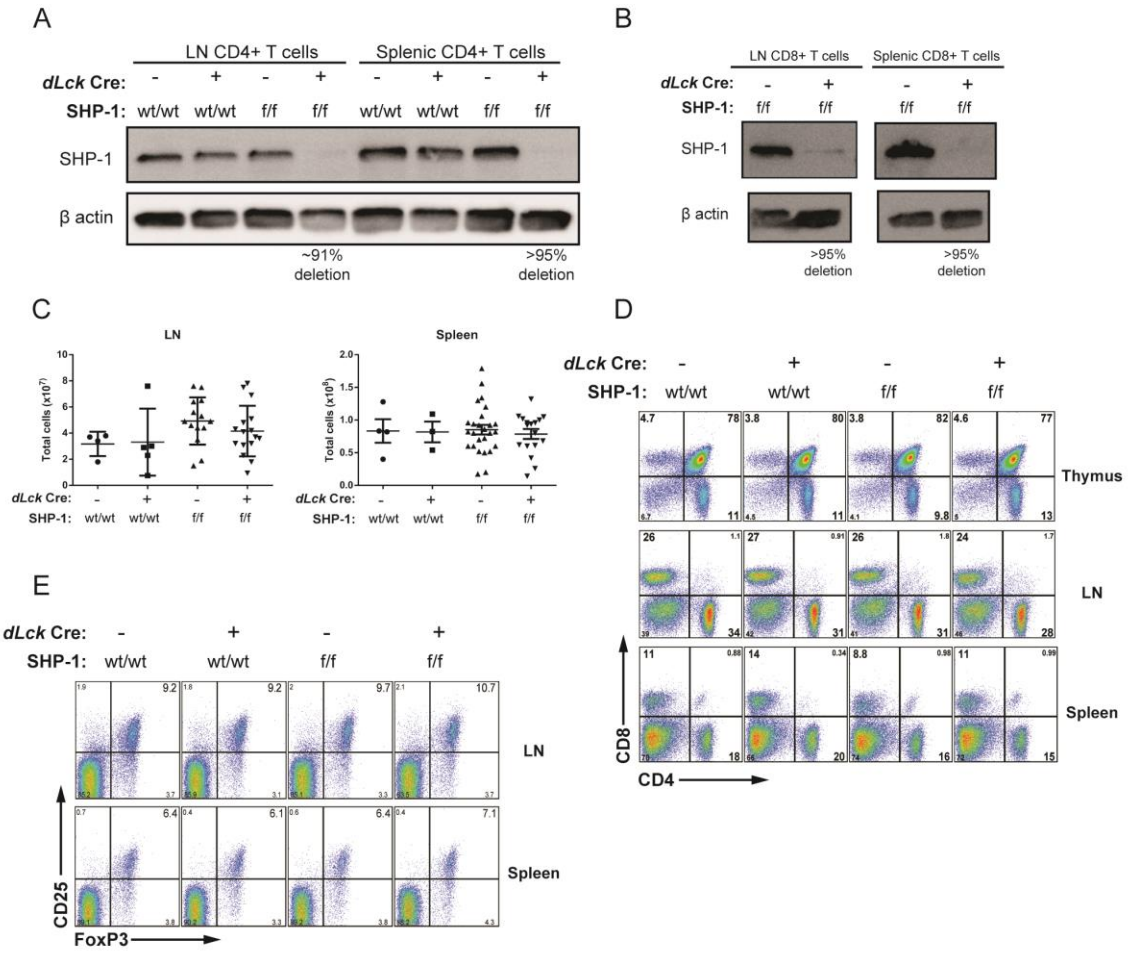

Figure 3.1. dLck-Cre SHP-$^{1f/f}$ mice display normal T cell compartment. (A) CD4+ T cells from spleens and peripheral lymph nodes of mice with indicated genotypes, were lysed in NP40, and run on an Any KD TGX gel (Bio-Rad) and immunoblotted with anti-SHP-1 and β-actin-HRP. SHP-1 levels were normalized to actin and percent SHP-1 deletion was calculated. **(B)** CD8+ T cells from spleens and peripheral lymph nodes of mice with indicated genotypes, lysed, run on an Any KD TGX gel and immunoblotted as in A. **(C)** Total numbers of lymph node cells and splenocytes isolated from mice of indicated genotypes. **(D)** Representative flow cytometric plot of CD4+ and CD8+ T cells composition in thymus, lymph nodes, and spleens of mice with indicated genotypes. **(E)**

Representative plot of percentage of Treg (CD4+CD25+Foxp3+) cells in spleens and lymph nodes of mice with indicated genotypes. Data in C were from 8 independent experiments, and a one-way ANOVA was performed.

$1^{-/-}$ Tcon cells was especially apparent at sub-optimal concentrations of anti-CD3 stimulation. (Fig. 3.2A). We considered three reasons for SHP-$1^{-/-}$ Tcon cells to display the observed increase in proliferation, which are not mutually exclusive: (1) an increase in the percentage of cells that initially become activated and go on to proliferate, (2) a decreased cell cycle time, and/or (3) an increased survival of cells in the culture. We first determined whether a greater proportion of SHP-$1^{-/-}$ Tcon cells responded to TCR stimulation by using the FlowJo Proliferation Platform algorithm, which takes into account the number of cells in each round of division relative to the input cells[184], and thereby estimates the fraction of T cells that initially responded to the stimulation. Based on this metric, we found a significant increase in the percentage of responding SHP-$1^{-/-}$ Tcon cells compared to SHP-$1^{+/+}$ Tcon cells, with the largest difference at the lowest stimulation dose (Fig. 3.2B). To complement this finding, we assessed the upregulation of CD25 (IL-2Rα) as a measure of early Tcon cell activation, and found that a significantly greater percentage of SHP-$1^{-/-}$ Tcon cells were CD25+ after 24 hours of stimulation compared to SHP-$1^{+/+}$ Tcon cells (Fig. 3.2C). Importantly, we observed no CD25 up-regulation on Tcon cells of either genotype in the absence anti-CD3 stimulation, indicating that any observed T cell activation was TCR/CD3 stimulation-dependent. Interestingly, SHP-$1^{+/+}$ Tcon cells reached a maximum percentage of CD25+ cells at 150ng/mL anti-CD3, with no further increase at 1000ng/mL anti-CD3, whereas the subpopulation of responding SHP-$1^{-/-}$ Tcon cells increased further at 1000ng/mL anti-CD3 compared to 150ng/mL. Up-regulation of CD69, another marker of activation, followed the same pattern (data not shown). These data suggest that there is a greater percentage of SHP-$1^{-/-}$ Tcon cells that respond and are activated by any given stimulation.

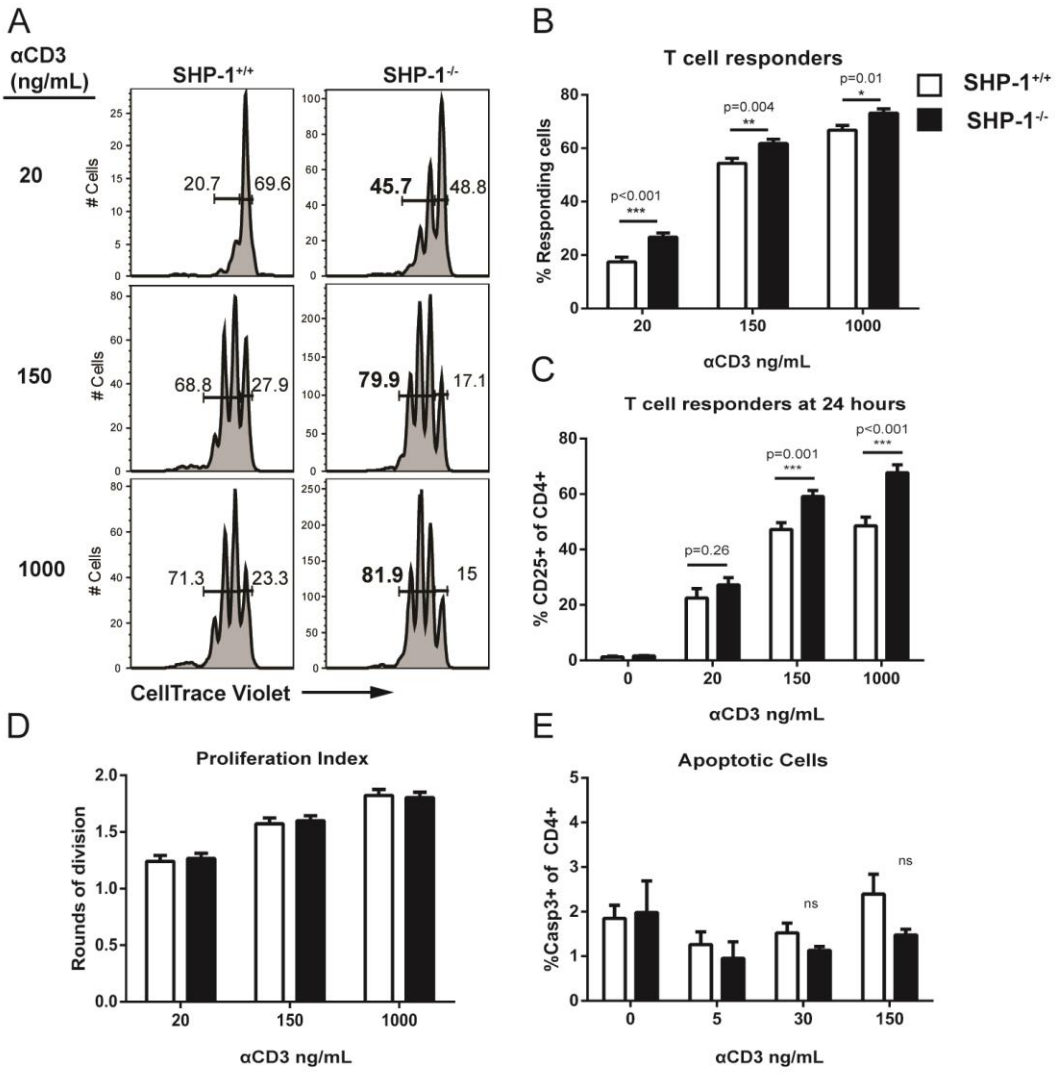

Figure 3.2. SHP-1 limits the number of T cells responding to TCR stimulation. (A) 72-hour proliferation of splenic CellTrace Violet-labeled CD4+CD25- T cells isolated from SHP-$1^{+/+}$ or SHP-$1^{-/-}$ mice. Proliferation was measured in response to indicated concentrations of anti-CD3 and irradiated CD4+ T cell-depleted splenocytes as APCs. Histograms shown are representative of 3 independent experiments, *n*=5-7 per genotype. Note that the number of cells on the y axis for histograms is greater for SHP-$1^{-/-}$ T cells than SHP-$1^{+/+}$ T cells. **(B)** Percent of CD4+ T cells within each culture initially responding to the indicated stimulation. Data were obtained from the proliferation assays

presented in A. Percent of T cell responders was calculated using the precursor frequency algorithm of the FlowJo Proliferation Platform, which takes into account the number of cells in each round of division relative to the input cells[184], and thereby estimates the fraction of T cells that initially responded to the stimulation. **(C)** Proliferation assays were set up as described in A, but cells were harvested after 24 hours and assessed for CD25 surface expression. Data represents 3 independent experiments, *n*=5-9 per genotype. **(D)** Proliferation index, which corresponds to the average rounds of division of T cells, was obtained from the proliferation assays presented in A using the FlowJo Proliferation Platform. **(E)** Proliferation assays were set up as described in A. Cells were stained for activated caspase-3 (Casp3) with Fitc-DEVD-FMK for the last hour of culture before harvest and flow cytometric analyses. Data represent percent of Casp3+ cells within CD4+ T cell population, *n*=3 per genotype. A standard regression ANOVA was performed for B-D**,** a two-way ANOVA with Sidak's multiple comparison post-test was used for statistical analysis of E. Error bars indicate ±SEM; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Second, when we calculated the proliferation index of each sample (using the FlowJo Proliferation Platform that provides the average number of cellular divisions of the cells that divided in culture), SHP-$1^{-/-}$ and SHP-$1^{+/+}$ Tcon cells underwent comparable rounds of divisions at any given dose of stimulation, with a slight increase in divisions at higher concentrations of stimulation (Fig. 3.2D). These data indicate that SHP-1 deficiency did not affect cell cycle time. To assess whether SHP-$1^{-/-}$ Tcon cells had an *in vitro* survival advantage over SHP-$1^{+/+}$ Tcon cells, we stained cells for activated caspase-3, a marker of apoptosis. After 24 hours in culture, we observed very little apoptosis among Tcon cells (<1%) with no significant difference between SHP-$1^{+/+}$ or SHP-$1^{-/-}$ Tcon cells in terms of apoptosis or cell death (Fig. 3.3A, B). By 72 hours, we still observed very low levels of apoptosis (≤ 2%) with no statistically significant differences between SHP-$1^{-/-}$ Tcon cells and SHP-$1^{+/+}$ Tcon cells (Fig. 3.2E). Taken together, these data demonstrate that SHP-1 controls the extent of TCR/CD3-driven proliferation by setting the threshold that determines the subpopulation of T cells responding to a given TCR stimulation.

CD4+ T cells lacking SHP-1 resist in vitro Treg suppression

Since our data indicated that SHP-1 lowered the threshold for Tcon cell activation and proliferation, we asked whether SHP-1 also regulated the susceptibility of Tcon cells to Treg-mediated suppression. Using an *in vitro* suppression assay, Tcon cells from SHP-$1^{-/-}$ and SHP-$1^{+/+}$ mice were assessed for their susceptibility to wild type Treg cell-mediated suppression (Fig. 3.4A). Strikingly, SHP-$1^{-/-}$ Tcon cells displayed ~3-fold

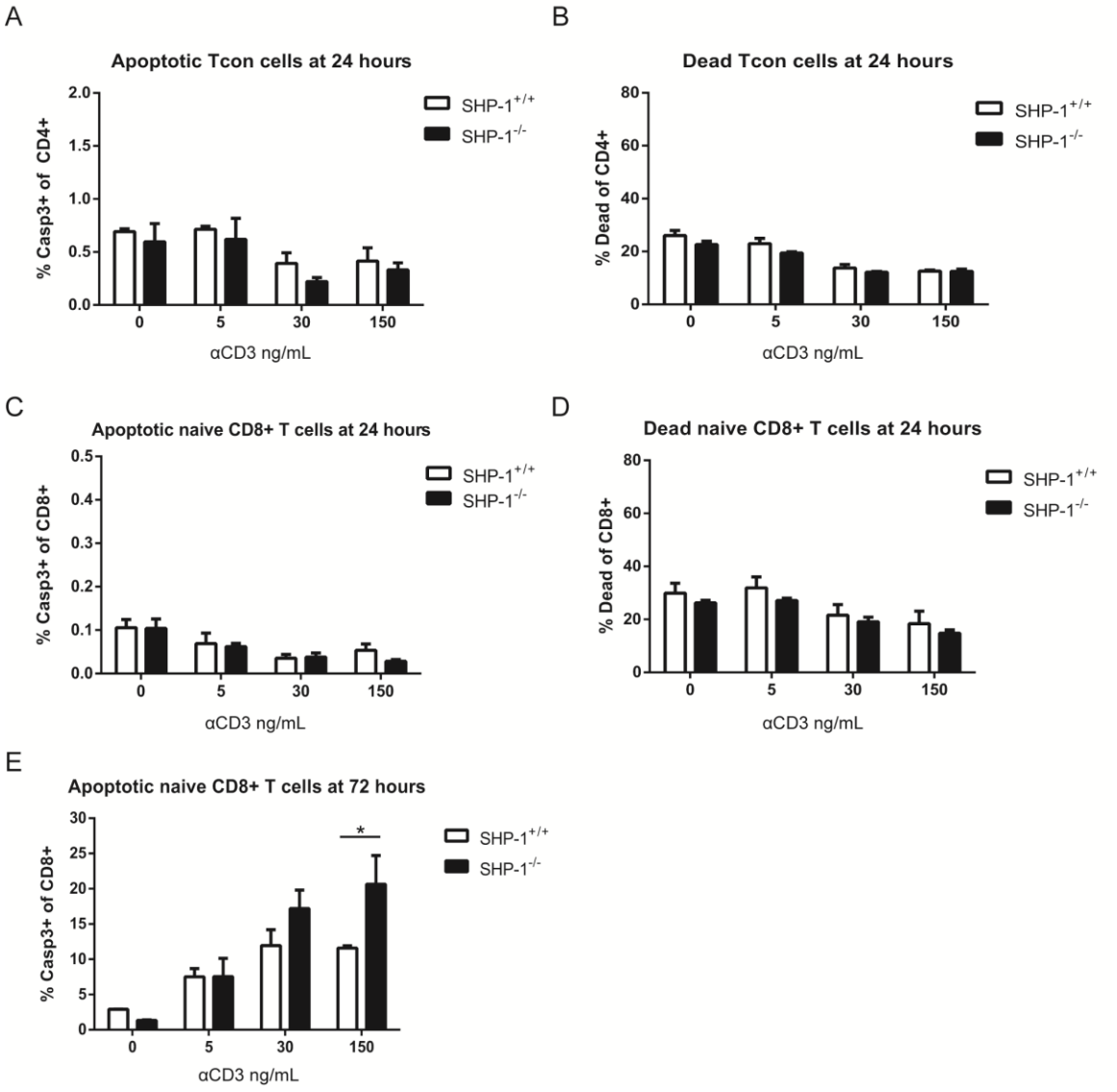

Figure 3.3. SHP-1 does not affect *in vitro* survival of T cells. CD4+CD25- Tcon cells were isolated from spleens of SHP-$1^{+/+}$ or SHP-$1^{-/-}$ mice and cultured for 24 or 72 hours in the presence of indicated concentrations of anti-CD3 and irradiated CD4+ T cell-depleted splenocytes as APCs. During the last hour of culture, cells were stained for caspase-3 with Fitc-DVED-FMK and then stained for flow cytometric analysis including staining for cell death by a live/dead dye. Data shown represent **(A)** percent Casp3+ cells

within CD4+ population or **(B)** percent dead cells within CD4+ population. **(C-E)** Naive ($CD44^{lo}$) CD8+ T cells were isolated from spleens of SHP-$1^{+/+}$ or SHP-$1^{-/-}$ mice and cultured as described above. Data shown represent (C) percent Casp3+ cells within the CD8+ population, (D) percent dead cells within the CD8+ population, or (E) percent Casp3+ cells within the CD8+ population after stimulation for 72 hours. *n*=3 mice each genotype and dose. Error bars represent ±SEM. A two-way ANOVA with Sidak's multiple comparison post-test was performed. No statistically significant differences were observed other than where indicated. *$p<0.05$.

greater responsiveness compared to SHP-$1^{+/+}$ Tcon cells, even at the maximally suppressive condition (Fig. 3.4A). Even after normalization to account for the increased baseline proliferation (no Treg condition in Fig. 3.4A), SHP-$1^{-/-}$ Tcon cells were significantly less suppressed by Tregs than SHP-$1^{+/+}$ Tcon cells (Fig. 3.4B), indicating that SHP-1 can influence the level of susceptibility to *in vitro* Treg-mediated suppression.

To determine whether the observed resistance to suppression in Tcon cells could be attributable to an expanded memory T cell population in mice with SHP-$1^{-/-}$ T cells [70,185], we first assessed whether there were any differences in the memory T cell compartment of *dLck*-Cre SHP-$1^{f/f}$ mice compared to SHP-$1^{+/+}$ mice, as has been described for *me/me* mice[171] and CD4-Cre SHP-$1^{f/f}$ mice[152,158]. However, we did not observe an increase in percentage (Fig. 3.5A, B) or absolute number (Fig. 3.5C) of Ag-experienced/memory-like $CD44^{hi}$ CD4+CD25-Foxp3- Tcon cells in the lymph nodes or spleens of *dLck*-Cre+ SHP-$1^{f/f}$ mice compared to control SHP-1-sufficient mice. As further indication that the composition of the CD4+ T cell compartment in the *dLck*-Cre+ SHP-$1^{f/f}$ mice was not altered, we detected no differences in the percentage of cells expressing activation markers CD69 or CD25 (data not shown). Furthermore, SHP-$1^{-/-}$ CD4+ T cells depleted of the $CD44^{hi}$ subpopulation (referred to here as naïve $CD44^{lo}$ T cells, Fig. 3.5D) retained a greater responsiveness to TCR stimulation (Fig. 3.5E, F), without any changes in cell cycle time. We also performed *in vitro* platebound anti-CD3 stimulation of naïve CD4+ T cells, with or without platebound CD28 costimulation, and found a similar trend, in which a great percentage of SHP-$1^{-/-}$ naïve CD4+ T cells respond to this stimulation (data not shown). This allowed a more direct comparison to results reported by Johnson et al.[152], in which naïve CD4-Cre SHP-$1^{f/f}$ CD4+ T cells did not show enhanced

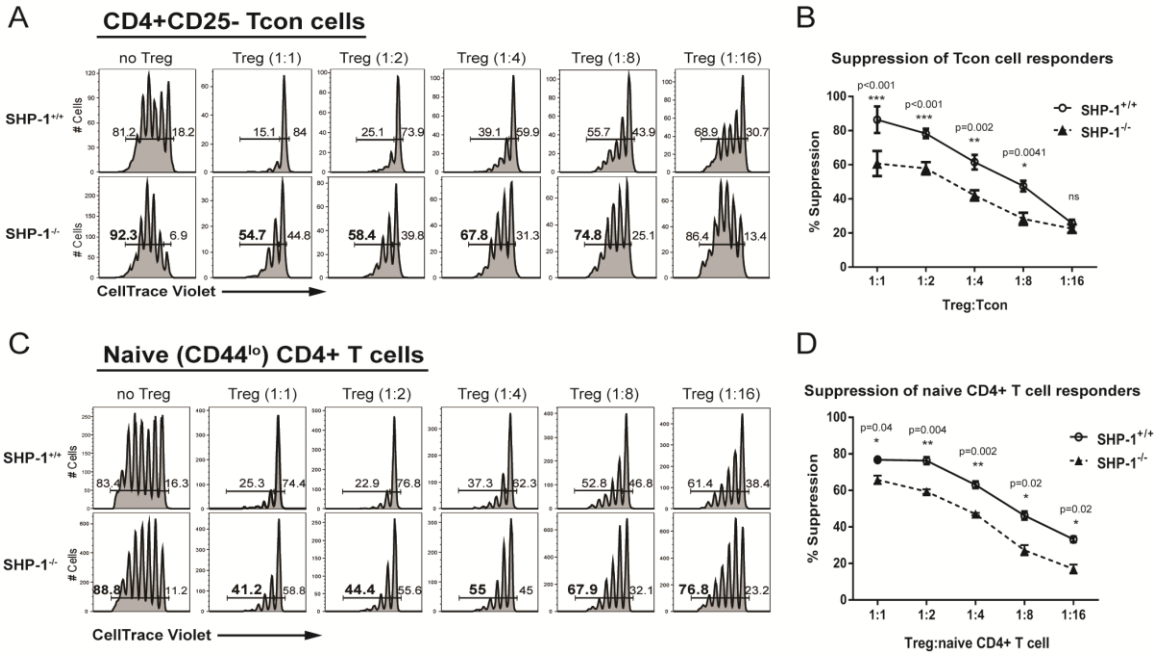

Figure 3.4. SHP-1$^{-/-}$ CD4+ T cells resist Treg-mediated suppression. (A) Splenic CD4+CD25- T (Tcon) cells were isolated from SHP-1$^{+/+}$ or SHP-1$^{-/-}$ mice, labeled with CellTrace Violet and cultured either alone or with wild type Tregs at the indicated ratios in the presence of 150ng/mL anti-CD3 and irradiated CD4+ T cell-depleted splenocytes as APCs, and proliferation was measured after 4 days. Histograms shown are representative of 5 independent experiments, *n*=8-10 mice per genotype. Bold numbers indicate most significant differences observed. **(B)** Suppression was calculated by normalizing each data point to the corresponding baseline proliferation (no Tregs, maximal response = 100% proliferation), which was then subtracted from 100 % proliferation. Note that as described in Fig. 1, proliferation was computed using FlowJo Proliferation Platform, which takes into account the number of cells in each round of division relative to the input cells[184], and thereby estimates the fraction of T cells that initially responded to the stimulation. A three-way ANOVA was performed. **(C)** Naïve

CD4+CD44^{lo} T cells were purified from spleens of SHP-1$^{+/+}$ or SHP-1$^{-/-}$ mice and suppression assays were performed as described in A in the presence of 30 ng/mL anti-CD3 and CD4+ T cell-depleted splenocytes as APCs, *n*=3 mice per genotype. **(D)** Suppression was calculated as in B. Student's *t* tests were performed for each Treg:T cell ratio. Error bars indicate ±SEM; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

responsiveness. Our data are consistent with SHP-1 regulating signaling downstream of the TCR in naïve T cell subsets. Moreover, SHP-$1^{-/-}$ naïve CD4+ T cells were resistant to Treg-mediated suppression *in vitro* (Figs. 3.4 C and D), confirming what we observed in the CD4+CD25- Tcon population. Together, these data suggest that SHP-1 regulates the susceptibility of CD4+ T cells to Treg-mediated suppression *in vitro*.

CD8+ T cells lacking SHP-1 also resist in vitro Treg suppression

Resistance of T cells to Treg-mediated suppression has not only been observed in CD4+ T cells, but also in CD8+ T cells[74,85,186,187], which has important clinical implications for cancer immunotherapy and chronic viral infection therapies. Like SHP-$1^{-/-}$ CD4+ T cells, SHP-$1^{-/-}$ CD8+ T cells exhibited greater responsiveness to TCR stimulation compared to SHP-$1^{+/+}$ CD8 T cells (Fig. 3.6A) without any detectable changes in cell cycle time (data not shown). SHP-$1^{-/-}$ CD8+ T cells also resisted Treg-mediated suppression (Fig. 3.6A, B). However, in contrast to the CD4+ T cell compartment, we did observe a substantial increase in the percentage and number of CD8+CD44^{hi} T cells in the lymph nodes and spleens of SHP-$1^{-/-}$ mice compared to SHP-$1^{+/+}$ mice (Figs. 3.7 A-C). To determine if SHP-1 deficiency also conferred naïve CD8+ T cells with resistance to Treg suppression, we isolated naïve CD8+ (CD44^{lo}) T cells and measured their suppression *in vitro* (Fig. 3.6C). A greater proportion of SHP-$1^{-/-}$ naïve CD8+ T cells responded to TCR stimulation in the absence of Treg cells (Fig. 3.6C). Similar to what we observed for CD4+ T cells, there were no significant differences in cell death or apoptosis between SHP-$1^{+/+}$ and SHP-$1^{-/-}$ naïve CD8+ T cells after 24 hours of stimulation across a range of anti-CD3 stimulation (Fig. 3.3C, D). There

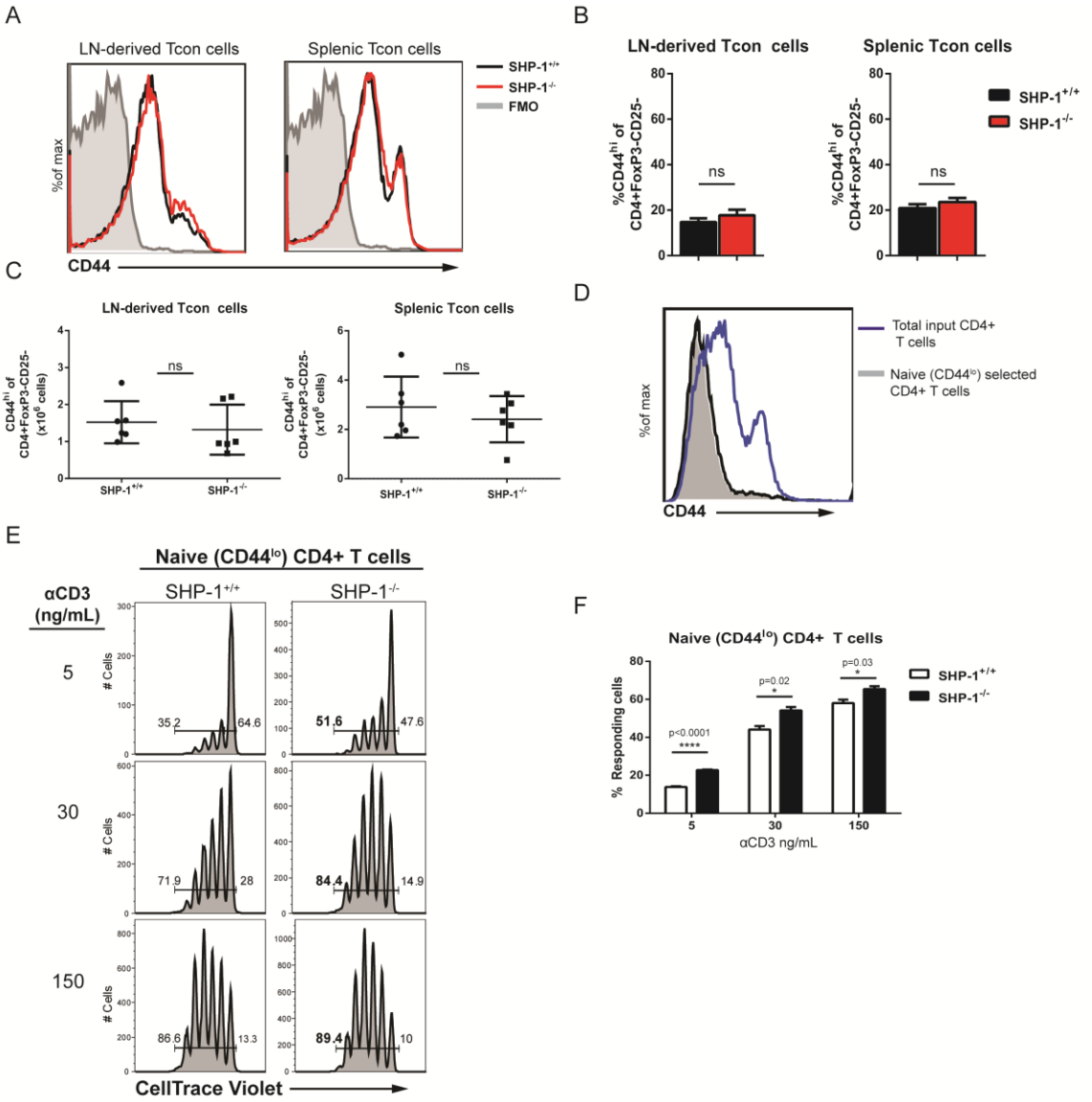

Figure 3.5. dLck-Cre SHP-1$^{f/f}$ mice show no expansion of memory CD4+ T cell compartment. (A) Representative histogram of CD44 expression on CD4+CD25-Foxp3-Tcon cells isolated from lymph nodes and spleens of SHP-1$^{+/+}$ or SHP-1$^{-/-}$ mice. **(B)** Quantification of percent CD4+CD25-FoxP3-CD44^{hi} Tcon cells. *n*=5-7 mice per genotype. **(C)** Total number of CD4+CD25-Foxp3-CD44^{hi} Tcon cells in lymph nodes and spleens of SHP-1$^{+/+}$ or SHP-1$^{-/-}$ mice. **(D)** CD44 surface expression levels on CD4+ T

cells pre- (input) and post- MACS selection of naive ($CD44^{lo}$) cells. **(E)** Naïve $CD4+CD44^{lo}$ T cells were isolated from spleens of SHP-$1^{+/+}$ or SHP-$1^{-/-}$ mice and labeled with CellTrace Violet and cultured in the presence of indicated concentrations of anti-CD3 with irradiated CD4+ T cell-depleted splenocytes as APCs. *n*=3 for each genotype. **(F)** Quantification of percent responding cells from D. A Student's *t* test was performed on data in B, C, and F; error bars indicate ±SEM.* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$

was also no observed survival advantage in SHP-$1^{-/-}$ naïve CD8+ T cells after 3 days of stimulation, and in fact at the highest dose of stimulation, SHP-$1^{-/-}$ naïve CD8+ T cells displayed enhanced apoptosis compared to SHP-$1^{+/+}$ cells, possibly due to an increase in activation-induced cell death (Fig. 3.3E). Furthermore, SHP-$1^{-/-}$ naïve CD8+ T cells exhibited resistance to suppression, similar to the total CD8+ T cell population (Fig. 3.6D), indicating that the phenotype was independent of the expanded antigen-experienced/memory-like CD8+ T cell subpopulation. These data demonstrate a role for SHP-1 in regulating susceptibility of not only CD4+ T cells, but also CD8+ T cells, to Treg-mediated suppression, likely by a similar mechanism in both T cell subsets.

SHP-1 regulates TCR signaling and susceptibility to Treg suppression in a cell-intrinsic manner

To further understand how SHP-1 regulates signaling downstream of TCR/CD3 stimulation and susceptibility to Treg suppression, we asked whether these phenotypes occurred in a cell-intrinsic and/or extrinsic manner. To investigate whether SHP-$1^{-/-}$ CD4+CD25- Tcon cells could transfer their enhanced TCR responsiveness to neighboring SHP-$1^{+/+}$ Tcon cells via a soluble mediator, we set up co-cultures (Fig. 3.8A); we labeled either SHP-$1^{+/+}$ or SHP-$1^{-/-}$ Tcon cells with CellTrace proliferation dye, mixed them at a 1:1 ratio with SHP-$1^{+/+}$ or SHP-$1^{-/-}$ Tcon cells, respectively, and assessed the proliferation of labeled cells (Fig. 3.8B). We found that adding SHP-$1^{-/-}$ Tcon cells to SHP-$1^{+/+}$ Tcon cells did not enhance the response of the SHP-$1^{+/+}$ Tcon cells (Fig. 3.8B), indicating that the enhanced responsiveness to TCR stimulation cannot be transmitted to neighboring T cells. We next asked whether SHP-$1^{-/-}$ T con cells could render their local environment

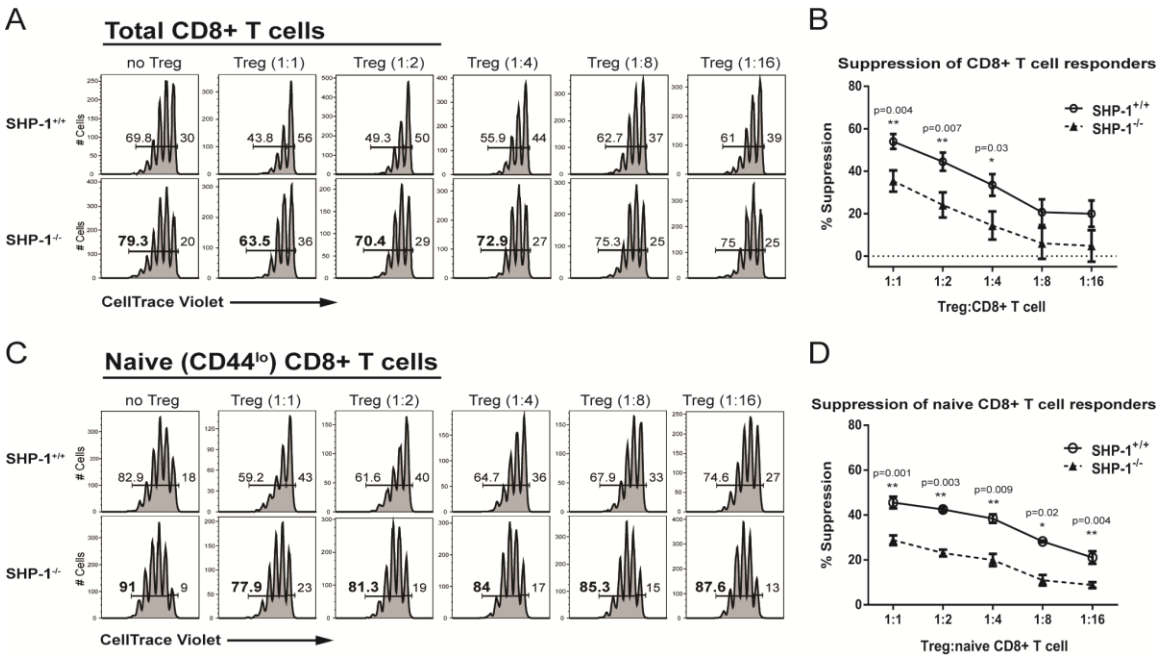

Figure 3.6. SHP-1$^{-/-}$ CD8+ T cells resist Treg-mediated suppression. (A) Splenic CD8+ T cells were isolated from SHP-1$^{+/+}$ or SHP-1$^{-/-}$ mice, labeled with CellTrace Violet and cultured either alone or with wild type Tregs at the indicated ratios in the presence of 10ng/mL anti-CD3 and CD4+ T cell-depleted splenocytes as APCs, and proliferation was measured after 3 days. Data are representative of 2 independent experiments; *n*=4 mice per genotype. **(B)** The percent responding cells was obtained using FlowJo Proliferation Platform as described in Figs. 1 and 2. Suppression was calculated by normalizing each data point to the corresponding baseline proliferation (no Tregs, maximal response = 100% proliferation), which was then subtracted from 100 % proliferation. A three-way ANOVA was performed. **(C)** Naïve CD8 T cells (CD8+CD44^{lo}) were isolated, labeled with CellTrace Violet, and cultured with Tregs as described in A. *n*=3 mice per genotype. **(D)** Percent suppression was obtained as in B**.**

Student's *t* tests were performed for each Treg:T cell ratio. Error bars indicate ±SEM. * $p \leq 0.05$, ** $p \leq 0.01$.

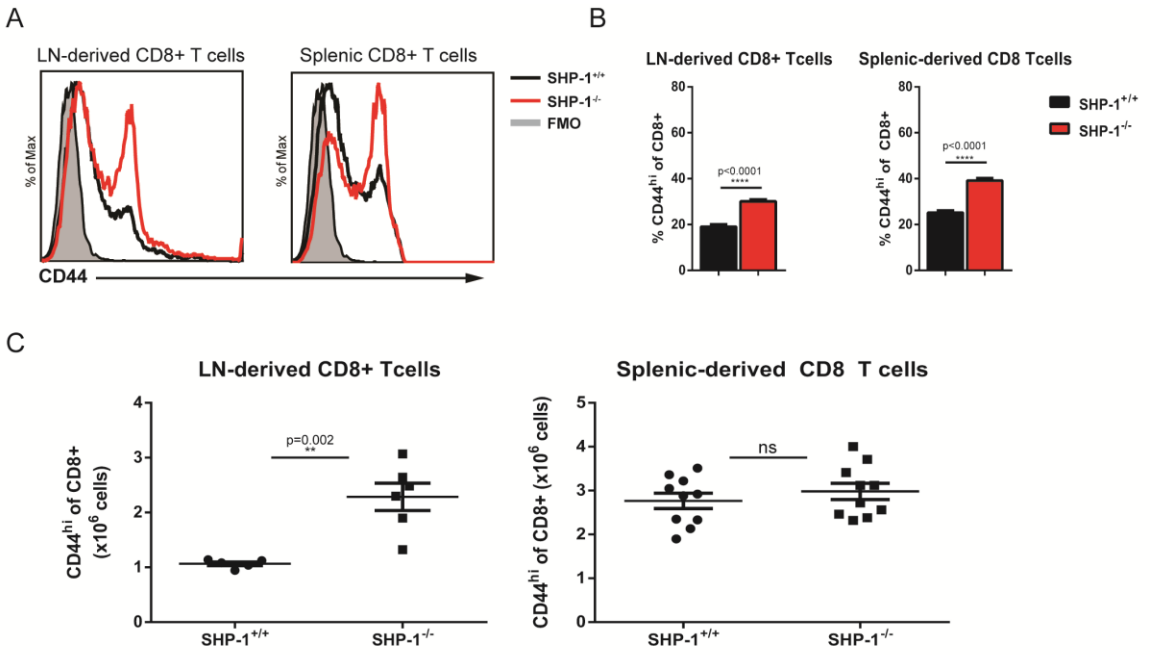

Figure 3.7. dLck-Cre SHP-1$^{f/f}$ mice show increase in CD44^{hi} CD8+ T cells. (A) Representative histogram of CD44 surface expression on CD8+ T cells in lymph nodes and spleens of SHP-1$^{+/+}$ or SHP-1$^{-/-}$ mice. **(B)** Quantification of percent CD44^{hi} CD8+ T cells. *n*=6-10 mice per genotype. **(C)** Total number of CD44^{hi} CD8+ T cells in lymph nodes and spleens of SHP-1$^{+/+}$ or SHP-1$^{-/-}$ mice. Note that these mice were 9-10 weeks old, at which time the average absolute number of splenocytes in SHP-1$^{-/-}$ mice was about 1.5fold lower than in SHP-1$^{+/+}$ mice ($p<0.0001$). A Student's t test was performed on data in (B) and (C); error bars indicate ±SEM. *$p\leq0.05$, **$p\leq0.01$, ***$p\leq0.001$, ****$p\leq0.0001$.

resistance-promoting for Treg mediated suppression, perhaps via the production of cytokines or other factors that could directly influence APCs. If this were the case, one would expect that SHP-1$^{-/-}$ Tcon cells would be capable of inducing "bystander resistance" in SHP-1$^{+/+}$ Tcon cells exposed to the same environment. Using the same experimental co-culture set-up as above, but in the presence of wild type Tregs, the addition of SHP-1$^{-/-}$ Tcon cells to SHP-1$^{+/+}$ Tcon cells did not induce any resistance to suppression in the SHP-1$^{+/+}$ Tcon cell population (Fig. 3.8C). These data suggest that SHP-1$^{-/-}$ Tcon cells resist Treg suppression by a cell-intrinsic mechanism, which does not affect neighboring cells or induce bystander resistance.

Consistent with SHP-1$^{-/-}$ T cells resisting suppression in a T cell-intrinsic manner, we also found that the cytokine IL-4 did not contribute to SHP-1$^{-/-}$ T cell resistance to suppression. Previous reports suggested that SHP-1$^{-/-}$ T cells, upon activation, become more T_H2-like and secrete a greater amount of IL-4[152,167]. Furthermore, IL-4 has been shown to be capable of inducing T cells to resist Treg-mediated suppression *in vitro*[91]. Therefore, we set up a suppression assay wherein Tcon cells were cultured in the presence of IL-4 neutralizing antibody, or in the presence of anti-IL-4R antibody (Fig. 3.9). Neither IL-4 neutralization nor IL-4R blockade affected the ability of SHP-1$^{-/-}$ CD4+ T cells to resist Treg-mediated suppression. We also investigated whether there were differences in expression of another negative regulatory of T cell signaling, Cbl-b (discussed in Chapter 4). Cbl proteins are E3 ubiquitin ligases that regulate the cellular localization and proteasomal degradation of signaling proteins, as well as enforcing the need for T cells to receive costimulation[188]. We previously found that *me/me* T cells

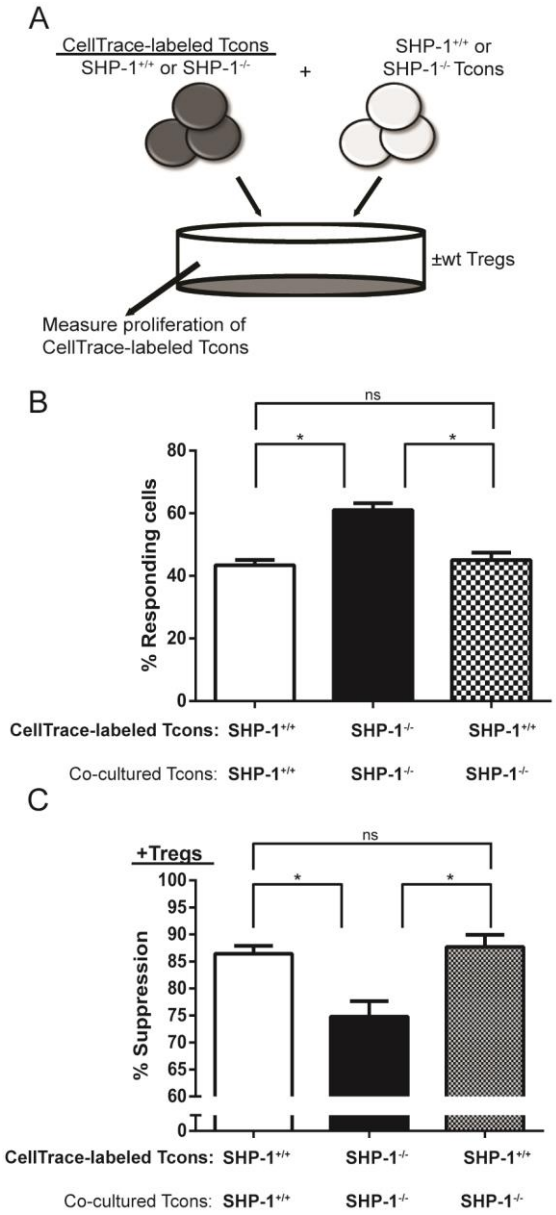

Figure 3.8. SHP-1-mediated T cell phenotypes are cell intrinsic. (A) Schematic representation of experimental setup. Splenic CD4+CD25- T cells (Tcon cells) were isolated from SHP-$1^{+/+}$ or SHP-$1^{-/-}$ mice and labeled with CellTrace dyes. Differently-labeled Tcon cells of indicated genotypes were co-cultured at 1:1 ratio in the presence of 30ng/mL anti-CD3 and irradiated CD4+ T cell-depleted splenocytes as APCs. **(B)** After

72 hours, the proliferation of the CellTrace Violet-labeled cells was measured and assessed using the FlowJo Proliferation platform. Graph shows percent responding cells of indicated genotype, compiled from two independent experiments; *n*= 6 mice per genotype. **(C)** Using the same setup as in B, with the addition of wild type Tregs at a ratio of 1:4 of Treg:total Tcon cells. After 96 hours, proliferation of CellTrace Violet-labeled cells was measured and analyzed as in B. Graph shows percent of suppression (calculated as in Figs. 2 and 3). A one-way ANOVA was performed on data in B and C. Error bars indicate ±SEM; * $p \leq 0.05$.

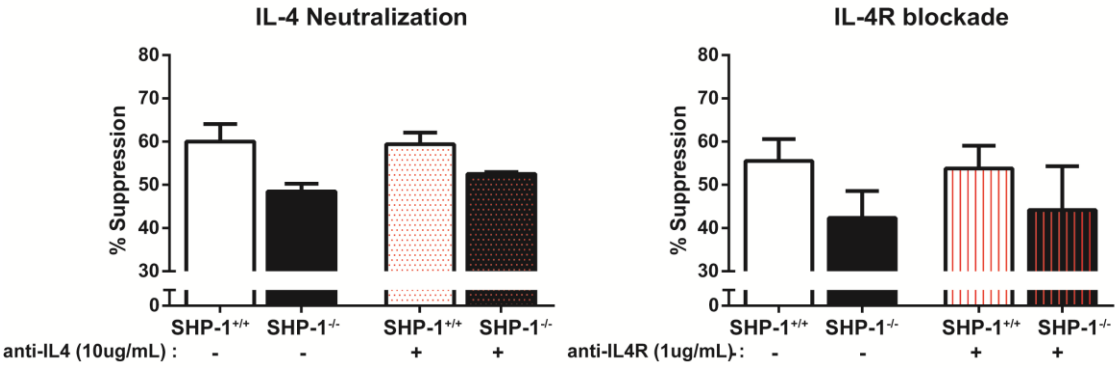

Figure 3.9. IL-4 and IL-4R blockade has no effect on SHP-1$^{-/-}$ Tcon cell resistance to Treg suppression. Tcon cells (CD4+CD25-) were isolated from spleens of SHP-1$^{+/+}$ or SHP-1$^{-/-}$ mice and Tregs (CD4+CD25+) were isolated from SHP-1$^{+/+}$ mice only. Tcon cells were labeled with CellTrace Violet and cultured with irradiated CD4-depleted splenocytes as APCs, 30ng/mL anti-CD3, and with Tregs at a 1:4 Treg:Tcon ratio. (*Left*) Cells were cultured in the absence of presence of 10μg/mL anti-IL4 antibody. (*Right*) Cells were cultured in the absence of presence of 1μg/mL anti-IL-4R (CD124) antibody. After 4 days, cells were harvested and assessed by flow cytometry. Percent responding cells was calculated by applying the FlowJo Proliferation Platform algorithm to CellTrace Violet dilution, and normalized to the condition without Tregs (maximal responsiveness). Percent suppression was calculated by subtracting percent responding cells from 100 percent. Error bars represent ± SEM.

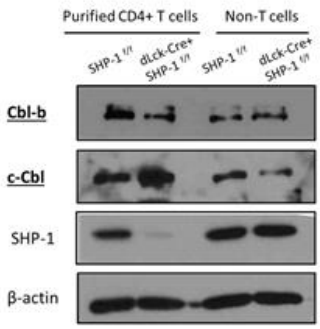

Figure 3.10. dLck-Cre SHP-1$^{f/f}$ T cells do not exhibit loss of Cbl proteins. CD4+ T cells were isolated from peripheral LN of SHP-1$^{+/+}$ or SHP-1$^{-/-}$ mice and non-T cells (remaining fraction) were lysed in NP40 lysis buffer and lysates were resolved on 8% SDS-gel and immunoblotted for c-Cbl and Cbl-b. Blots were re-probed for β-actin as loading control, and for SHP-1.

lacked expression of Cbl-b and its related isoform, c-Cbl (Chapter 4). However, we observed normal levels of Cbl protein expression in T cells from dLck-Cre+ SHP-$1^{f/f}$ mice (Fig 3.10), suggesting that Cbl is not responsible for resistance to suppression in this model.

SHP-1 deficiency enhances activation of the Akt pathway

Our data suggested that SHP-1 deficiency led to intracellular changes in the signaling pathways downstream of the TCR, which might ultimately mediate T cell resistance to suppression. A number of reports have implicated enhanced PI3K/Akt in conferring T cells with resistance to suppression[70,73,74,78,85,133,134]. Moreover, it was previously demonstrated that SHP-1 negatively regulates the PI3K/Akt pathway in *me/me* thymocytes[149,150]. We therefore assessed the phosphorylation of Akt at T308 as a measure of Akt activation[189] in response to TCR/CD3 stimulation. At 24 hours post-stimulation, SHP-$1^{-/-}$ CD4+ T cells displayed enhanced Akt phosphorylation over a range of TCR stimulation conditions compared to SHP-$1^{+/+}$ CD4+ T cells (Fig. 3.11 A and B). Additionally, there was also a slightly higher baseline activation of Akt in SHP-$1^{-/-}$ CD4+ T cells that received no TCR/CD3 stimulation. We observed the same enhanced Akt activation in SHP-$1^{-/-}$ CD8+ T cells compared to SHP-$1^{+/+}$ CD8+ T cells, both at baseline as well as following TCR/CD3 stimulation. (Fig. 3.11 C and D). Taken together, these data suggest that enhanced activation of the PI3K/Akt pathway in the SHP-$1^{-/-}$ T cells may provide one component of resistance to Treg-mediated suppression, similar to what has been described for T cells isolated from patients with lupus[78], multiple sclerosis[74], and juvenile idiopathic arthritis[70,73].

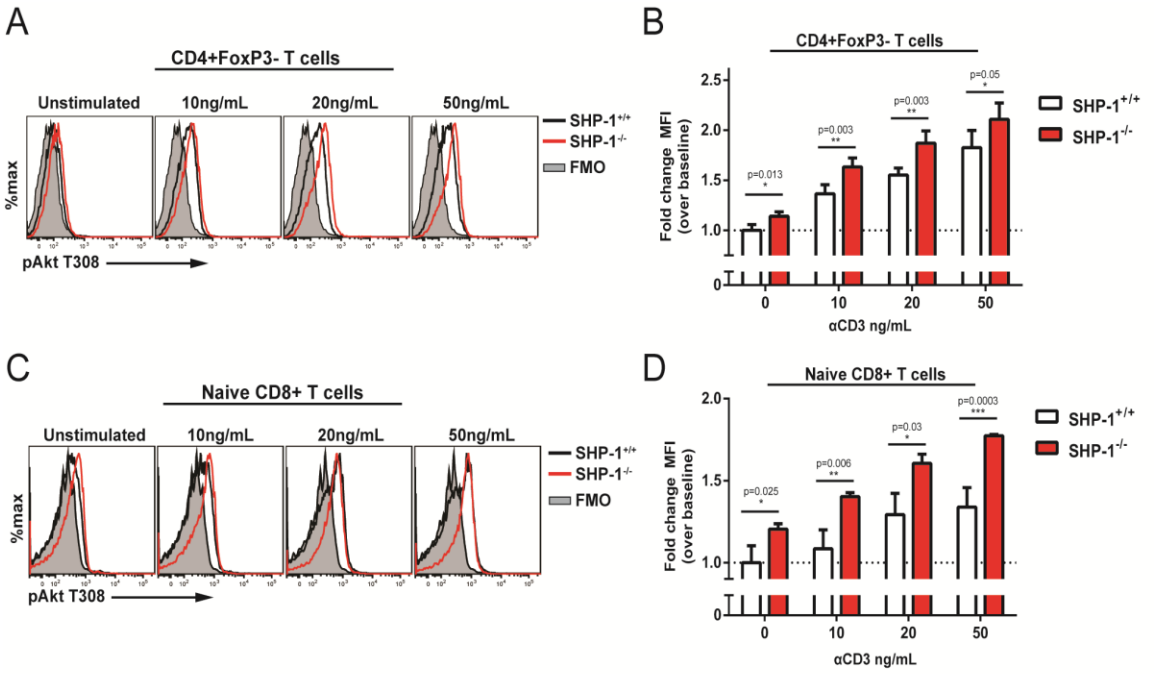

Figure 3.11. SHP-1$^{-/-}$ T cells exhibit enhanced activation of Akt. **(A)** Splenic CD4+CD25- T (Tcon) cells were isolated from SHP-1$^{+/+}$ or SHP-1$^{-/-}$ mice and cultured in the presence of indicated concentrations of anti-CD3 and irradiated CD4+ T cell-depleted splenocytes as APCs. After 24 hours, intracellular levels of phospho-Akt (T308) were assessed by flow cytometry. Histograms represent phospho-Akt (T308) levels within live CD4+Foxp3- SHP-1$^{+/+}$ or SHP-1$^{-/-}$ T cells compared to FMO control. Data represent 3 independent experiments, *n*=6-9 mice per genotype. **(B)** Bar graph represents compiled relative increase in phospho-Akt MFI compared to baseline (unstimulated SHP-1$^{+/+}$ Tcon cells). **(C)** Splenic naïve (CD44^{lo}) CD8+ T cells were isolated from SHP-1$^{+/+}$ or SHP-1$^{-/-}$ mice and cultured in the presence of indicated concentrations of anti-CD3 and irradiated CD4+ T cell-depleted splenocytes as APCs. After 24 hours, intracellular levels of phospho-Akt (T308) were assessed by flow cytometry. Histograms represent phospho-

Akt (T308) levels within live CD8+ SHP-$1^{+/+}$ or SHP-$1^{-/-}$ T cells compared to FMO control. *n*=3 mice per genotype. **(D)** Bar graph represents relative increase in phospho-Akt MFI compared to baseline (unstimulated SHP-$1^{+/+}$ CD8+ T cells)**.** A one-column *t* test with a null hypothesis of 1 was applied to fold change MFI values in B and D, obtained by comparing MFI of SHP-$1^{-/-}$ cells to the MFI of the SHP-$1^{+/+}$ cells at each dose. Error bars indicate ±SEM; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.00.1$

Tcon cells lacking SHP-1 resist Treg-mediated suppression *in vivo*

To assess whether SHP-1 regulates the susceptibility to Treg mediated suppression *in vivo*, we used a murine model of Treg-mediated control of homeostatic expansion[190]. We intravenously injected SHP-$1^{+/+}$ or SHP-$1^{-/-}$ CD4+CD25- T cells (CD45.2) at a 1:1 ratio with CD45.1 wild type CD4+CD25- T cells into *Rag1*$^{-/-}$ mice, with or without wild type Tregs. After 10 days, we assessed the expansion of CD4+ T cells (non-Treg, Foxp3-) in the spleens of *Rag1*$^{-/-}$ recipient mice (Fig. 3.12A). In the absence of Tregs, we observed no significant differences in the expansion of SHP-$1^{+/+}$ or SHP-$1^{-/-}$ CD4+ T cells, when compared to the percentages (Fig. 3.12B) and absolute numbers of co-injected wild type CD45.1 CD4+ T cells, suggesting that SHP-1 does not regulate homeostatic expansion. In the presence of Tregs, we observed a substantial reduction in absolute number of T cells recovered, indicating Treg-mediated suppression of homeostatic expansion (Fig. 3.12C). There was no difference in the extent of suppression between SHP-$1^{+/+}$ T cells and wild type CD45.1 T cells co-injected with SHP-$1^{+/+}$ or SHP-$1^{-/-}$ T cells. However, SHP-$1^{-/-}$ T cells exhibited significantly greater relative and absolute homeostatic expansion (~2.5fold) in the presence of Tregs compared to SHP-$1^{+/+}$ T cells or co-injected wild type CD45.1 T cells, indicating a resistance to Treg-mediated suppression (Fig. 3.12B and C). Taken together, these data strongly suggest that SHP-1 regulates the susceptibility of CD4+ T cells to Treg-mediated suppression *in vitro* as well as *in vivo*.

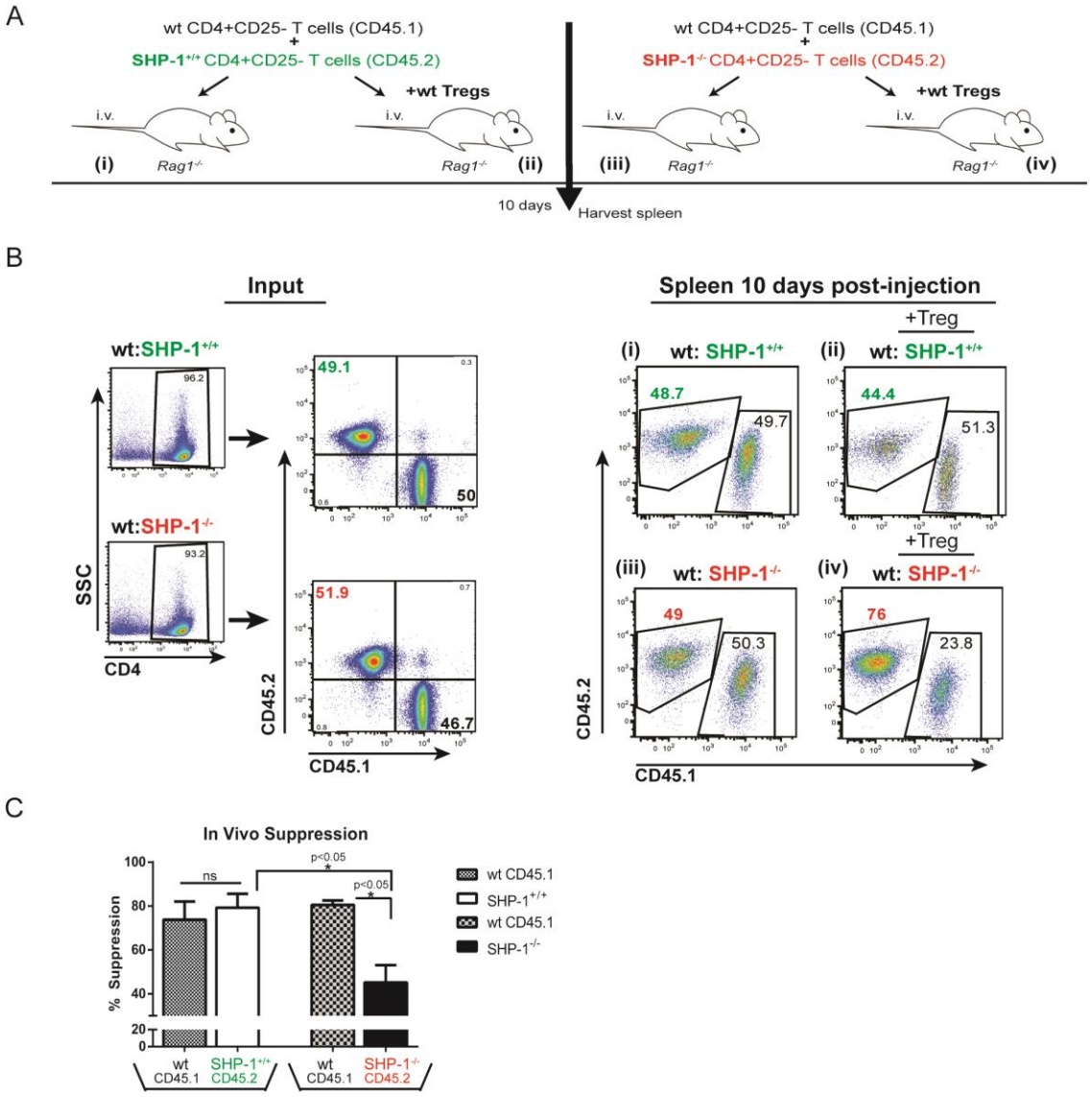

Figure 3.12. SHP-1$^{-/-}$ CD4+ T cells resist Treg suppression *in vivo*. (A) Schematic representation of experimental setup. Splenic CD4+CD25- T (Tcon) cells were isolated from wild type CD45.1 mice or SHP-1$^{+/+}$ or SHP-1$^{-/-}$ CD45.2 mice and labeled with CellTrace Violet. Wild type Tregs (CD4+CD25+) were isolated from spleens of SHP-1$^{+/+}$ mice. 3x10^{6} total Tcon cells were injected i.v. via the tail vein into *Rag1*$^{-/-}$ recipient mice, at a 1:1 ratio of either CD45.2 SHP-1$^{+/+}$:CD45.1 wild type Tcon cells or CD45.2 SHP-1$^{-/-}$

:CD45.1 wild type Tcon cells. Half the recipients received Tcon cells only, and the other half received Tcon cells along with $7.5x10^5$ SHP-$1^{+/+}$ Tregs (1:4 Treg:Tcon ratio). After 10 days, spleens of recipient mice were harvested and stained for analysis by flow cytometry. **(B)** (*Left*) Representative flow plots of CD4+CD25- input Tcon cells. (*Top)* Input for conditions i and ii: CD45.2 SHP-$1^{+/+}$ with CD45.1 wild type CD4+ Tcon cells; (*Bottom*) input for conditions iii and iv: CD45.2 SHP-$1^{-/-}$ with CD45.1 wild type CD4+ Tcon cells. (*Right*) Plots show percentages of splenic CD45.1+ and CD45.2+ CD4+Foxp3- T cells recovered 10 days post-injection; experimental conditions (with or without Tregs) as indicated. **(C)** Percent suppression was computed by subtracting the percent relative expansion for each indicated genotype from 100%. Percent relative expansion was calculated by dividing the absolute number of CD4+Foxp3- T cells recovered in the presence of co-injected Tregs over the absolute number of CD4+Foxp3- T cells recovered in the absence of Tregs (maximal expansion), multiplied by 100. *n*=3-4 recipient mice per donor condition. Error bars indicate ±SEM; * $p \leq 0.05$.

3.3 DISCUSSION

For T cells to mount a productive response against a pathogen, they must be able to transiently overcome constraints imposed by Treg cells. Environmental factors as well as strong antigenic signals through the TCR in the presence of co-stimulation have been shown to allow T cells to become refractory to Treg suppression[93,94,177,179,180]. However, the intracellular signaling pathways that result in resistance to suppression are not well-defined. Here, we identify the tyrosine phosphatase SHP-1 as one of the intracellular regulators of conventional T cells that influence their susceptibility to Treg suppression. Both SHP-$1^{-/-}$ CD4+ and CD8+ T cells resisted Treg suppression of proliferation *in vitro*, and SHP-$1^{-/-}$ CD4+ T cells resisted Treg suppression of homeostatic expansion *in vivo*. Moreover, SHP-$1^{-/-}$ T cells resisted Treg suppression in a T cell-intrinsic manner, as co-culture (Fig. 3.8) or co-injection (Fig. 3.12) of SHP-$1^{+/+}$ (wild type) and SHP-$1^{-/-}$ CD4+ T cells could not induce SHP-$1^{+/+}$ (wild type) CD4+ T cells to become resistant to suppression. SHP-$1^{-/-}$ T cells have been reported to produce increased amounts of IL-4 when stimulated *in vitro*, and SHP-1 additionally negatively regulates the subsequent downstream phosphorylation of STAT6, suggesting that SHP-$1^{-/-}$ T cells are hyper-responsive to IL-4 signaling[152]. Since IL-4 has been shown to induce resistance to Treg suppression *in vitro*[91], it raised the possibility that IL-4 might play a role in mediating the observed resistance to suppression in SHP-$1^{-/-}$ T cells. However, we found that neither IL-4 neutralizing antibodies nor antibody blockade of IL-4Rα-mediated signaling altered the resistance of SHP-$1^{-/-}$ T cells to Treg suppression (Fig. 3.9), indicating that the resistance reported here is IL-4-independent. This is consistent with a T cell-intrinsic mechanism and likely mediated by alterations in intracellular signaling events.

Previous studies demonstrated that deficiency of Cbl-b[128] and TRAF6[133], two other negative regulators of T cell activation, also resulted in T cells that resist Treg suppression. A recent study suggested that SHP-1 regulates the degradation of Cbl-b, such that SHP-1-deficient T cells have decreased levels of Cbl-b protein after TCR stimulation alone[167]. While there are striking similarities between SHP-$1^{-/-}$ and Cbl-$b^{-/-}$ T cells, our proliferation and suppression assays included costimulatory signals from irradiated APCs, which lead to Cbl-b degradation[122] in both SHP-$1^{+/+}$ and SHP-$1^{-/-}$ T cells, and therefore would not account for the observed resistance to Treg suppression. Moreover, we did not detect any differences in Cbl-b protein expression between SHP-$1^{-/-}$ T cells and SHP-$1^{+/+}$ T cells (Fig. 3.10). We did, however, observe enhanced activation of the Akt pathway in SHP-$1^{-/-}$ CD4+ T cells and naïve CD8+ T cells, both basally and upon TCR stimulation. The PI3K/Akt pathway is primarily activated downstream of the TCR and CD28 costimulatory signaling, and the resultant signaling cascade allows T cells to proliferate by increasing cell size and glucose metabolism, inactivating cell cycle inhibitors, and enhancing cellular survival[137]. An important mechanism of Treg suppression is depriving T cells of costimulatory signals via downregulation of costimulatory molecules CD80/CD86 (B7.1/B7.2) on APCs and upregulation of inhibitory molecules like CTLA-4 and LAG3[2]. CTLA-4 can outcompete CD28 for binding of B7 molecules on APCs, and LAG3 can prevent maturation of APCs to adequately engage T cells[191]. Previous work suggested that SHP-1-deficient T cells have a reduced requirement for costimulation[181]. Since SHP-$1^{-/-}$ T cells show enhanced Akt activation upon TCR stimulation, they likely resist Treg suppressive mechanisms that specifically inhibit costimulation, as their need for costimulation is reduced by the

enhancement in Akt activation (Fig. 3.13). Interestingly, many of the environmental factors shown to induce suppression-refractory T cells have been linked to enhancing activation of the PI3K/Akt pathway[50].

Our work also helps to clarify recent discrepancies reported on SHP-1 function in negative regulation of TCR signaling due to the use of CD4-Cre mediated deletion. Using the *distal Lck*-Cre line, in which SHP-1 deletion is temporally distinct from early stages of thymic selection, minimized developmental or potential repertoire changes to the T cell compartment. Importantly, *dLck*-Cre+ SHP-$1^{f/f}$ mice did not display any detectable differences in the composition of the thymic or peripheral T cell compartments compared to SHP-1-sufficient control mice, nor the expansion of CD4+ memory ($CD44^{hi}$) T cells. However, consistent with data published by others and us (reviewed in [19]), we observed increased responsiveness to TCR stimulation in SHP-$1^{-/-}$ CD4+ and CD8+ T cells (Fig. 3.14), which was directly attributable to loss of SHP-1 within the T cells rather than an expansion of antigen-experienced T cells.

Aside from gaining insight into the molecular mechanisms of Treg resistance, which has been linked to the pathophysiology of autoimmune diseases, our findings might also be applicable toward tumor immunotherapy. Tumors actively recruit and generate Tregs to maintain a suppressive microenvironment[192]. Thus, the goal of current adoptive cell transfer and/or chimeric antigen receptor (CAR)-T cell therapies is to modify or create CD8+ T cells with enhanced responsiveness toward tumor antigen[193]. Many of the signaling components being incorporated into CAR-T cells are from costimulatory molecules, which have also been found to induce resistance to Treg suppression. For example, both 4-1BB and OX40 signaling in T cells has been found to induce Treg-

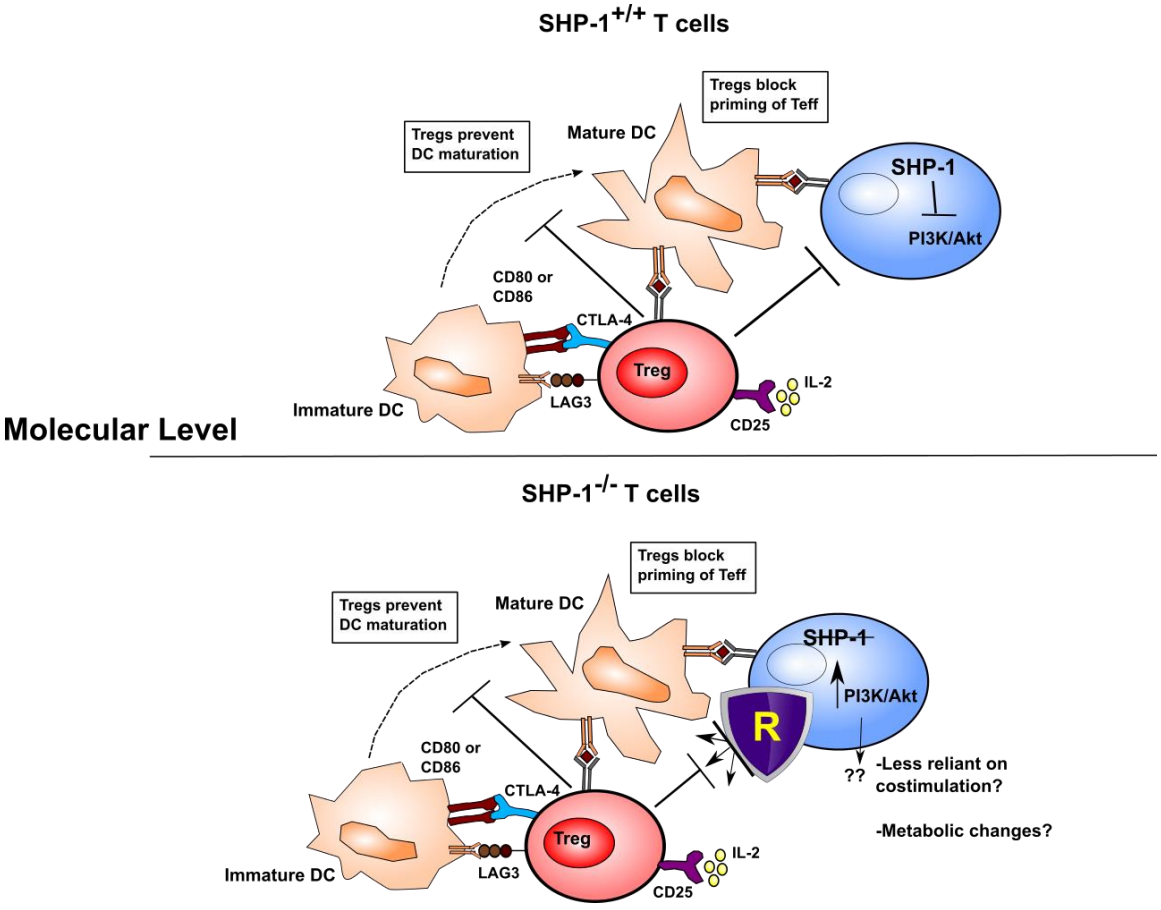

Figure 3.13. Model of how SHP-1 deficiency allows T cells to resist Treg suppression at the molecular level. (*Top*) SHP-$1^{+/+}$ T cells are suppressed by Tregs through various mechanisms, whereas (*bottom*) SHP-$1^{-/-}$ T cells (naïve CD4+ and CD8+, CD4+CD25- Tcon cells, and total CD8+ T cells) resist wildtype Treg-suppression (represented by shield with R for resistance). As a result of SHP-1 deficiency, there is enhanced activation of the PI3K/Akt pathway. How this allows a T cell to resist Treg suppression remains unknown, but may make the T cells less reliant on costimulation by an APC or may result in metabolic changes to the T cell, overcoming some Treg suppressive mechanisms.

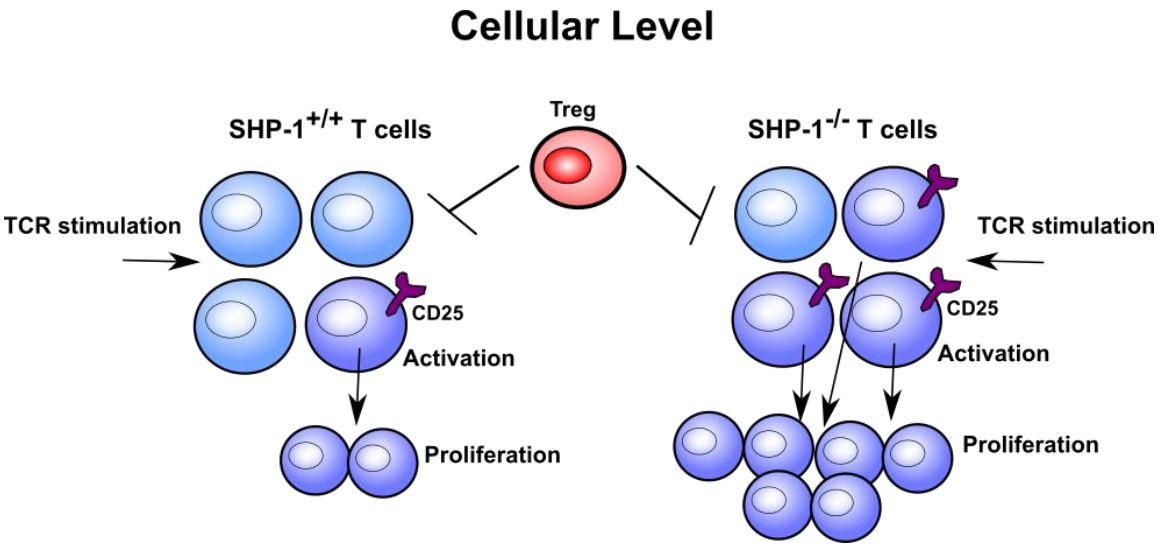

Figure 3.14. Model of how SHP-1 deficiency allows T cells to resist Treg suppression at the cellular level. This model depicts the difference in responsiveness to TCR/CD3-mediated stimulation between SHP-$1^{+/+}$ and SHP-$1^{-/-}$ T cells in the presence of Tregs. A greater proportion of SHP-$1^{-/-}$ T cells respond to TCR stimulation by upregulating CD25, becoming activated, and proliferating, despite competing suppression by Tregs.

resistance[112–117], and components of both have been used in second generation CAR-T cells[194]. Along these lines, adoptive transfer of SHP-1$^{-/-}$ or SHP-1 knockdown (via siRNA) CD8+ T cells improved tumor control in a mouse model of disseminated leukemia[170]. However, whether CD8+ T cell resistance to Treg suppression played a role in tumor control was not examined. Our findings suggest that incorporating SHP-1 ablation could be useful in current CAR-T cell or adoptive cell transfer therapies to allow CD8+ cytotoxic T lymphocytes to overcome Treg suppression and better control tumor outgrowth. Signaling through many of the costimulatory molecules being used currently in CAR-T cell trials also enhance Akt activation. Directly enforced constitutive Akt activation induced human CD8+ T cells to resist Treg suppression and led to enhanced cytotoxicity toward a neuroblastoma cell line[195]. Not only are these findings translatable to tumor immunotherapy, but also for treatment of chronic viral infections. It has been shown that chronic viral infection induces Tregs to suppress the function of CD8+ T cells, preventing viral clearance[196]. Stimulation of CD8+ T cells with the costimulatory molecule 4-1BB rendered T cells resistant to Treg suppression and able to clear a chronic viral infection in mice[114]. Therefore, our data reveal SHP-1 as a possible target to modulate the activation and function of T cells for tumor and chronic viral infection immunotherapies, and provide more evidence pointing to the critical nature of the PI3K/Akt pathway in regulating the balance between T cells and Treg cells.

CHAPTER 4

Additional models of SHP-1 deficiency in T cells: Studies in *motheaten* and CD4-Cre SHP-1$^{f/f}$ mice, and pharmacological inhibition of SHP-1 via sodium stibogluconate

This chapter contains unpublished data generated by Emily Mercadante, and Drs. Tessy Iype and Mohan Sankarshanan, where indicated.

4.1 RATIONALE

Prior to the work in Chapter 3, we utilized three other approaches to study the role of SHP-1 in T cells: *me/me* mice, CD4-Cre SHP-1$^{f/f}$ mice, and an inhibitor of SHP-1. For reasons discussed below, we ultimately chose to use the dLck-Cre SHP-1$^{f/f}$ mouse model for the majority of our studies, since it was superior to the models in this chapter. While these three approaches had various flaws, they nevertheless provided independent confirmation of results found in dLck-Cre SHP-1$^{f/f}$ mice. Furthermore, the use of a pharmacological inhibitor of SHP-1 allowed confirmation that phenotypes observed in SHP-1-deficient T cells were related to the loss of SHP-1 enzymatic function rather than the absence of SHP-1 protein expression. Thus, in this study we made a preliminary characterization of the role of SHP-1 in regulating susceptibility of T cells to Treg-mediated suppression in three independent model systems.

For many years, despite their systemic inflammation and early demise, *motheaten* (*me/me*) mice were the standard model for studying the role of the tyrosine phosphatase SHP-1 in T cell development and function[19]. Lack of SHP-1 in *me/me* thymocytes and peripheral CD4+ and CD8+ T cells results in cells that are hyper-responsive to TCR stimulation. A greater proportion of *me/me* thymocytes and/or peripheral T cells become activated and proliferate, and produce greater amounts of IL-2 than wildtype counterparts[155,157,181,182,197]. Furthermore, upon TCR stimulation, *me/me* thymocytes synthesize IL-2 without reliance upon costimulation[181]. However, *me/me* mice lack SHP-1 in all hematopoietic cell lineages, leading to an inflammatory, hyper-activating environment. Thus, it is difficult to determine if T cell phenotypes in *me/me* mice are cell-intrinsic or caused by external stimuli from other SHP-1-deficient immune cells. To

overcome the complications imposed by the total loss of SHP-1 in all hematopoietic cells in *me/me* mice, we generated a mouse model in which SHP-1 is deleted specifically in T cells under the control of CD4-Cre[175]. As a complementary approach to genetic deletion of SHP-1, we employed a pharmacological inhibitor of SHP-1, sodium stibogluconate (SSG). Using these three models of SHP-1-deficiency, we investigated a possible role for SHP-1 in regulating the susceptibility of T cells to Treg-mediated suppression. T cell susceptibility to Treg suppression depends, in part, upon the strength of activation signals it receives[93,94,179], implicating a role for SHP-1, since it regulates the strength of TCR signaling[154,157,169,197]. The balance between T cell activation and suppression by Tregs impacts the overall immune response (see Appendix A for further discussion).

4.2 RESULTS AND DISCUSSION

4.2A *me/me* Tcon cells resist Treg-mediated suppression *in vitro*

Given that a greater proportion of *me/me* T cells respond to and become activated by TCR stimulation without reliance upon costimulation, we asked whether this would affect their susceptibility to suppression by Tregs. It has been shown that strong TCR activation and costimulation causes T cells to become refractory to Treg suppression[93,94,179]. Thus, we hypothesized that *me/me* T cells might overcome suppression by wildtype Tregs. We assessed the ability of *me/me* Tcon (CD4+CD25-) cells to proliferate in the presence of wildtype Tregs by two methods: CFSE dilution and ^{3}H thymidine incorporation. wt, *me/+*, and *me/me* DO11.10 TCR Tg Tcon cells were stimulated with OVA peptide and irradiated APCs in the presence of various ratios of

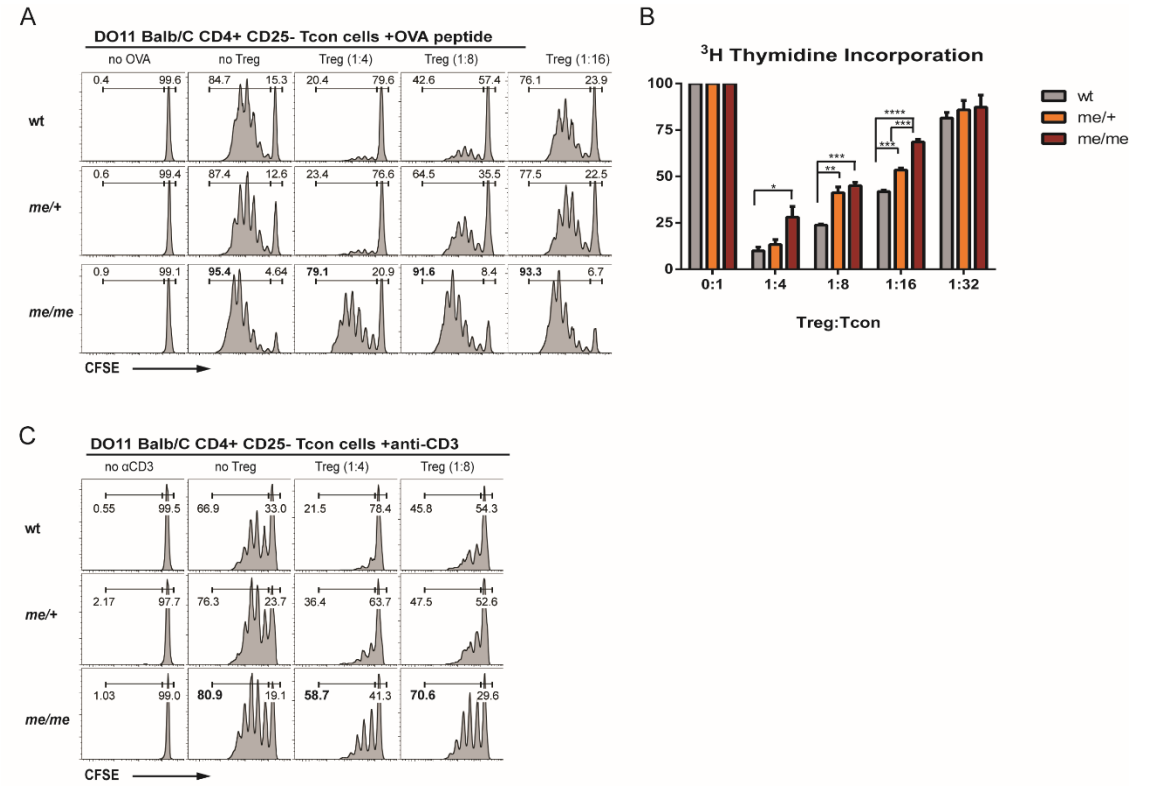

Figure 4.1. *me/me* Tcon cells resist *in vitro* Treg suppression in response to Ag-specific and non-specific stimulation. CD4+CD25- Tcon cells were isolated from spleens of wt, *me/+* or *me/me* DO11.10 TCR Tg mice and Tregs (CD4+CD25+) were isolated from spleens of wt mice only. **(A, C)** Tcon cells were stained with CFSE and cultured for 4 days with irradiated APCs, indicated ratios of Tregs, and 125ng/mL OVA peptide (A) or anti-CD3 (C). After 4 days, cells were stained for flow cytometric analysis. **(B)** Tcon cells were cultured as described with irradiated APCs, OVA peptide, and indicated ratios of Tregs for 72 hours. Cells were then pulsed with [^{3}H] thymidine for 18 hours and thymidine incorporation was measured. A, C represent *n*=1 each genotype; B represents *n*=3 each genotype, ±SEM. A one-way ANOVA with a Tukey's multiple

comparison post-test was applied to data in B, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. Data generated by Tessy Iype and Mohan Sankarshanan.

Tregs (Fig. 4.1A). As previously demonstrated, *me/me* Tcon cells hyper-proliferate in the absence of Tregs (no Treg condition), with *me/+* Tcon cells showing an intermediate hyper-responsive phenotype (Fig. 4.1A-C). *me/me* Tcon cells resisted Treg suppression at all ratios tested, as evidenced by their ability to proliferate to a greater extent than wt or *me/+* Tcon cells at the same ratio (Fig. 4.1A). These results were recapitulated using ^{3}H thymidine incorporation as a measurement of cellular division (Fig. 4.1B). Thymidine uptake was normalized for each genotype to the uptake in the no Treg condition (set to 100% uptake). Even accounting for this increased baseline division, *me/me* Tcon cells showed greater thymidine uptake at most Treg ratios tested, while *me/+* Tcon cells showed an intermediate increase in division (Fig. 4.1B). Finally, we also assessed the ability of *me/me* Tcon cells to resist suppression in response to non-specific TCR stimulation via anti-CD3. Again, *me/me* Tcon cells resisted Treg suppression and *me/+* Tcon cells showed an intermediate resistance (Fig. 4.1C). These results indicate that *me/me* peripheral Tcon cells resist wt Treg-mediated suppression *in vitro*.

4.2B SHP-1 deletion mediated by CD4-Cre induces Tcon cells to resist Treg suppression

Since *me/me* mice display systemic inflammation and autoimmune-like disease, as well as hyper-activation of myeloid cells[141–143], it is difficult to determine if T cell phenotypes are cell-intrinsic or caused by external stimuli in the inflammatory environment. Thus, we wanted to utilize a model in which SHP-1 is deleted specifically in T cells. We generated CD4-Cre SHP-$1^{f/f}$ mice, in which CD4-Cre mediates recombination at the DN4 thymocyte stage, prior to thymocyte selection (Figure 1.9)[175].

Furthermore, we crossed CD4-Cre SHP-1$^{f/f}$ mice to the DO11.10 Balb/C background, in order to directly compare these mice to DO11.10 *me/me* mice. We did not observe overt disease in CD4-Cre+ SHP-1$^{f/f}$ mice out to 7 months of age.

First, we assessed whether Tcon (CD4+CD25-) cells isolated from spleens of CD4-Cre+ SHP-1$^{f/f}$ (C57BL/6) mice exhibited hyper-responsiveness to TCR stimulation. Stimulation of Tcon cells with different doses of anti-CD3 and irradiated APCs demonstrated that a greater percentage of CD4-Cre+ SHP-1$^{f/f}$ Tcon cells proliferate after 2 and 4 days of culture, with larger differences noticeable after 2 days (Fig. 4.2A, B). Next, we assessed whether CD4-Cre+ SHP-1$^{f/f}$ Tcon cells were also resistant to Treg suppression *in vitro*. CD4-Cre- and CD4-Cre+ SHP-1$^{f/f}$ Tcon cells were cultured with anti-CD3, irradiated APCs, and varying ratios of wt Tregs in a suppression assay. CD4-Cre+ SHP-1$^{f/f}$ Tcon cells resisted Treg suppression (Fig. 4.2C, D). Even after normalizing to the increased baseline proliferation of CD4-Cre+ SHP-1$^{f/f}$ Tcon cells in the absence of Tregs, CD4-Cre+ SHP-1$^{f/f}$ Tcon cells were less suppressed (proliferated more) in the presence of Tregs (Fig. 4.2D). Therefore, the resistance to Treg-suppression observed in *me/me* Tcon cells is likely T-cell intrinsic, as deletion of SHP-1 specifically in T cells via CD4-Cre also induces resistance to suppression.

4.2C Pharmacological inhibition of SHP-1 *in vivo* induces Tcon cells to resist *in vitro* Treg suppression

To complement the aforementioned genetic models of SHP-1 deletion, we also utilized a specific pharmacological inhibitor of SHP-1, sodium stibogluconate (SSG). SSG is used clinically to treat leishmaniasis[198], and has been tested in phase I clinical

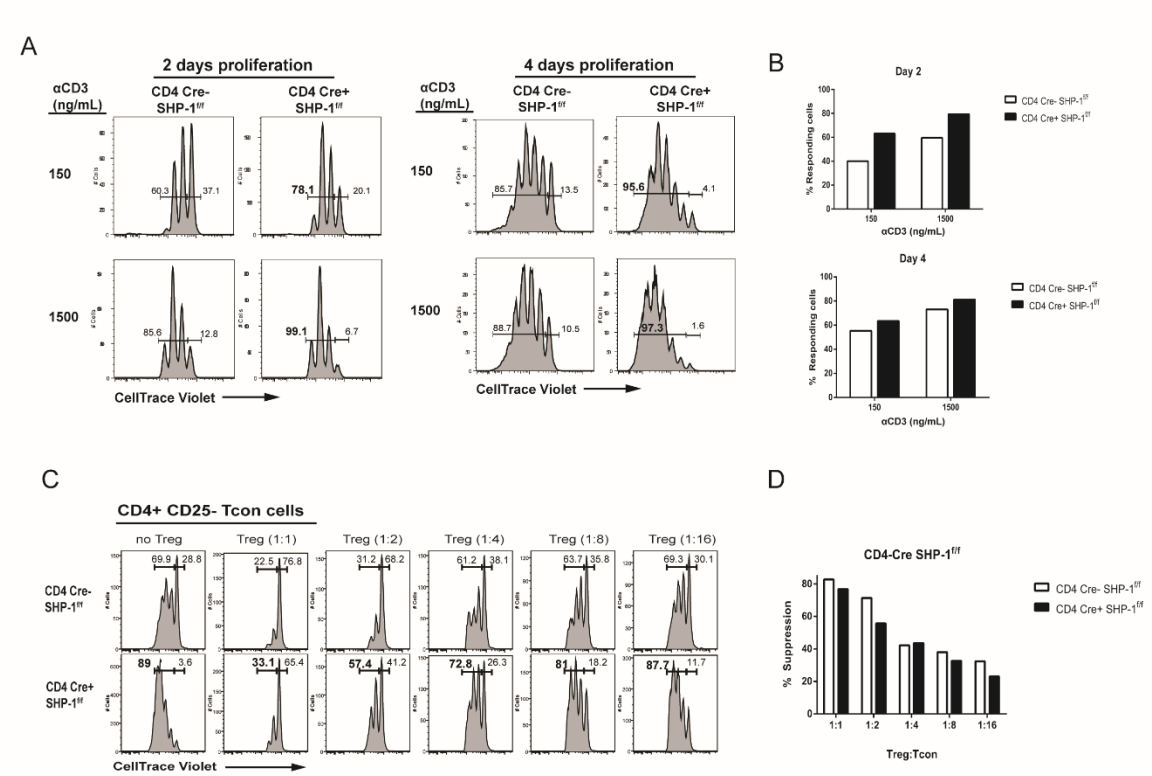

Figure 4.2. CD4-Cre+ SHP-1$^{f/f}$ Tcon cells are hyper-responsive to TCR stimulation and resist Treg-mediated suppression *in vitro*. (A) CD4+CD25- Tcon cells were isolated from spleens of CD4-Cre- or CD4-Cre+ SHP-1$^{f/f}$ mice and labeled with CellTrace Violet. Tcon cells were cultured for 2 or 4 days with 150 or 1500ng/mL anti-CD3 and irradiated APCs. **(B)** Percent responding cells was obtained using the FlowJo Proliferation Platform algorithm. *n*=1 each genotype. **(C)** Tcon cells were isolated from CD4-Cre- or CD4-Cre+ SHP-1$^{f/f}$ mice and labeled with CellTrace Violet. Tcon cells were cultured with CD4-Cre- SHP-1$^{f/f}$ Tregs, irradiated APCs, and anti-CD3 for 96 hours, and were then assessed by flow cytometric analysis. **(D)** Percent responding cells was obtained using the FlowJo Proliferation algorithm, and normalized to the no Treg

condition for each genotype. Percent suppression was calculated by subtracting percent responding cells from 100. *n*=1 each genotype.

trials for its efficacy in combination with IFNα against solid tumors[199]. At 10μg/mL, SSG has been reported to specifically inhibit 99% of SHP-1 activity, with minimal inhibition of other related phosphatases[200]. The inhibitory activity of SSG is mediated by its binding to the catalytic domain of SHP-1[200]. We took advantage of this inhibitor to determine if it could phenocopy the Treg-resistance observed in *me/me* and CD4-Cre SHP-$1^{f/f}$ Tcon cells.

Wildtype DO11.10 Balb/C mice were injected i.p. with SSG (10mg/mouse)[172,201], and Tcon cells were isolated from spleens of mice 48 hours post-injection. Tcon cells from either PBS- or SSG-treated mice were cultured 4 days in an *in vitro* suppression assay with irradiated APCs and Tregs isolated from PBS-treated mice (Fig. 4.3A), along with indicated concentrations of OVA peptide. Importantly, Tcon cells from SSG-treated mice were not hyper-responsive to stimulation in the absence of Tregs (Fig. 4.3B, C), which is likely because as cells undergo proliferation they synthesize new SHP-1 that will no longer be inhibited by SSG. Despite this, Tcon cells from SSG-treated mice appeared to resist Treg suppression (Fig. 4.3B). The same results were observed when Tcon cells were stimulated in a non-specific manner with anti-CD3, with a trend toward resistance to Treg suppression (Fig. 4.3C).

Therefore, the initial inhibition of SHP-1 *in vivo* was sufficient to render Tcon cells resistant to Treg suppression *in vitro*, despite the ability of proliferating cells to synthesize new, uninhibited SHP-1. This is consistent with the notion that resistance to suppression occurs early (within the first 16 hours) of interaction between a Treg and a Tcon cell, during activation of the Tcon cell[180] (see Appendix A for further discussion). One limitation to the use of SSG is its unreliable activity and potential off-target effects.

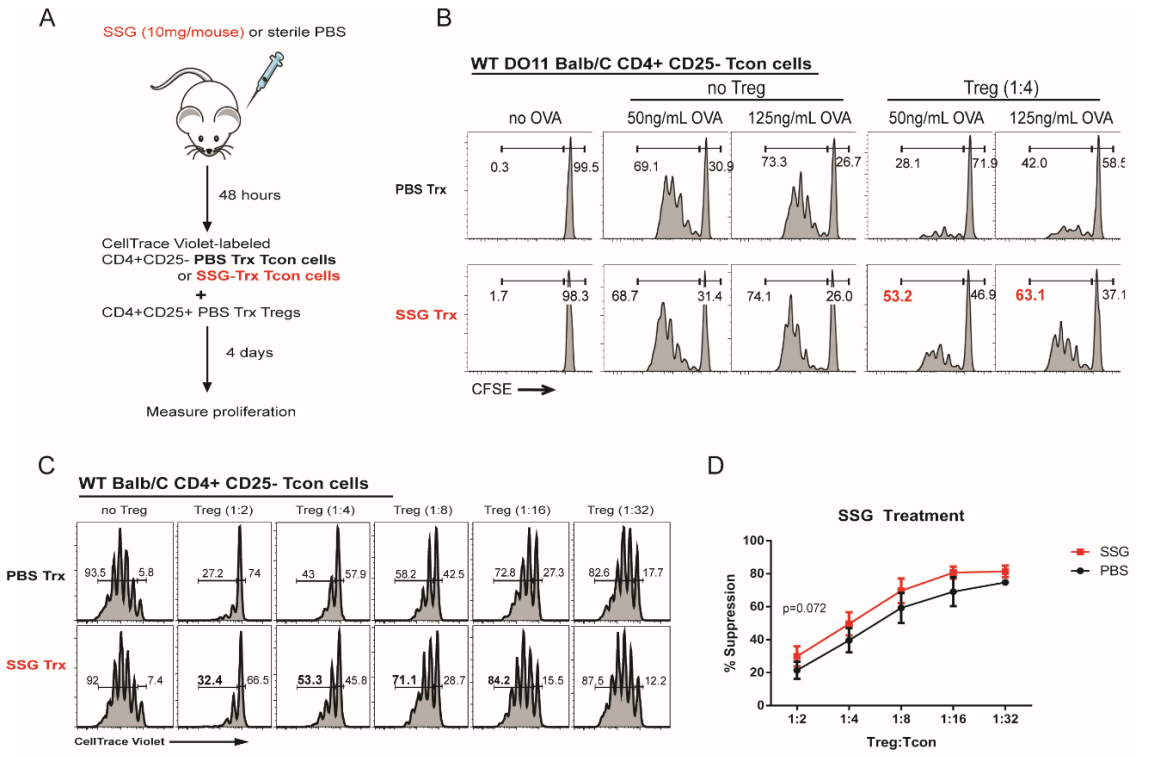

Figure 4.3. *In vivo* pharmacological inhibition of SHP-1 induces Tcon cells to resist Treg suppression *in vitro*. (A) Schematic of experimental setup. Wildtype DO11.10 Balb/C or non-Tg Balb/C mice were injected i.p. with 10mg/mouse of SSG or sterile PBS as a control. 48 hours-post-injection, Tcon cells were isolated and labeled with CellTrace Violet or CFSE, and Treg cells and APCs were isolated from PBS-treated mice only. Tcon cells were cultured with Tregs and irradiated APCs, along with indicated concentrations of OVA peptide (**B)** or soluble anti-CD3 (**C)** for 4 days, then assessed by flow cytometry. **(D)** Percent responding cells in (C) was obtained from FlowJo Proliferation Platform algorithm and normalized to the no Treg condition. Percent suppression was calculated by subtracting percent responding cells from 100. *n*=4-5 each genotype. A three-way ANOVA was performed on data in (D). *n*=1 each genotype in (B); error bars indicate ±SEM. (B) generated by Tessy Iype and Mohan Sankarshanan.

To control for off-target effects, we treated *me/me* mice (which lack SHP-1) with SSG, and observed no further effect on Tcon cells (data not shown). The activity of SSG is not tested by the manufacturer, and it cannot be easily dissolved in solution, posing technical reproducibility problems. Use of clinical grade SSG (known as Pentostam) is restricted in the U.S. Treatment of mice with SSG also inhibits SHP-1 in other immune cell types, which could impact the Treg-resistant phenotype, making it difficult to determine if the observed result was truly T cell-intrinsic. Therefore, while the use of SSG has severe limitations, the data here suggest that inhibition of SHP-1 activity is sufficient to confer Tcon cells with Treg resistance.

4.2D Resistance to suppression in SHP-1-deficient Tcon cells is not mediated by loss of Cbl-b protein

Cbl-b is an E3 ubiquitin ligase which acts as a negative regulator of T cell signaling by enforcing the need for costimulatory signals[122–124]. Like *me/me* mice, Cbl-b KO mice develop systemic autoimmunity due to hyper-activation of lymphocytes, especially because T cells are fully activated in the absence of costimulation[127]. Cbl-b KO T cells were shown to be resistant to Treg suppression *in vitro* and *in vivo*[128–131], leading us to investigate if SHP-1 might regulate Cbl-b, and its related and partially redundant isoform, c-Cbl[202]. We isolated total T cells from peripheral LN of wt, *me/+*, and *me/me* (C57BL/6) mice and immunoblotted for c-Cbl and Cbl-b (Fig. 4.4A, B). *me/me* T cells exhibited a complete loss of both c-Cbl and Cbl-b protein, whereas *me/+* T cells had similar levels of Cbl protein compared to wt T cells. Thymocytes from *me/me* mice exhibited a slight reduction in Cbl protein levels, but not to a significant extent (data not shown). To

determine if loss of Cbl proteins was occurring at the post-transcriptional level, we analyzed mRNA transcript for c-Cbl and Cbl-b and found no significant differences in mRNA levels between genotypes (Fig. 4.4C). These data suggested that loss of Cbl proteins occurred via post-transcriptional modifications, consistent with possible regulation by SHP-1. However, when we immunoblotted for c-Cbl (Fig. 4.4D) and Cbl-b (data not shown) protein levels in T cells from CD4-Cre+ SHP-$1^{f/f}$ mice, we observed no difference in protein expression compared to wildtype BL6 or CD4-Cre- SHP-$1^{f/f+}$ T cells (Fig. 4.4D). Therefore, while *me/me* T cells lack Cbl-b and c-Cbl, loss of Cbl proteins seems to occur in a cell-extrinsic manner, since CD4-Cre+ SHP-$1^{f/f}$ T cells have normal levels of Cbl protein. We further confirmed that this result was not due to altered cellular localization of Cbl proteins by use of two different lysis buffers (RIPA vs. NP40, data not shown). Furthermore, these data indicate that resistance to Treg suppression in SHP-1-deficient Tcon cells occurs independent of alterations in Cbl-b protein expression.

Interestingly, a recent paper suggested that SHP-1 is recruited to Cbl-b and dephosphorylates tyrosine residues in Cbl-b, inhibiting its ubiquitin ligase activity, and potentially preventing its ubiquitination and subsequent degradation[167]. This study used naïve CD4+ T cells from CD4-Cre SHP-$1^{f/f}$ mice and showed that over the course of 30 minutes of TCR stimulation with anti-CD3, Cbl-b expression was reduced in SHP-1-deficient T cells[167]. There also seemed to be a slight reduction of Cbl-b expression in CD4-Cre+ SHP-$1^{f/f}$ CD4+ T cells in the absence of TCR stimulation (steady state), which is not what we observed in total T cells. It is possible that we would observe a similar reduction of Cbl-b expression in T cells from CD4-Cre+ SHP-$1^{f/f}$ mice in the presence of TCR stimulation. However, since costimulation through CD28 has been shown to lead to

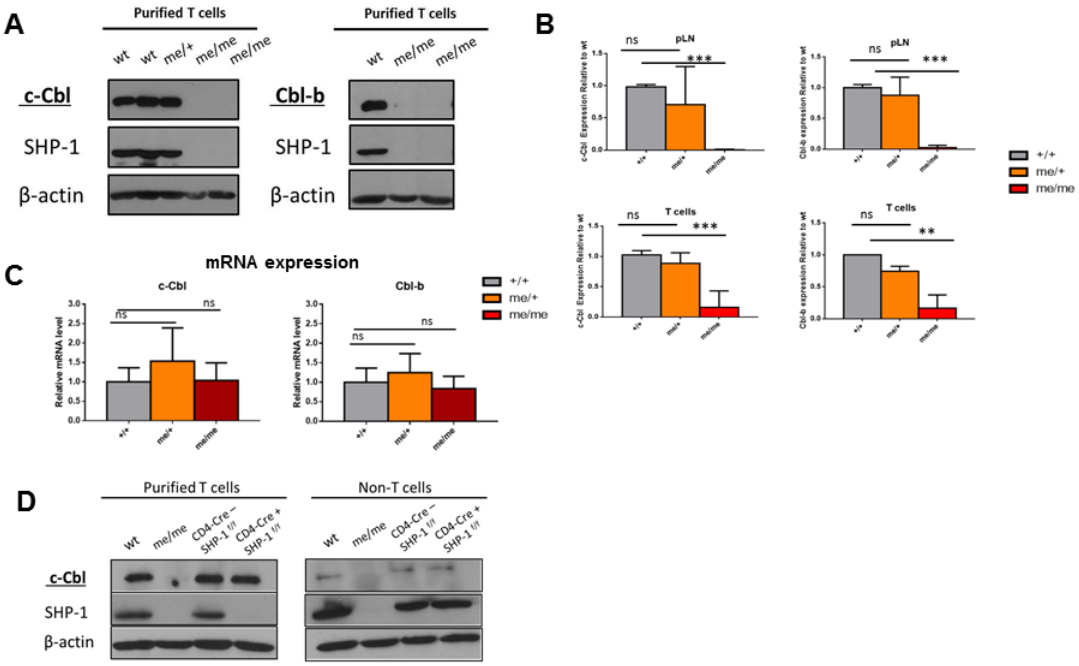

Figure 4.4 Loss of Cbl-b and c-Cbl protein in *me/me* T cells is SHP-1-independent and does not occur in CD4-Cre+ SHP-1$^{f/f}$ T cells. (A) Total T cells were purified from the LN of wt, *me/+* and *me/me* mice. Cells were lysed in NP40 lysis buffer and lysates were resolved on 8% SDS-gel and immunoblotted for c-Cbl and Cbl-b. Blots were re-probed for β-actin as loading control, and for SHP-1. **(B)** Band densities were determined using ImageJ software, and normalized to β-actin. *n*=3-6 each genotype. **(C)** Total T cells were isolated from wt, *me/+* and *me/me* mice. Total RNA was extracted and cDNA was synthesized. qRT-PCR was performed using murine *c-Cbl* primers (Mm00519552_m1) and *Cbl-b* primers (Mm01343092_m1). The mRNA levels were normalized to *Hprt*. The *c-cbl* and *cbl-b* mRNA level of wt T cells is set as 1.0. *n=3-5* each genotype. **(D)** Total T cells and non-T cells (unlabeled fraction) were isolated from peripheral LN of wt, *me/me* (BL6), CD4-Cre- and CD4-Cre+ SHP-1$^{f/f}$ mice and immunoblotted as described. *n*=2

each genotype. An independent one-group t test was run on *me/me* T cells normalized to wt in (B). A Student's *t* test was used to compare normalized *me/+* to *me/me* T cells (B). A Kruskal-Wallis analysis with a Dunn's multiple comparison post-test was applied to (C). ***$p<0.0001$, **$p=0.004$**.** Error bars indicate ±SEM.

the ubiquitination and degradation of Cbl-b[126], and our suppression assay setup includes irradiated APCs to provide co-stimulation, Cbl-b degradation/loss is unlikely to account for resistance to Treg suppression in SHP-1-deficient T cells. Overexpression of Cbl-b in CD4-Cre+ SHP-$1^{f/f}$ T cells apparently reduced hyper-proliferation in response to TCR/CD28 stimulation[167], but since Cbl-b is itself a negative regulator of costimulation and enforces T cell anergy[122], it is not possible to determine from this setup whether Cbl-b overexpression reverses the effects of SHP-1 deficiency or simply adds a further layer of regulation. The same is true for experiments suggesting that Cbl-b overexpression reverses the previously reported tendency of CD4-Cre+ SHP-$1^{f/f}$ T cells to skew to T_H2 cell *in vitro*[152]. Since Cbl-b KO T cells also have a greater disposition to skew to T_H2[203], it is not possible to say whether overexpression of Cbl-b has mitigated the effects of SHP-1 deficiency. Rather, a more thorough analysis would need to be performed including overexpression of Cbl-b in wildtype T cells (CD4-Cre-) to determine the extent to which Cbl-b, by itself, reduces proliferative responses and T_H2 skewing.

Both *me/me* and Cbl-b KO T cells do not require costimulation for full activation; it is possible that the lack of Cbl-b expression in *me/me* T cells at steady state accounts for their reduced need for costimulation. It remains to be determined whether CD4-Cre+ SHP-$1^{f/f}$ T cells have reduced need for costimulation comparable to *me/me* T cells. Further investigation is needed to clarify why *me/me* T cells but not CD4-Cre+ SHP-$1^{f/f}$ T cells lack c-Cbl and Cbl-b expression at steady state. It is possible that secondary effects of the *me/me* phenotype, such as the exposure of *me/me* T cells to a hyper-activating environment, affects Cbl-b expression. For example, APCs deficient in SHP-1 might express higher levels of costimulatory molecules, leading to greater T cell activation, and

explaining the expansion of the CD44^{hi} memory T cell compartment. The Cbl immunoblots were performed on total T cells, and there may be a greater percentage of memory-like T cells in *me/me* mice compared to CD4-Cre+ SHP-1$^{f/f}$ mice, and these cells might preferentially lack Cbl. Analysis of Cbl-b expression specifically in naïve (CD44^{lo}) *me/me* T cells would be necessary to demonstrate whether non-Ag experienced cells, which presumably have not received costimulation in the periphery, also show a loss of Cbl-b. However, skewed representation of T cell subsets would be unlikely to account for the complete loss of Cbl protein as observed, but rather a reduction in Cbl protein.

4.2E CD4-Cre+ SHP-1$^{f/f}$ mice have T cell compartment abnormalities

It has been well-described that *me/me* mice have lymphadenopathy and splenomegaly[141,142], as well as premature involution of the thymus[204]. Additionally, thymocyte selection is altered in *me/me* mice, with increased negative and positive selection[154,155,157]. Therefore, we wanted to assess whether there were changes in the overall cellularity of the peripheral LN and spleen in CD4-Cre+ SHP-1$^{f/f}$ mice or any alterations in the T cell compartment, since SHP-1 deletion occurs before positive and negative selection. While we did not observe any overall changes in total cellularity of the LN and spleen, there was a trend for slightly lower cellularity in the spleens of CD4-Cre+ SHP-1$^{f/f}$ mice (Fig. 4.5A). Importantly, we observed a decreased percentage of CD4+ T cells in the LN, spleen, and thymus of both C57BL/6 and DO11.10 Balb/C CD4-Cre+ SHP-1$^{f/f}$ mice compared to CD4-Cre- SHP-1$^{f/f}$ mice (Fig. 4.5B). Therefore, the ratio of CD4:CD8 T cells was shifted in SHP-1-deficient mice, with an intermediate phenotype in CD4-Cre+ SHP-1$^{f/+}$ mice. The difference in CD4:CD8 ratio was likely mitigated in

DO11.10 mice, which selects for CD4+ T cells. We observed a similar trend in *me/me* spleen and LN, with a decreased percentage of CD4+ T cells compared to wt or *me/+* mice (Fig. 4.5C). The reduction of CD4+ T cells in the periphery in CD4-Cre+ SHP-$1^{f/f}$ mice suggests that thymocyte selection is altered as has been observed in *me/me* mice, and that these changes in thymocyte development affect the composition of the peripheral T cell compartment.

We also observed an accumulation of Tregs in the periphery of older CD4-Cre+ SHP-$1^{f/f}$ mice, which could be an effect of altered thymic selection. Selection of thymic Tregs depends on relatively high-affinity signals from self-peptide/MHC complexes[6]. As such, *me/me* mice have been shown to have an increased percentage of Tregs within the CD4+ T population in the thymus[182]. Likewise, *me/me* mice (DO11.10) also showed increased percentages of Tregs (about 2-fold more) in the spleen and LN (Fig. 4.5E). CD4-Cre+ SHP-$1^{f/f}$ mice at 8 weeks of age did not show a difference in percentage of Tregs in the LN, however at 7 months of age, both CD4-Cre+ SHP-$1^{f/+}$ and SHP-$1^{f/f}$ mice had increased percentages of Tregs in the spleen and LN (Fig. 4.5D), suggesting perhaps an accumulation of Tregs over time. We also assessed the overall activation status of T cells *ex vivo*. There were no differences in expression of CD25 on CD4+Foxp3- cells from *me/me* or CD4-Cre+ SHP-$1^{f/f}$ mice (data not shown), suggesting that T cells are not activated upon isolation but rather hyper-sensitive to subsequent stimulation. Importantly, however, we observed a substantial increase in the percentage of $CD44^{hi}$ Tcon (CD4+FoxP3-) cells in spleens and LN of CD4-Cre+ SHP-$1^{f/f}$ mice, and even more in *me/me* mice (Fig. 4.5F, G).

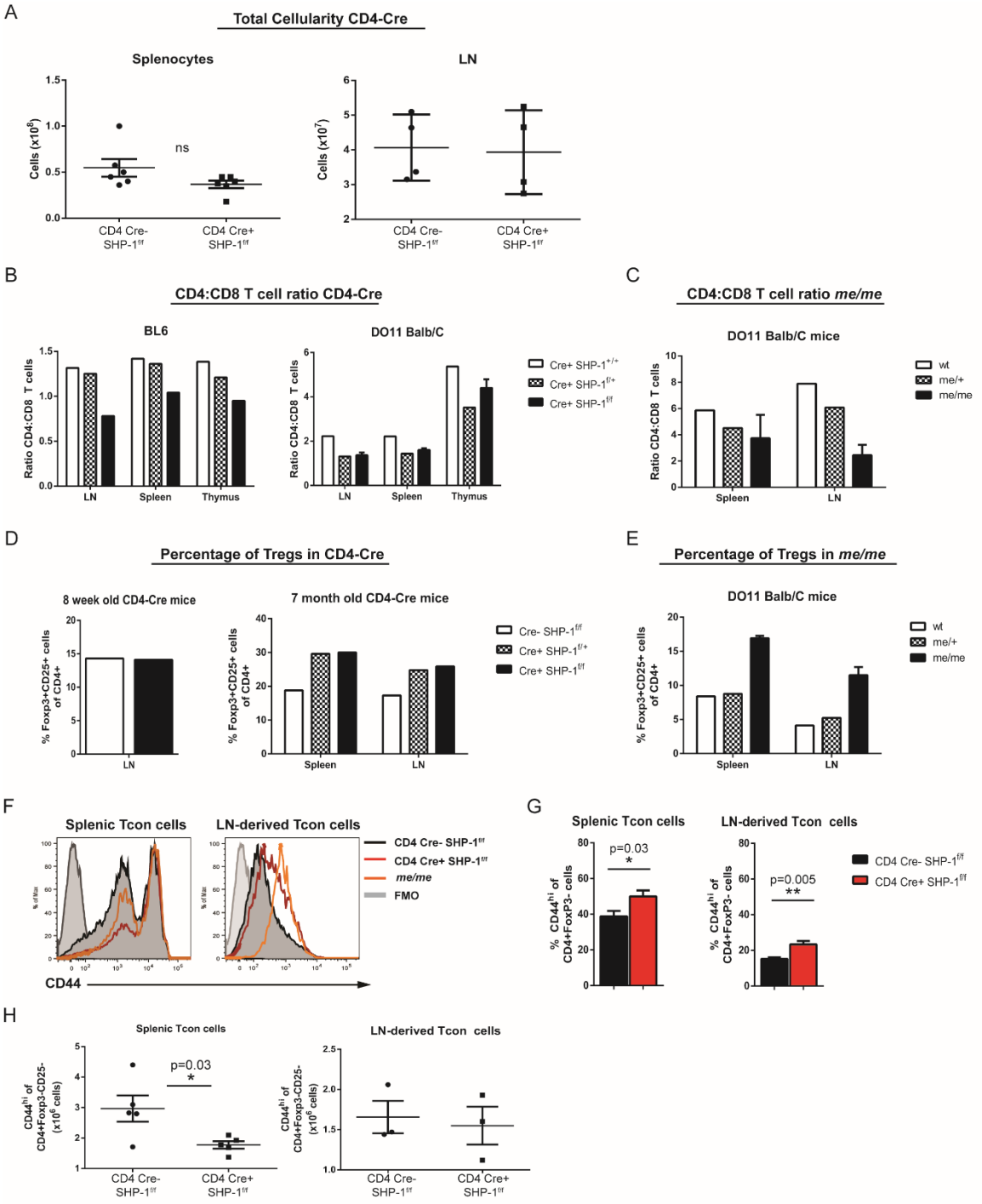

Figure 4.5. T cell compartment abnormalities in *me/me* and CD4-Cre+ SHP-1$^{f/f}$ mice. **(A)** Total cellularity in spleens and LN of CD4-Cre- or CD4-Cre+ SHP-1$^{f/f}$ mice

(C57BL/6) at 6 weeks of age, *n*=5-6 each genotype. **(B)** Percentages of CD4+ and CD8+ T cells in spleens, LN, and thymuses of CD4-Cre- SHP-$1^{f/f}$, CD4-Cre+ SHP-$1^{f/+}$ or CD4-Cre+ SHP-$1^{f/f}$ mice (C57BL/6, *left*, DO11.10 Balb/C, *right*) at 6 weeks of age were assessed by flow cytometry. CD4:CD8 ratio was calculated, *n=1-2* each genotype. **(C)** Percentages of CD4+ and CD8+ T cells in spleens and LN of wt, *me/+*, or *me/me* mice (DO11.10 Balb/C) at 2.5 weeks of age were assessed by flow cytometry, and CD4:CD8 T cell ratio was calculated. *n*=1-2 each genotype. **(D)** Percentage of Foxp3+CD25- cells of CD4+ T cells in LN of 8 week old C57BL/6 (*left)* CD4-Cre- or CD4-Cre+ SHP-$1^{f/f}$ mice or (*right*) spleens and LN of 7 month old CD4-Cre- SHP-$1^{f/f}$, CD4-Cre+ SHP-$1^{f/+}$ or CD4-Cre+ SHP-$1^{f/f}$ mice were assessed by flow cytometry, *n*=1 each genotype. **(E)** Percentage of Foxp3+CD25- cells of CD4+ T cells in spleen and LN of wt, *me/+*, or *me/me* mice (DO11.10 Balb/C) at 2.5 weeks of age were assessed by flow cytometry, *n=1-2* each genotype. **(F)** Representative CD44 expression on CD4+Foxp3- Tcon cells from spleens and LN of wt, *me/me*, CD4-Cre- or CD4-Cre+ SHP-$1^{f/f}$ mice assessed by flow cytometry. **(G)** Percentage of $CD44^{hi}$ CD4+Foxp3- cells in spleens and LN of CD4-Cre- or CD4-Cre+ SHP-$1^{f/f}$ mice, *n*=3-5 each genotype. **(H)** Absolute numbers of $CD44^{hi}$ CD4+Foxp3- cells in spleens and LN of CD4-Cre- or CD4-Cre+ SHP-$1^{f/f}$ mice *n*=3-5 each genotype. Error bars indicate ±SEM. A Student's *t* test was performed on data in (A), (G), and (H), * $p \leq 0.05$, ** $p \leq 0.01$.

Overall, there was a lower absolute number of $CD44^{hi}$ CD4+Foxp3- T cells in CD4-Cre+ SHP-$1^{f/f}$ spleens, owing to the decreased percentage of CD4+ T cells and slightly less total splenocytes in these mice (Fig. 4.5H). CD44 indicates Ag-experienced, memory-like T cells, suggesting that there are less naïve ($CD44^{lo}$) T cells and that more have become activated at some point.

Despite the fact that CD4-Cre+ SHP-$1^{f/f}$ mice showed no overt disease phenotype at steady state, their T cell compartments demonstrated substantial alterations similar to *me/me* mice. Several studies have shown that SHP-1 is involved in negative and positive thymocyte selection, and that the absence of SHP-1 during this process significantly alters the proportion of CD4+ SP and CD8+ SP thymocytes depending on the TCR Tg model used[154,155,157]. Increased selection of thymocytes in Tg models also leads to increased accumulation of either CD8+ or CD4+ T cells in the LN (depending on whether the TCR is specific for MHCI/II). It was previously reported that DO11.10 *me/me* mice have a greater percentage of CD4+ cells in the thymus because of increased positive selection[154]. However, at 3 weeks of age, we observed less CD4+ T cells in the spleen and LN of DO11.10 *me/me* mice. It is possible that peripheral CD4+ T cells have become activated and trafficked to tissues, or died from activation-induced cell death. The same trend was observed in CD4-Cre+ SHP-$1^{f/f}$ mice, wherein the thymus, LN, and spleen contained a lower percentage of CD4+ T cells. A recent report using CD4-Cre+ SHP-$1^{f/f}$ mice showed lower percentages and numbers of CD4+ and CD8+ cells in the thymus and spleen[158]. This study used more specific flow markers to differentiate developmental stages of thymocytes, which could explain the reduction in CD8+ T cell percentages, which we did not observe. Consistent with our findings, this study showed

an increased percentage of CD44^{hi} CD4+ and CD8+ T cells in the spleen, but only significant increases in the number of CD44^{hi} CD8+ T cells[158]. There was a concomitant decrease in naïve (CD44^{lo}) CD4+ and CD8+ T cells, suggesting that a greater proportion of naïve T cells become Ag-experienced memory-like cells, perhaps because of lowered TCR activation thresholds. Finally, CD4-Cre+ SHP-1$^{f/f}$ thymocytes also exhibited increased negative selection[158] similar to *me/me* mice.

Since SHP-1 is deleted before positive and negative selection occurs in CD4-Cre SHP-1$^{f/f}$ mice, this indicates that like *me/me* mice, the T cell repertoire is potentially skewed. Indeed, we observed increased accumulation of Tregs in older CD4-Cre+ SHP-1$^{f/f}$ mice, which is likely due to a lower threshold for Treg development in the absence of SHP-1[182]. Thus, while deletion of SHP-1 under the control of CD4-Cre allows for T cell-specific SHP-1 deletion and avoids the systemic inflammation of *me/me* mice caused by SHP-1-deficient myeloid cells, it does not avoid changes to the T cell compartment as a whole. The altered T cell repertoire would have consequences for immune responses to various pathogens, and therefore warrants caution when using this model. An additional complication to using the CD4-Cre SHP-1$^{f/f}$ mouse model is that CD4 is expressed by plasmacytoid dendritic cells (pDCs) and some NKT cells, and that CD4-Cre in fact mediates recombination in a proportion of these cells (>82% in pDCs and ~10% NK1.1+ cells)[205–207]. Since SHP-1 plays important roles in DCs, this off-target deletion could potentially mimic some of the hyper-activating characteristics of *me/me* DCs. Therefore, these preliminary observations of T cell alterations in CD4-Cre SHP-1$^{f/f}$ mice suggested that a T cell-specific Cre system that mediates SHP-1 deletion post-thymic selection is superior, and avoids effects on T cell development (see Chapter 3).

Chapter 5

SHP-1-deficient Tregs from dLck-Cre SHP-1$^{f/f}$ mice and Foxp3-Cre SHP-1$^{f/f}$ mice are more suppressive than wildtype Tregs

This chapter contains unpublished data.

5.1 INTRODUCTION

Tregs rely on stimulation through their TCR to be fully functional, as well as for their homeostasis and trafficking[93,208,209]. Many studies have demonstrated the necessity of TCR and costimulatory signaling in promoting Treg suppressive function by influencing Treg gene signatures, metabolism, and cellular adhesion[210,211]. Since SHP-1 regulates TCR signaling, we had previously investigated whether SHP-1 could regulate Treg suppressive function[172]. We found that Tregs from *me/me* mice, which lack SHP-1, are more suppressive than wildtype Tregs, both *in vitro* and *in vivo*[172]. In the absence of SHP-1, a greater percentage of Tregs expressed markers characteristic of an activated Treg, in particular CD103 and ICAM-1. Moreover, *me/me* Tregs were more efficient in forming conjugates with APCs, thereby inhibiting the upregulation of costimulatory molecules CD80/CD86 on APCs to suppress conventional T cell activation[172].

As discussed in Chapters 3 and 4, *me/me* mice develop systemic inflammation, which can influence T cell phenotypes in a cell-extrinsic manner. Thus, to determine if SHP-1 regulates Treg function in a cell-intrinsic manner, we utilized dLck-Cre+ SHP-$1^{f/f}$ mice, wherein SHP-1 is deleted in all T cells, to assess the function of SHP-1-deficient Tregs. Furthermore, we generated Foxp3-Cre+ SHP-$1^{f/f}$ mice in which SHP-1 is deleted specifically in Tregs upon Foxp3 expression[212]. Using these two mouse models, we show preliminary data suggesting that SHP-1-deficient Tregs are more suppressive than wildtype Tregs *in vitro*. We also observed superior suppression by Foxp3-Cre+ SHP-$1^{f/f}$ Tregs compared to dLck-Cre+ SHP-$1^{f/f}$ Tregs, raising the possibility that the timing of SHP-1 deletion may influence the extent to which SHP-1 deficiency augments Treg suppressive function.

5.2 RESULTS AND DISCUSSION

In what we termed a "crossover" suppression assay, we assessed the ability of SHP-$1^{+/+}$ and SHP-$1^{-/-}$ Tregs to suppress SHP-$1^{+/+}$ or SHP-$1^{-/-}$ Tcon cells (all from dLck-Cre SHP-$1^{f/f}$ mice) (Fig. 5.1). First, we compared suppression of SHP-$1^{+/+}$ Tcon cells by SHP-$1^{-/-}$ versus SHP-$1^{+/+}$ Tregs. SHP-$1^{-/-}$ Tregs seemed to be more suppressive than SHP-$1^{+/+}$ Tregs, which was more apparent at lower Treg:Tcon ratios (1:8, 1:16, 1:32). We had previously shown that SHP-$1^{-/-}$ Tcon cells are resistant to suppression by SHP-$1^{+/+}$ Tregs (Chapter 3). Thus, we also compared the ability SHP-$1^{-/-}$ versus SHP-$1^{+/+}$ Tregs to suppress "resistant" SHP-$1^{-/-}$ Tcon cells. SHP-$1^{-/-}$ Tregs suppressed SHP-$1^{-/-}$ Tcon cells more strongly than SHP-$1^{+/+}$ Tregs. However, SHP-$1^{-/-}$ Tcon cells were still slightly less suppressed than SHP-$1^{+/+}$ Tcon cells by SHP-$1^{-/-}$ Tregs, suggesting SHP-$1^{-/-}$ Tregs do not fully negate the resistance phenotype of SHP-$1^{-/-}$ Tcon cells. These data had high variability between conditions, and will need to be repeated to conclusively determine whether SHP-$1^{-/-}$ Tregs fully overcome the resistance of SHP-$1^{-/-}$ Tcon cells.

Next, we assessed the differences in suppressive function of Foxp3-Cre- versus Foxp3-Cre+ SHP-$1^{f/f}$ Tregs (Fig. 5.2). *Foxp3* is an X-linked gene, and therefore when using Foxp3-Cre+ mice, female mice were homozygous for the Foxp3-Cre allele to ensure full deletion of SHP-1. We observed a statistically significant increase in the suppressive ability of Foxp3-Cre+ SHP-$1^{f/f}$ Tregs compared to Foxp3-Cre- SHP-$1^{f/f}$ Tregs, particularly at higher Treg:Tcon ratios. We also compared the suppressive ability of Foxp3-Cre+ SHP-$1^{f/f}$ Tregs to that of dLck-Cre+ SHP-$1^{f/f}$ Tregs in the same assay (Fig. 5.3). We found that Foxp3-Cre+ SHP-$1^{f/f}$ Tregs were significantly more suppressive

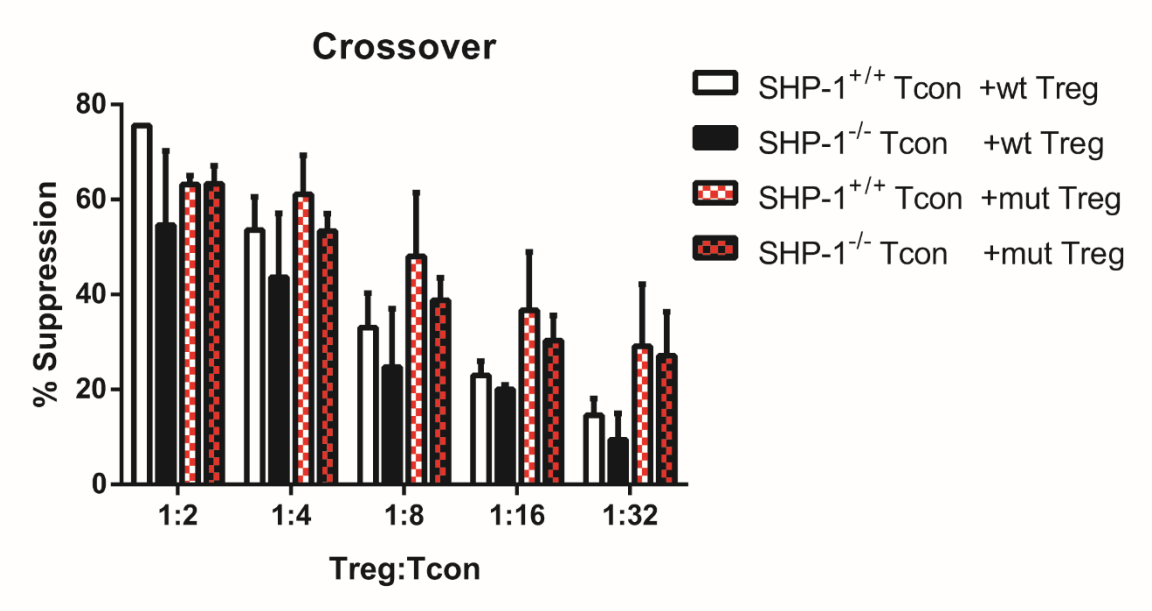

Figure 5.1. dLck-Cre+ SHP-1$^{f/f}$ Tregs are more suppressive than dLck-Cre- SHP-1$^{f/f}$ Tregs. Tcon cells (CD4+CD25-) and Tregs (CD4-CD25+) were isolated from spleens of dLck Cre- SHP-1$^{f/f}$ (SHP-1$^{+/+}$) or dLck Cre+ SHP-1$^{f/f}$ (SHP-1$^{-/-}$) mice. Tcon cells were stained with CellTrace Violet and plated with irradiated CD4-depleted splenocytes as APCs and soluble anti-CD3. Tregs were cultured with Tcon cells at indicated ratios. After 4 days, cells were harvested and assessed by flow cytometry. The percent responding cells was calculated using the FlowJo Proliferation Platform algorithm applied to CellTrace Violet dilution. Percent responding cells was normalized to the condition without Tregs (maximal responsiveness) for each culture combination, and then percent suppression was calculated by subtracting percent responding cells from 100 percent. Red bars indicate Tcon cells that were cultured with "mut", SHP-1$^{-/-}$ Tregs. *n*=2 mice each condition, error bars indicate ±SEM.

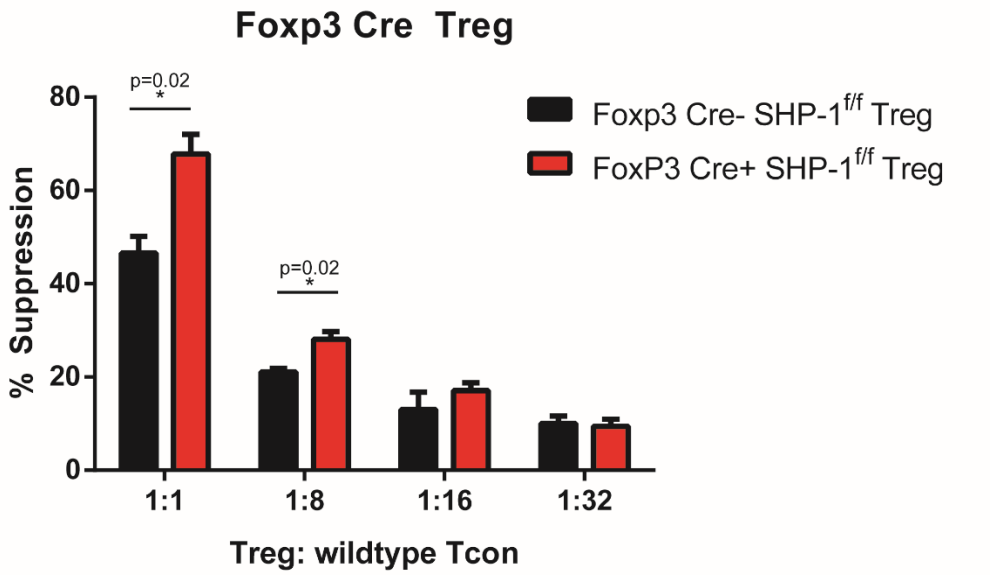

Figure 5.2. Foxp3-Cre+ SHP-1$^{f/f}$ Tregs are more suppressive than Foxp3-Cre- SHP-1$^{f/f}$ Tregs. Tcon cells (CD4+CD25-) were isolated from spleens of Foxp3 Cre- SHP-1$^{f/f}$ mice only and stained with CellTrace Violet. Tregs (CD4+CD25+) were isolated from Foxp3 Cre- SHP-1$^{f/f}$ mice or Foxp3 Cre+ SHP-1$^{f/f}$ (homozygous for Cre if female) mice. Tcon cells were plated with irradiated CD4-depleted splenocytes as APCs and soluble anti-CD3. Tregs were cultured with Tcon cells at indicated ratios. After 4 days, cells were harvested and assessed by flow cytometry. The percent responding cells was calculated using the FlowJo Proliferation Platform algorithm applied to CellTrace Violet dilution. Percent responding cells was normalized to the condition without Tregs (maximal responsiveness) for each culture combination, and then percent suppression was calculated by subtracting percent responding cells from 100 percent. *n*=3 mice each condition, error bars indicate ±SEM. A one-way ANOVA with a Tukey's multiple comparison post-test was performed on each Treg:Tcon ratio.

of SHP-$1^{+/+}$ Tcon cells than wildtype (dLck-Cre- SHP-$1^{f/f}$) Tregs, and tended to be slightly more suppressive than dLck-Cre+ SHP-$1^{f/f}$ Tregs (top graph). This difference was even more apparent when we assessed the suppression of SHP-$1^{-/-}$ Tcon cells (bottom graph). These data will need to be repeated for more statistical power and to overcome the high variability within and between experiments. However, these results indicate that Foxp3-mediated deletion of SHP-1 results in Tregs with increased functional capacity compared to Tregs that undergo SHP-1 deletion regulated by the distal Lck promoter.

The differences in suppressive capacity between dLck-Cre+ and Foxp3-Cre+ SHP-$1^{f/f}$ Tregs could be due to differences in SHP-1 deletion efficiency. We have not investigated the extent to which SHP-1 is deleted in Tregs from either mouse strain, and a direct comparison would provide insight into whether functional differences can be attributed to incomplete SHP-1 deletion or rather the timing of SHP-1 deletion.

Based on these results, it is possible that the lack of disease we observed in dLck-Cre+ SHP-$1^{f/f}$ mice is due to enhanced Treg suppression negating the effects of hyper-responsive Tcon cells. Even though SHP-$1^{-/-}$ Tcon cells are resistant to wildtype Treg suppression, they are likely controlled more adequately by SHP-$1^{-/-}$ Tregs *in vivo*, preventing the development of autoimmune or inflammatory disease. Induction of EAE in dLck-Cre+ SHP-$1^{f/f}$ mice via administration of MOG peptide and adjuvant would reveal whether there is enhanced Treg function *in vivo,* and if so, whether it exerts a protective effect or is negated by suppression-resistant Tcon cells.

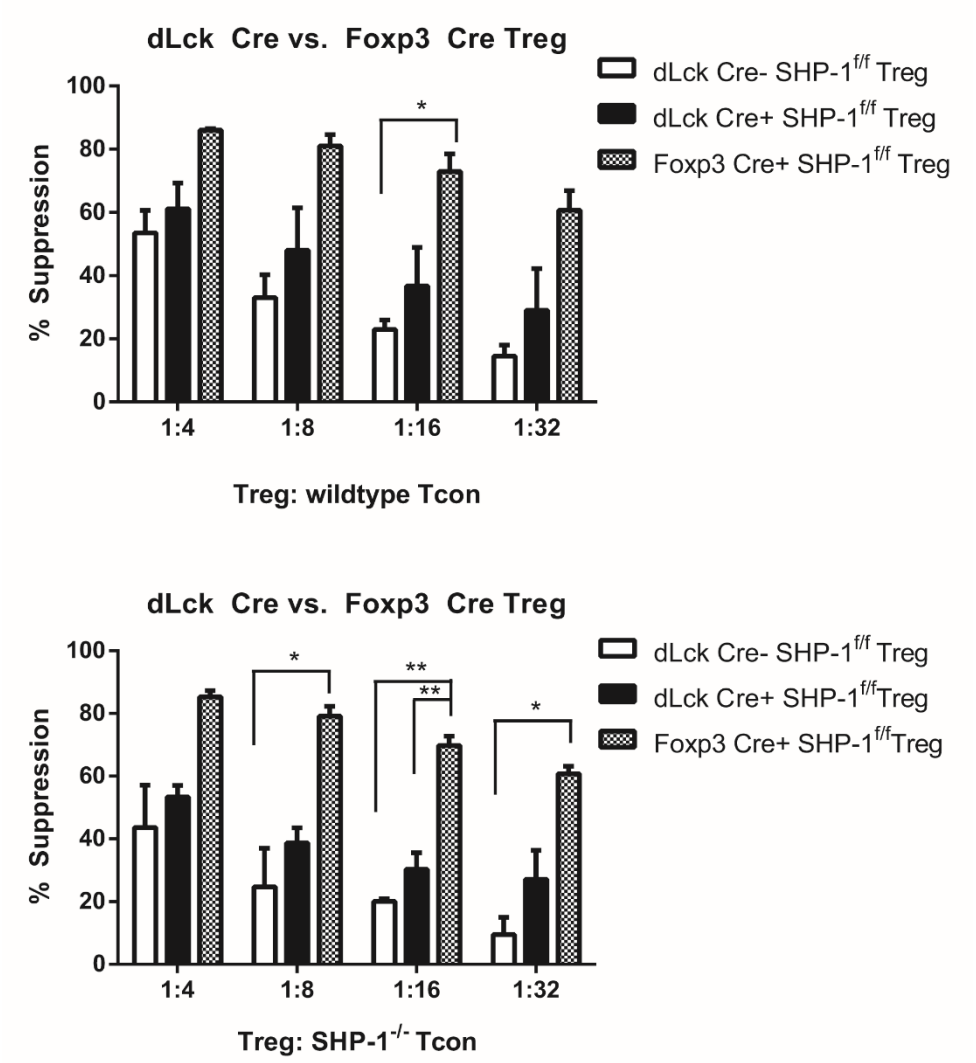

Figure 5.3. Foxp3-Cre+ SHP-1$^{f/f}$ Tregs are more suppressive than dLck-Cre+ SHP-1$^{f/f}$ Tregs. Tcon cells (CD4+CD25-) were isolated from spleens of dLck Cre- SHP-1$^{f/f}$ mice or dLck Cre+ SHP-1$^{f/f}$ mice and stained with CellTrace Violet. Tregs (CD4+CD25+) were isolated from dLck Cre-, dLck Cre+, or Foxp3 Cre+ SHP-1$^{f/f}$ mice (homozygous for Cre if female). Tcon cells were plated with irradiated CD4-depleted splenocytes as APCs and soluble anti-CD3. (*Top*) Tregs were cultured with dLck Cre- SHP-1$^{f/f}$ Tcon cells or (*bottom*) dLck Cre+ SHP-1$^{f/f}$ Tcon cells at indicated ratios. After 4 days, cells were harvested and assessed by flow cytometry. The percent responding cells

was calculated using the FlowJo Proliferation Platform algorithm applied to CellTrace Violet dilution. Percent responding cells was normalized to the condition without Tregs (maximal responsiveness) for each culture combination, and then percent suppression was calculated by subtracting percent responding cells from 100 percent. *n*=2 mice each condition, error bars indicate ±SEM. A one-way ANOVA with a Tukey's multiple comparison post-test was performed on each Treg:Tcon ratio. *$p \leq 0.05$, **$p \leq 0.01$.

Chapter 6

Conclusions, Future Directions, and Significance

6.1 Summary of major findings

In the past decade, T cell resistance to Treg-mediated suppression has been identified as a prominent pathophysiological characteristic of autoimmune disease. Our work addresses how dysregulation of signaling downstream of the TCR can allow T cells to become refractory to Treg suppression. These findings contribute to a better understanding of a paradigm in which integration of activation signals and environmental cues by a T cell inform its susceptibility to Treg suppression. T cells must transiently overcome Treg-imposed constraints when responding to a pathogen, but return to being suppressible to limit ongoing inflammation. Thus, T cells must have means through which they can gauge the necessity of overcoming Treg suppression. We identify an intracellular signaling pathway that serves this rheostat function. Importantly, these results provide new immunotherapeutic targets to modulate the balance between T cells and Tregs in the contexts of autoimmune disease and cancer.

First, we clarified the previously controversial role of SHP-1 in regulating the response to TCR stimulation. Our data shows that in T cells, SHP-1 does not regulate cellular survival or cellular division time, but rather influences the ability of a cell to respond to TCR stimulation by becoming activated. Second, we identified a novel role for SHP-1 in regulating T cell susceptibility to Treg suppression both *in vitro* and *in vivo*. The use of four independent model systems of SHP-1-deficiency strongly reinforced the concept that SHP-1 sits at the balance of T cell activation or suppression. Third, SHP-1 exerts this function in naïve CD4+ and naïve CD8+ T cells. Fourth, SHP-1 regulates resistance to suppression in a cell-intrinsic manner, such that SHP-1-deficient T cells cannot transfer resistance to neighboring wildtype T cells through soluble factors.

Finally, SHP-1 negatively regulates the PI3K/Akt pathway in T cells, and enhancement of this pathway is correlated with resistance to Treg suppression.

In addition to SHP-1 regulating T cell susceptibility to Treg suppression, we also demonstrated that SHP-1 regulates the suppressive function of Tregs in a cell-intrinsic manner. This differential role of SHP-1 in conventional T cells versus Tregs has important implications for immunotherapy strategies for autoimmune disease and cancer. Presented below are critical future studies, applications of our findings toward autoimmune disease and cancer immunotherapies, and the broader significance of our work.

6.2 Future directions

Future studies to determine the molecular and cellular mechanisms of T cell resistance to Treg suppression

In this work, we have demonstrated that SHP-1 negatively regulates the PI3K/Akt pathway downstream of TCR stimulation, and that enhanced activation of the PI3K/Akt pathway seems to be correlated with allowing T cells to resist suppression (Chapter 3). We had also identified Cbl-b as a possible candidate for regulation by SHP-1 and a mediator of resistance to Treg suppression (Chapters 3, 4). We did not, however, observe changes in Cbl-b expression in SHP-1-deficient T cells (dLck-Cre or CD4-Cre SHP-$1^{f/f}$) at steady state. Furthermore, Cbl-b expression is reduced upon costimulatory signaling through CD28, and because we provided irradiated APCs as costimulation in our

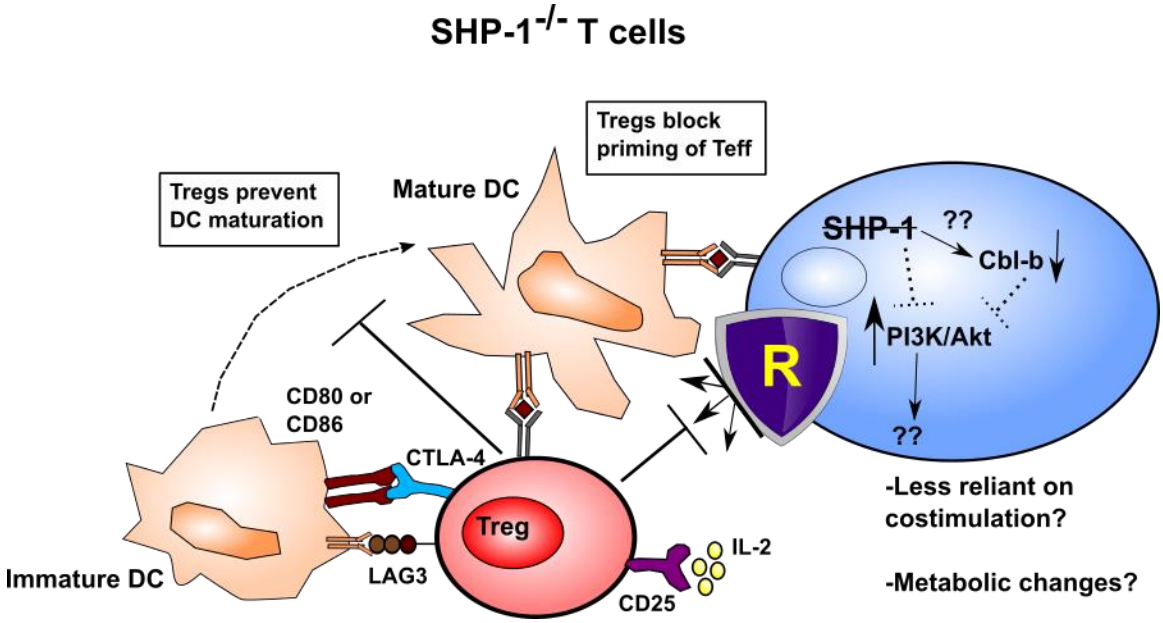

Figure 6.1 Remaining questions about whether SHP-1 regulates Cbl-b and what happens downstream of PI3K/Akt pathway. In the absence of SHP-1, T cells exhibit heightened activation of the PI3K/Akt pathway and resist Treg-mediated suppression. It remains to be determined whether SHP-1 regulates Cbl-b, such that SHP-$1^{-/-}$ T cells have reduced Cbl-b expression upon TCR stimulation. Reduction of Cbl-b could also result in heightened activation of PI3K/Akt through relief of Cbl-b's negative regulation of PI3K/Akt. The changes that occur downstream of enhanced PI3K/Akt activation remain unknown, but might result in an altered metabolic profile of the T cell as well as reduced need for costimulatory signals.

suppression assays, we did not assess Cbl-b levels in this context. Based on our findings, there are two primary areas that require further investigation to elucidate the molecular mechanism of T cell resistance to Treg suppression in our model: signaling events upstream of PI3K/Akt - namely the possible role of Cbl-b - and signaling events downstream of PI3K/Akt and the resultant changes to the T cell (Fig. 6.1).

Cbl-b

As discussed in Chapter 3, a recent study using CD4-Cre SHP-1$^{f/f}$ T cells suggested that SHP-1 regulates Cbl-b activity and expression. The proposed model suggests that SHP-1 is recruited to Cbl-b upon TCR stimulation and dephosphorylates it, inhibiting its ubiquitin ligase activity and preventing its autoubiquitination, thereby maintaining its expression in the cell. Therefore, in the absence of SHP-1, Cbl-b becomes rapidly phosphorylated upon TCR stimulation and undergoes autoubiquitination resulting in a reduction of Cbl-b expression[167]. However, CD28 costimulation disrupts the association between SHP-1 and Cbl-b.

Prior to this work, it was shown that Cbl-b is necessary to enforce T cell anergy in the absence of costimulation. Thus, upon TCR stimulation alone, Cbl-b expression is upregulated. Cbl-b targets other signaling molecules, such as PI3K, for ubiquitination, resulting in their proteasomal degradation or altered localization in the cell, thereby inhibiting T cell activation[122]. However, once a costimulatory signal is received through CD28, PKC-θ associates with Cbl-b and phosphorylates it[126] (Fig. 6.2). This phosphorylation either allows Cbl-b to change conformation and undergo autoubiquitination, or enables its ubiquitination by another E3 ligase, Nedd4[213]. The ubiquitination of Cbl-b leads to its proteasomal degradation so that there is a reduction in

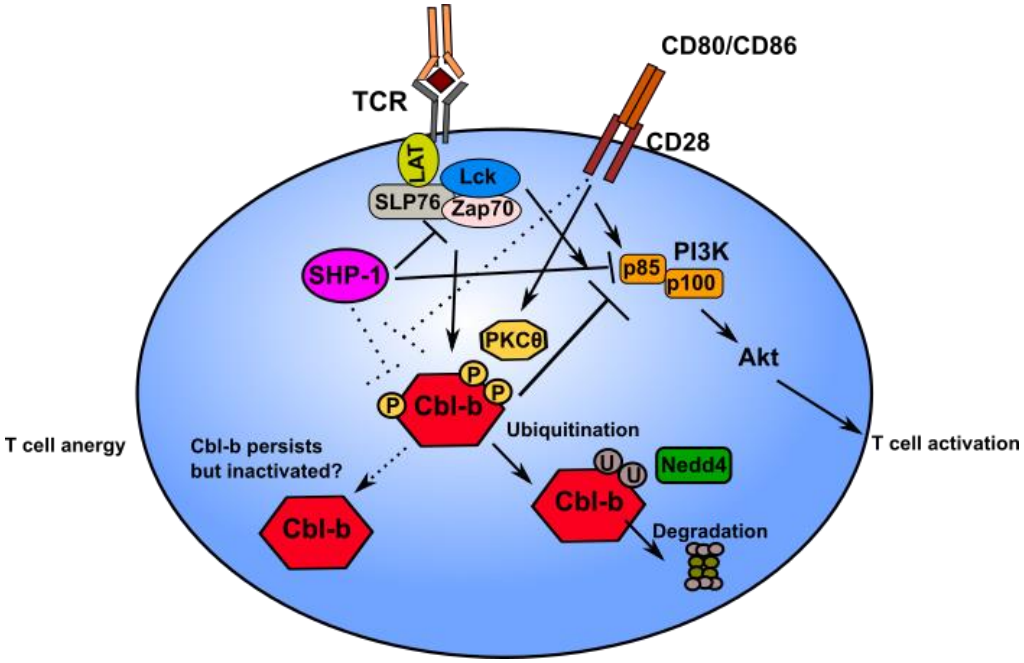

Figure 6.2. Role of Cbl-b in enforcing T cell anergy and proposed mechanism of regulation by SHP-1. Prior studies defined the mechanism by which Cbl-b enforces the need for costimulatory signals to avoid T cell anergy. In the absence of CD28 costimulation, Cbl-b expression is upregulated in response to TCR stimulation. Cbl-b ubiquitinates the p85 subunit of PI3K, inhibiting its translocation to the TCR/CD28 complex, ultimately preventing T cell activation. Upon CD28 costimulation, however, PKCθ is activated, and phosphorylates Cbl-b. This phosphorylation is thought to recruit another ubiquitin ligase, Nedd4, which ubiquitinates Cbl-b, targeting it for proteasomal degradation. The reduction in Cbl-b relieves its inhibition of PI3K and T cells can become activated. A new model proposed by Xiao et al. suggests that upon TCR stimulation, SHP-1 dephosphorylates Cbl-b tyrosine residues, preventing a conformational change that would otherwise lead to its autoubiquitination and degradation (through a PKCθ-independent mechanism that remains to be defined). Thus,

Cbl-b persists in the cell but is inactivated by dephosphorylation by SHP-1. This seems to be at odds with the role of Cbl-b in exerting its ubiquitin ligase function to inhibit PI3K when TCR stimulation occurs in the absence of costimulation. Xiao et al. go on to show that CD28 costimulation dissociates the interaction between SHP-1 and Cbl-b, so that SHP-1 does not seem to regulate Cbl-b when costimulatory signals are received. Dashed lines indicate proposed regulation by the Xiao et al. study that have yet to be further investigated.

Cbl-b and it no longer inhibits T cell activation signals. Thus, T cells given CD28 costimulation show reduced (but not completely absent) Cbl-b protein expression[126,167]. Importantly, it has yet to be assessed whether Cbl-b expression in SHP-$1^{-/-}$ T cells that receive TCR stimulation and costimulation is even further reduced compared to SHP-$1^{+/+}$ T cells. Presumably, costimulatory signals negate the regulatory effects of SHP-1 on Cbl-b, since Xiao et al[167]. demonstrated that CD28 costimulation dissociates SHP-1 from Cbl-b. However, if Cbl-b expression is further reduced in SHP-$1^{-/-}$ T cells upon TCR stimulation and costimulation, this may represent another component of the molecular mechanism of resistance to Treg suppression in our model.

It is unclear why, upon TCR stimulation, SHP-1 would enable the continued maintenance of Cbl-b expression but also inactivate its ligase function, as proposed by Xiao et al. One would hypothesize that Cbl-b must remain functional to target other molecules for degradation, inhibiting TCR signals and inducing anergy. Therefore, while this study provides evidence of a role for SHP-1 in regulating Cbl-b function and expression, much remains unclear. It would be useful to determine whether we observe reduced Cbl-b expression in dLck-Cre+ SHP-$1^{f/f}$ T cells upon TCR stimulation alone, which would agree with the notion that SHP-1 regulates Cbl-b degradation in response to TCR stimulation. As noted in Chapter 3, Xiao et al. observed reduced Cbl-b expression in CD4-Cre+ SHP-$1^{f/f}$ T cells in the absence of any stimulation, which is not consistent with our observations.

Furthermore, Xiao et al. imply that the phenotype of SHP-1-deficient T cells (hyper-responsiveness to TCR stimulation and a tendency to skew to T_H2 cells) is a result of the loss of Cbl-b, rather than loss of SHP-1 regulation of other signaling elements. To

address this, the group retrovirally transfected SHP-1-deficient T cells with Cbl-b and showed a reversal of hyper-proliferation and T_H2 skewing. However, because Cbl-b itself negatively regulates T cell proliferation and T_H2 skewing[123,127,203], it is necessary to include a control wherein wildtype T cells were transfected with Cbl-b. By simply overexpressing a negative regulator of T cell functions, one cannot conclude that this represents a reversal of SHP-1-deficient phenotypes without knowing the extent to which overexpression of Cbl-b impacts wildtype T cell proliferation and skewing. Rather, pharmacological inhibition of the proteasome would eliminate degradation of Cbl-b and allow one to determine whether increased Cbl-b reduction plays a role in the hyper-responsive phenotype of SHP-1-deficient T cells. Then, one could assess whether SHP-1-deficient T cells retain their ability to hyper-proliferate in response to TCR stimulation and costimulation independent of Cbl-b.

It remains to be determined whether T cells from CD4-Cre or dLck-Cre SHP-$1^{f/f}$ mice are less reliant on costimulation, as has been observed in *me/me* T cells[181]. It will be important to determine whether TCR stimulation alone at sub-optimal concentrations can induce anergy in SHP-1-deficient T cells (no proliferation, low IL-2 production, increased Cbl-b expression) or whether the T cells show reduced Cbl-b expression and the ability to become activated and proliferate. Direct comparison of Cbl-b expression and phospho-Akt in response to TCR stimulation with and without CD28 costimulation would be informative.

It is possible that SHP-1 does regulate the stability of Cbl-b protein expression and that SHP-1-deficient T cells have reduced need for costimulation because of reduced Cbl-b. Both SHP-1 and Cbl-b negatively regulate PI3K through different mechanisms; SHP-1

dephosphorylates the p85 subunit of PI3K, inactivating it[149,150], while Cbl-b ubiquitinates the p85 subunit of PI3K, altering its ability to interact with CD28 and TCRζ and become activated[125]. Thus, it is possible that the observed enhancement of PI3K/Akt activation in our SHP-1-deficient T cells could be due to SHP-1 deficiency by itself, or SHP-1 deficiency *and* reduction in Cbl-b, both amplifying the activation of the PI3K/Akt pathway. In either case, enhanced activation of PI3K/Akt could bypass the need for costimulation, providing a mechanism for T cells to resist Treg suppression.

Downstream of Akt

The finding that SHP-1$^{-/-}$ T cells show enhanced PI3K/Akt activation and resist Treg suppression is consistent with several other studies in which T cells that resist Treg suppression show enhanced activation of the PI3K/Akt pathway[50]. To more firmly establish that enhanced activation of PI3K/Akt is linked to resistance to Treg suppression, reversal of the enhanced activation of PI3K/Akt is necessary. We attempted to inhibit Akt in T cells *in vitro* through the use of two different inhibitors: Akt VIII, and MK-2206. However, we did not observe any effect of these inhibitors on resistance to suppression of SHP-1$^{-/-}$ T cells, nor were we able to confirm their inhibitory activity. We also used wortmannin, an inhibitor of PI3K, and did not observe effects on resistance. Previous reports using Akt VIII in human T cell cultures successfully reversed resistance to Treg suppression without affecting the ability of the T cells to proliferate in the absence of Tregs[70,74,78]. We hypothesize that murine T cells may have different susceptibility to the inhibitors tested than human T cells, and may also require longer pre-treatment with the inhibitors to achieve adequate inhibition. Future studies will need to quantify the

inhibitory activity of Akt and/or PI3K inhibitors to determine whether the absence of effects we observed was due to lack of inhibition. To circumvent issues with inhibitors, one could transfect T cells to overexpress the endogenous inhibitor of PI3K, PTEN. This strategy was successfully employed in murine T cells deficient in TRAF6, and their resistance to suppression was reversed compared to control T cells transfected with PTEN[133]. We hypothesize that transfection of SHP-1$^{-/-}$ T cells with PTEN would reduce the hyper-activation of the PI3K/Akt pathway and thereby reverse resistance to Treg suppression.

While we provide yet more evidence that the PI3K/Akt pathway is important in resistance to Treg suppression, to date it remains unknown how enhancement of the PI3K/Akt pathway allows a T cell to resist Treg suppression. As discussed in the review article in Appendix A, the PI3K/Akt pathway impacts cellular metabolism, cell cycle entry, cell survival, and proliferation[137]. Since this pathway has pleiotropic effects on T cells, it is difficult to pinpoint what specific Treg suppressive mechanisms PI3K/Akt activation can overcome. As we still do not know which or how many Treg suppressive mechanisms are in play *in vitro*, we can only speculate how T cells overcome resistance to these suppressive mechanisms. Enhanced survival *in vitro* does not seem to play a role in resistance to suppression, as Tregs do not seem to induce apoptosis of T cells in this setting[85,127,133]. We also observed no survival advantage of SHP-1$^{-/-}$ T cells *in vitro*. Instead, Tregs do appear to mediate suppression through contact with APCs *in vitro*, as well as through secretion of suppressive cytokines[49]. Therefore, *in vitro*, Tregs may downregulate costimulatory molecules on APCs to deprive T cells of adequate priming. To gain more insight into what pathways downstream of Akt are important for resistance

to Treg suppression *in vitro*, we could perform RNA-seq analysis on SHP-$1^{+/+}$ and SHP-$1^{-/-}$ T cells that receive TCR stimulation alone, TCR stimulation with costimulation, and after exposure to Tregs for various times (12, 24, 48, 72, 96 hours). This would allow us to understand more about what pathways are being repressed by Tregs, and which are less suppressed in SHP-$1^{-/-}$ T cells.

A previous study sought to determine how different lengths of pre-activation of Tregs could impact their ability to suppress T cells[214]. Interestingly, this study dissected the effect of Treg suppression on T cell activation pathways in T cells that escaped suppression versus those that were suppressed. They did so by performing phosphoflow and DNA content analysis on T cells and using CFSE dilution to gate specifically on cells remaining in generation 0 (suppressed) versus those that proliferated (generations 1+). This would be a useful approach to apply to our SHP-$1^{-/-}$ T cells, and could potentially identify further differences in other T cell activation pathways as a result of SHP-1 deficiency.

Activation of Akt in response to TCR stimulation and CD28 costimulation has been shown to drive increases in cellular size and metabolic activity in T cells[137]. T cells that are activated require energy for the biosynthesis of new proteins as they undergo proliferation and differentiation, and therefore undergo a switch from oxidative phosphorylation to glycolysis[215]. T cell metabolism is therefore fine-tuned to meet the needs of a T cell under different circumstances. It is possible that the enhanced activation of the PI3K/Akt pathway results in enhanced glycolytic activity, and that this allows T cells to resist Treg suppression. To address this possibility, metabolic analysis via Seahorse of SHP-$1^{-/-}$ T cells pre- and post- exposure to Tregs would be informative. This,

in addition to information gained from RNA-seq analyses, would identify whether increased metabolic function is a mechanism of resistance to Treg suppression.

Another candidate mechanism of SHP-1$^{-/-}$ T cell resistance to Treg suppression is increased expression of integrins. Intriguingly, Tcon cells from both *me/me* and CD4+Cre SHP-1$^{f/f}$ mice showed higher expression levels of the adhesion molecule ICAM-1. Additionally, *me/me* Tcon cells were found to form more stable, long-lasting conjugates with APCs. Interaction time with APCs is an important factor in the priming and activation of a Tcon cell and ICAM-1/LFA-1 interactions are crucial for long-lived APCs contacts[216]. It is possible that one of the downstream consequences of SHP-1 deficiency is an upregulation of adhesion molecules like ICAM-1 (no changes in LFA-1 were observed). These results are consistent with a study in which a greater proportion of CD8+ T cells from *me/me* mice were found to form stable conjugates with peptide-pulsed APCs[169]. In fact, ICAM-1 expression has been found to be regulated in T cells by protein tyrosine phosphatase activity upon T cell activation[217]. Further studies are needed to determine if upregulated expression of ICAM-1 in SHP-1-deficient T cells allows more stable conjugate formation, which in turn allows enhanced T cell priming and helps promote resistance to Treg suppression. Enhanced expression of adhesion molecules does not fully account for the ability of SHP-1-deficient T cells to hyper-respond to TCR stimulation; stimulation of *me/me* and dLck-Cre+ SHP-1$^{f/f}$ T cells (Chapter 3) with plate-bound anti-CD3 alone leads to enhanced responsiveness, in the absence of conjugate formation with APCs. Flow cytometric imaging of conjugate formation between dLck-Cre+ SHP-1$^{f/f}$ T cells and APCs would clarify a possible role for enhanced adhesion molecule expression and conjugate formation in resistance to suppression[172]. Intravital

imaging of TCR Tg SHP-1$^{-/-}$ T cell conjugate formation with APCs in lymph nodes upon administration of OVA^{218} would allow us to determine if this mechanism is also relevant *in vivo*.

Another remaining question is whether the pathways downstream of SHP-1 that regulate resistance to Treg suppression are the same as those that regulate T cell activation and proliferation. In our *in vivo* studies, T cells transferred into a lymphopenic host underwent homeostatic expansion, which was not regulated by SHP-1. Homeostatic expansion in lymphopenic hosts is driven primarily by IL-7 and to a lesser extent, engagement of T cells with self-peptide/MHC[219]. Therefore, we did not observe enhanced expansion of SHP-1$^{-/-}$ T cells at baseline (in the absence of Tregs) but did observe resistance to Treg suppression of their expansion (Chapter 3). These data indicate that perturbation of intracellular signaling pathways downstream of the TCR are sufficient to allow a T cell to resist Treg suppression of expansion that is driven through cytokine signals. Thus, further investigation is needed to determine how SHP-1 deficiency results in T cells that resist Treg suppression in the absence of concurrent hyper-proliferation (Fig. 6.3).

While it is difficult to determine the specific mechanism(s) of Treg suppression that SHP-1$^{-/-}$ T cells overcome, the involvement of enhanced activation of the PI3K/Akt pathway in resistance represents a possible immunotherapeutic target for autoimmune disease. Inhibition of Akt *in vitro* can reverse T cell resistance to Treg suppression[70,74,78]. Use of PI3K inhibitors *in vivo* have shown mixed results in the context of autoimmunity. Some studies reported that PI3K inhibitors had an inhibitory effect on Treg function

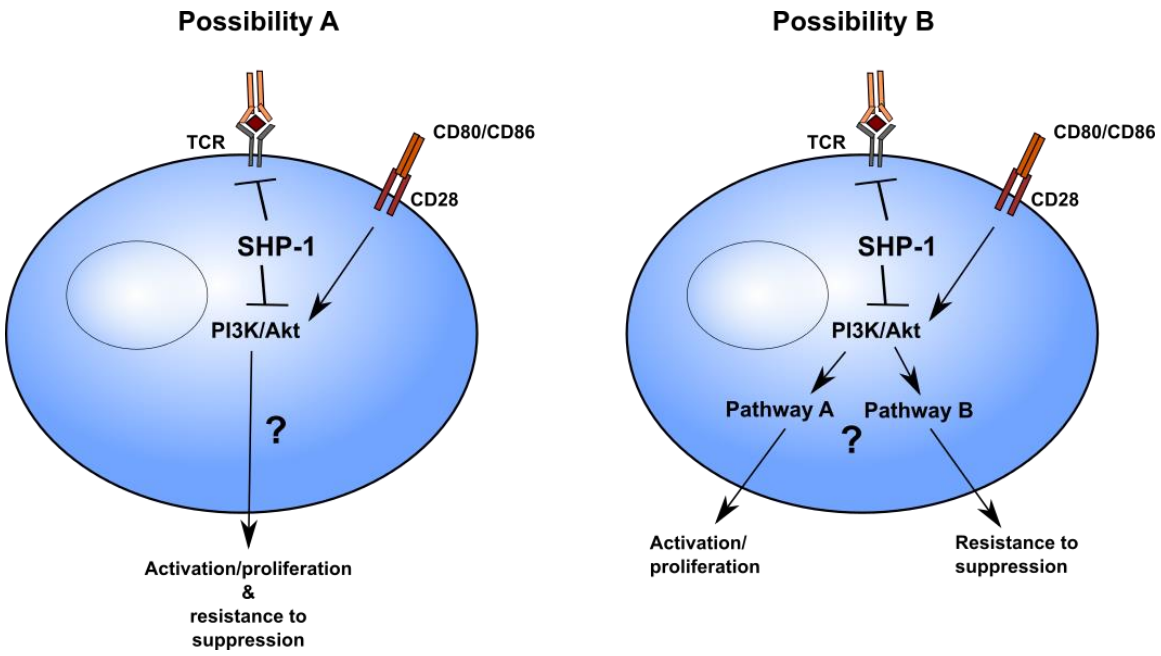

Figure 6.3. Model of dependent or independent pathways regulating T cell activation and proliferation versus resistance to suppression. In possibility A, a T cell that is capable of resisting suppression by a Treg is also hyper-responsive to TCR stimulation/costimulation in the absence of Tregs. This indicates that overlapping pathway(s) regulate both resistance to Treg suppression and T cell activation and proliferation. In this scenario, T cells that do not demonstrate hyper-activation and hyper-proliferation in response to TCR stimulation/costimulation are not able to resist Treg suppression either. In possibility B, the pathways which regulate resistance to Treg suppression versus T cell activation and proliferation are independent. This means that although they may have common upstream regulators, like SHP-1 and PI3K/Akt, there is a downstream divergence that allows one outcome in the absence of another. Therefore, T cells which do not hyper-respond to TCR stimulation/costimulation in the absence of Tregs could still be capable of resisting Treg suppression.

rather than inhibiting effector T cells[220,221], whereas others showed amelioration of disease via inhibitory effects on effector T cells[222,223]. As of now, no studies have addressed whether inhibition of PI3K/Akt *in vivo* can ameliorate autoimmune disease through increasing T cell susceptibility to Treg suppression. mTOR is a downstream target of Akt, and inhibition of mTOR with rapamycin is used for treatment of transplant rejection. Rapamycin has been found to promote anergy in conventional T cells while also inducing an increase in the frequency of Tregs[224]. However, it is unknown whether rapamycin could reverse resistance to Treg suppression in autoimmune disease. Pre-treatment of Tcon cells *in vitro* with rapamycin followed by assessment via a suppression assay may aid in answering this question. Further studies are needed to determine if specific pathways downstream of Akt are involved in resistance, and whether they could be specifically targeted for autoimmune disease therapies.

Future studies on the role of SHP-1 in T cell resistance to Treg suppression of effector function

Throughout this work, we have measured T cell resistance to Treg suppression of activation and proliferation. Physiologically, Treg suppression of T cell priming might occur in the context of a naïve T cell encountering antigen in a secondary lymphoid organ. We demonstrated that SHP-1 regulates resistance to suppression in naïve CD4+ and CD8+ T cells, as well as CD4+CD25- Tcon cells and total CD8+ T cells. Both the Tcon cell and total CD8+ T cell populations contain Ag-experienced effector T cells. We

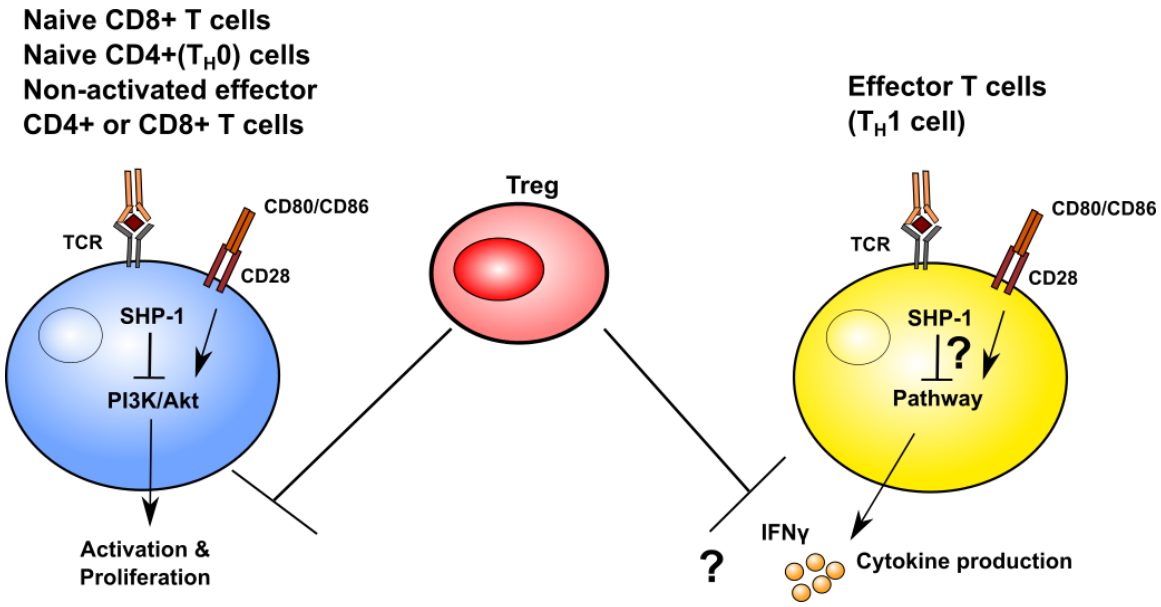

Figure 6.4. Future studies on the role of SHP-1 in regulating susceptibility of effector T cells to Treg suppression of effector function. (*Left*) We have shown in this work that SHP-1 regulates Treg suppression of activation and proliferation of naïve CD4+ and CD8+ T cells, as well as Ag-experienced cells present in the CD4+CD25- Tcon cell and total CD8+ T cell populations. (*Right*) Further studies are needed to determine whether SHP-1 also regulates susceptibility of effector T cells, for example T_H1 cells, to Treg suppression of cytokine production (here, IFNγ). Treg suppression of cytokine production may occur through a different mechanism than suppression of T cell activation and proliferation. Furthermore, studies suggest that TCR activation and costimulation of effector T cells induces slightly different signaling pathways to enable cytokine production compared to the signaling in naïve T cells that enables activation and proliferation. Thus, it will be worthwhile to determine if the SHP-1/PI3K/Akt signaling axis also plays a role in regulating suppression of effector T cell function.

have yet to investigate whether SHP-1 also regulates the resistance of differentiated effector T cells to suppression of effector function (Fig. 6.4). Therefore, we skewed naïve T cells *in vitro* into T_H1 cells and assessed whether IFNγ production by SHP-$1^{-/-}$ T_H1 cells was less suppressed by Tregs compared to that of SHP-$1^{+/+}$ T_H1 cells. Interestingly, we did not find any differences in the proportion of SHP-$1^{-/-}$ T cells that skewed to T_H1 cells compared to SHP-$1^{+/+}$ T cells, which is in contrast to previous work suggesting that a greater proportion of *me/me* T cells skew toward T_H1[153,164]. This apparent discrepancy may be explained by the systemic inflammatory environment in *me/me* mice, which is absent in dLck-Cre SHP-$1^{f/f}$ mice and therefore better reflects SHP-1-dependent effects. We used an IFNγ ELISA to measure the IFNγ produced by T cells in the supernatant after coculture with Tregs. Initial results suggested that SHP-$1^{-/-}$ T cells produced less IFNγ in the presence of Tregs compared to SHP-$1^{+/+}$ T cells. However, using ELISA to measure IFNγ in the supernatant did not account for the fact that SHP-$1^{-/-}$ T cells proliferate to a greater extent and therefore may "eat up" IFNγ at a greater rate. Thus, future studies must measure IFNγ production via flow cytometry in response to PMA/ionomycin stimulation of T cells before and after exposure to Tregs.

Previous studies suggest that Treg suppression of cytokine production can occur independently of suppression of proliferation[225]. CD4+ T cells were skewed *in vitro* to T_H1 cells and given TCR stimulation and CD28 costimulation, which allowed them to proliferate despite the presence of Tregs. However, Tregs suppressed production of IFNγ through a cell-contact dependent mechanism[225]. Thus, SHP-1 may or may not regulate the ability of T cells to resist suppression of effector function/cytokine production, since this appears to occur through a different pathway than suppression of T cell

activation/proliferation. The role of SHP-1 in regulating the susceptibility of effector T cells to Treg suppression of cytokine production could also be addressed *in vivo*. In a delayed-type hypersensitivity model, TCR Tg (OTII) T_H1 T cells are injected into a mouse foot pad along with APCs and OVA peptide, which causes a local swelling response from IFNγ secretion. Co-injection with Tregs can suppress the swelling[172], unless T_H1 cells are resistant to suppression.

Less is known about Treg suppression of CD8+ T cell effector function than suppression of CD4+ effector T cells. Chronic retroviral infections induce Tregs, which suppress CD8+ T cells, preventing clearance of virus[114,196,226]. CD8+ T cells are able to become activated and proliferate in response to viral Ag, but cannot differentiate effectively into CTLs and therefore do not produce perforin, granzyme-B, or IFNγ[196]. Previous work has shown that treatment of mice with anti-GITR or anti-4-1BB antibodies restores the ability of CD8+ T cells to exert cytolytic functions, resisting suppression by Tregs and clearing virus. Both GITR and 4-1BB provide costimulatory signals to the CD8+ T cells, suggesting that provision of greater costimulation allows CD8+ T cells to overcome Treg suppression. It is therefore possible that SHP-$1^{-/-}$ CD8+ T cells would be able to resist Treg suppression *in vivo* in a chronic viral setting. If so, adoptive cell transfer therapy of CD8+ T cells that have undergone siRNA knockdown of SHP-1[170] might be useful for clearing chronic viral infections, such as HIV, HSV, and HCV.

Future studies on the role of SHP-1 in regulating Treg function

In addition to defining the role of SHP-1 in regulating T cell susceptibility to Treg suppression, we have also found that SHP-1 regulates the suppressive function of Tregs.

Previous work from our lab showed that *me/me* Tregs are more suppressive than wildtype Tregs, both *in vitro* and *in vivo*[172]. To follow up on this work, we generated Foxp3-Cre SHP-$1^{f/f}$ mice, which mediates SHP-1 deletion specifically in Tregs. Preliminary data suggests that Foxp3-Cre+ SHP-$1^{f/f}$ Tregs are more suppressive than wildtype Tregs *in vitro*, but further investigation is required. Tregs from dLck-Cre+ SHP-$1^{f/f}$ mice also seemed to be more suppressive than dLck-Cre- SHP-$1^{f/f}$ Tregs, but were not as potent as Foxp3-Cre+ SHP-$1^{f/f}$ Tregs (Chapter 5). It remains to be determined whether differences in Treg suppressive ability between these two mouse models is due to the timing of SHP-1 deletion or differences in SHP-1 deletion efficiency.

Increased Treg suppressive capacity in dLck-Cre+ SHP-$1^{f/f}$ mice has important implications for using this model to study autoimmune disease responses. Based on the findings that dLck-Cre+ SHP-$1^{f/f}$ T cells are resistant to Treg suppression, one might hypothesize that induction of EAE, for example, would be more severe. However, if dLck-Cre+ SHP-$1^{f/f}$ Tregs have increased suppressive capacity, this may negate the effects of "Treg resistant" T cells, resulting in overall no difference in disease severity compared to wildtype mice. It is also possible that the Tregs in dLck-Cre+ SHP-$1^{f/f}$ mice would "win out" over SHP-$1^{-/-}$ T cells, and result in protection from EAE. Enhanced Treg function may also explain why we did not observe any overt signs of inflammation or development of disease in dLck-Cre+ SHP-$1^{f/f}$ mice. Induction of EAE in Foxp3-Cre+ SHP-$1^{f/f}$ mice would also be a useful model to determine whether Foxp3-Cre+ SHP-$1^{f/f}$ Tregs are more suppressive *in vivo*, resulting in protection from disease.

The mechanism by which SHP-$1^{-/-}$ Tregs exert enhanced suppression remains unclear, but our previous work showed that a greater percentage of *me/me* Tregs

upregulate expression of adhesion molecules and form longer-lasting conjugates with APCs[172]. It will be important to characterize the phenotype of Foxp3-Cre+ SHP-1$^{f/f}$ Tregs, since unlike *me/me* Tregs, the phenotype is T cell-intrinsic and arises in the absence of systemic inflammation. It would also be informative to compare the phenotype of Foxp3-Cre+ SHP-1$^{f/f}$ Tregs to dLck-Cre+ SHP-1$^{f/f}$ Tregs to determine if there are differences that would account for the apparent superiority of Foxp3-Cre+ SHP-1$^{f/f}$ Tregs. RNA-seq on Tregs from these mouse models at steady state as well as after TCR stimulation and costimulation may identify pathways that regulate Treg function and are enhanced by SHP-1-deficiency.

Future studies on efficacy of SHP-1 ablation in conjunction with adoptive cell therapy:

Cancer immunotherapy

Understanding the dual role that SHP-1 plays in different subsets of T cells is an important consideration for immunotherapy. For example, sodium stibogluconate (SSG), an inhibitor of SHP-1, was tested in phase I clinical trials for its efficacy in solid tumors[227,228]. While it worked in synergy with IFNα treatment, investigators did not assess whether SSG treatment augmented Treg function, which would be counterproductive to anti-tumor immunity. Furthermore, this may preclude future therapies that would attempt to inhibit SHP-1 directly in a tumor, e.g. via intratumoral injection. Tumors recruit Tregs[192] and therefore inhibition of SHP-1 would only enhance Treg suppression of tumor-specific T cells. Thus, therapies must aim to specifically inhibit SHP-1 in one subset of T cells depending on the context. Adoptive cell transfer

(ACT) therapy in conjunction with SHP-1 ablation would be the ideal approach for immunotherapies. CD8+ T cells can be isolated from patients, transfected with siRNA/shRNA to knockdown SHP-1, and re-introduced to the patient. This strategy has been successfully employed in a mouse model of leukemia. CD8+ TCR Tg T cells were transfected with shRNA against SHP-1, showing efficient knockdown[170]. SHP-1 KD CD8+ T cells were injected into mice, and demonstrated improved control of leukemia[170].

Preceding use of SHP-1 ablation in conjunction with ACT for tumor immunotherapy, further studies of the anti-tumor efficacy of SHP-$1^{-/-}$ T cells are needed. Only one report has investigated how SHP-$1^{-/-}$ CD8+ T cells provide improved tumor control, and it did not address the contribution of resistance to Treg suppression in controlling tumor outgrowth. The study suggested that SHP-$1^{-/-}$ CD8+ T cells exhibited enhanced short-term accumulation and greater cytolytic effector function against tumor cells[170]. The approach used for *in vivo* studies in chapter 3 could be applied here. *Rag1*$^{-/-}$ mice can be injected with B16-OVA melanoma followed by transfer of congenically labeled SHP-$1^{+/+}$ or SHP-$1^{-/-}$ OTI CD8+ T cells in the presence or absence of wildtype Tregs. Tumor control is expected to be greater with SHP-$1^{-/-}$ T cells, however co-transfer with Tregs will assess whether resistance to Treg suppression also impacts tumor control relative to wildtype T cells co-transferred with Tregs. It will also be important to determine whether SHP-$1^{-/-}$ polyclonal CD8+ T cells, rather than TCR Tg tumor-specific T cells, can adequately recognize and respond to tumor Ag, as this is clinically relevant. Importantly, SHP-$1^{-/-}$ CD8+ T cells did not induce autoimmune responses in leukemic mice, and ultimately formed the same number of long-lived memory T cells as

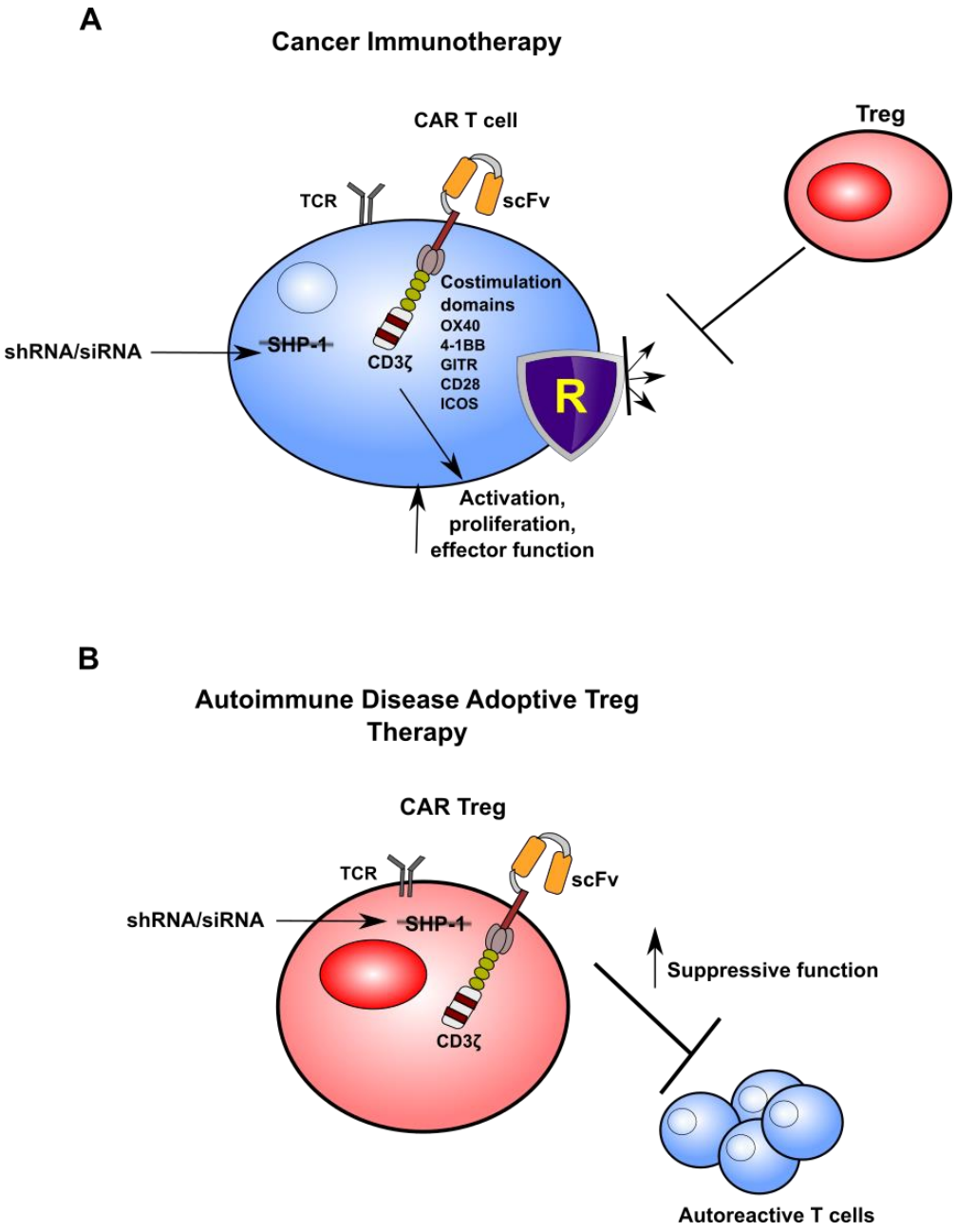

Figure 6.5. Adoptive T cell/Treg transfer immunotherapies for cancer and autoimmune disease incorporating SHP-1 ablation. (A) Current adoptive T cell transfer therapy for cancer utilizes engineered CAR-T cells to enable enhanced anti-tumor responses. Third generation CARs incorporate two costimulatory domains and the

CD3ζ domain fused to a scFv that will recognize tumor-associated antigens. Use of additional molecules/domains for CAR-T cell is currently being tested, but our work suggests that incorporating knockdown of SHP-1 through shRNA/siRNA would further enhance CAR-T cell anti-tumor efficacy. **(B)** Adoptive Treg cell transfer therapy for autoimmune disease relies on the isolation and expansion of a patient's Tregs. The success of CAR-T cell technology for cancer immunotherapy has led to the proposal of generating CAR-Tregs. We propose the incorporation of shRNA/siRNA knockdown of SHP-1 in Tregs to augment their suppressive function. More potently suppressive Tregs, when reintroduced to the patient, would be better able to suppress autoreactive T cell responses.

wildtype T cells[170]. Overall, SHP-1 ablation in T cells promises to be a viable cancer treatment strategy (Fig. 6.5A).

Autoimmune disease immunotherapy

Along the same lines, use of adoptive Treg therapy[229] could be combined with SHP-1 inhibition to augment Treg suppressive ability for autoimmune disease treatment. Thus far, several clinical trials have been undertaken for use of Treg adoptive therapy in the treatment of Type 1 diabetes[229] and GVHD in solid organ transplant[230]. Tregs are isolated and expanded *ex vivo* via anti-CD3, anti-CD28, and IL-2, then re-introduced into the patient. It remains unclear what the optimal number of Tregs for adoptive therapy is; too many Tregs can result in immunosuppression of responses against infection and cancer, while too few can be ineffective to treat autoimmune disease. As of now, trials have simply tested the use of different numbers of Tregs for therapy[229]. By augmenting Treg function through inhibition of SHP-1, patients may require a lower number of Tregs to effectively suppress autoimmune responses. This strategy may also overcome the problem of resistant effector T cells, which are a feature of many autoimmune diseases[50] (Fig. 6.5B).

6.3 Perspective

The field of immunology is constantly evolving in our understanding of the molecular and cellular bases of disease. We can now think of autoimmune disease as one end of a spectrum, on which the other end is cancer. This spectrum encompasses the

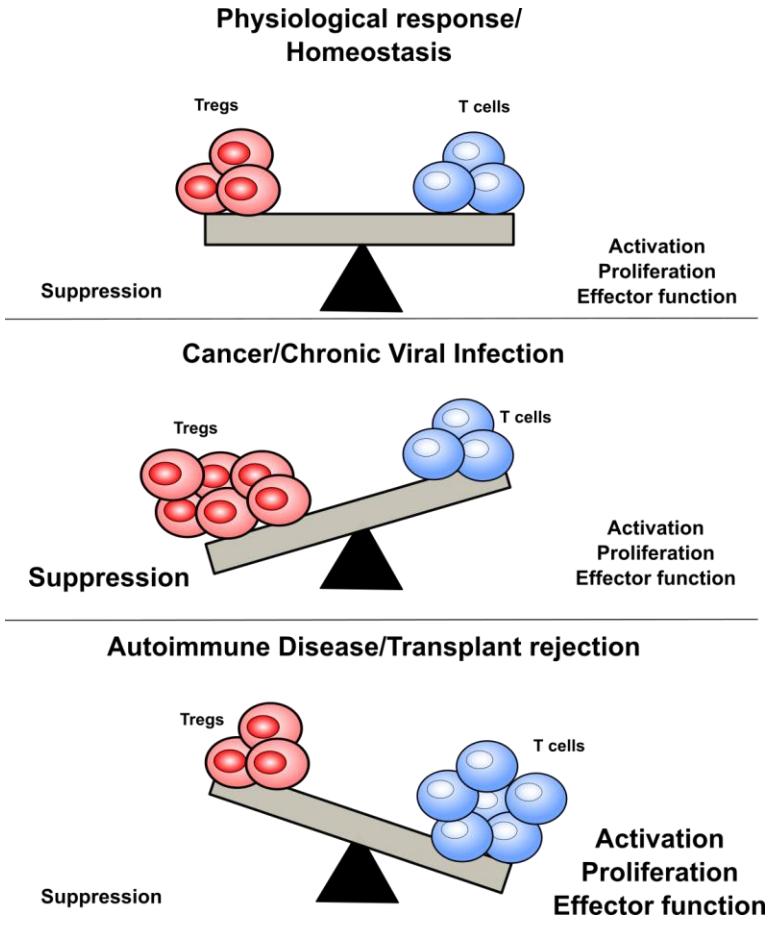

Figure 6.6. Balance between Treg suppression and T cell activation, proliferation, and effector function maintains immune homeostasis. During a physiological immune response, T cells transiently overcome Treg-imposed constraints, but ultimately become suppressible again. This balance is necessary to maintain immune homeostasis. In cancer and chronic viral infections, Tregs gain the upper hand and prevent anti-tumor T cell responses or viral clearance. In autoimmune disease, Tregs cannot adequately suppress autoreactive T cells, whether because of decreased Treg number, defects in function, or because T cells are resistant to suppression. Although the balance here is simplified to be represented by equal Treg and T cell numbers, there are many other factors that influence this balance.

ability of the immune system, in particular T cells, to recognize Ag and mount a response. In autoimmune disease, a break in tolerance allows autoreactive T cells to respond and attack self, and become insensitive to suppression by Tregs. Thus, the precarious "balance" is tipped too far in the favor of effector T cells (Fig 6.6). This is also seen in the case of GVHD, where although the response is not against self, effector T cells cannot be adequately controlled and mediate organ transplant rejection[231].

In cancer, effector T cells cannot respond and clear tumor cells for various reasons, among which is the establishment of an immunosuppressive environment dominated by Tregs[192]. Thus, the balance in this case is tipped too far in the favor of immunosuppression/Tregs (Fig. 6.6). Chronic viral infections also lie on this side of the spectrum, with these viruses inducing Tregs to maintain suppression of CD8+ T cell responses that might otherwise clear virus[232].

It is important to conceptualize this balance between T cells and Tregs when implementing treatment strategies, and use greater precision in attempting to modulate the balance. The "gold standard" therapy for autoimmune disease is immunosuppressants, which may ameliorate autoimmune disease symptoms but also impair the patient's ability to stave off pathogenic infections[233]. Unfortunately, these medications swing the balance too far in the suppressive direction and the patient will be more susceptible to infection and potentially, cancer. The same is true of cancer therapies that over-activate T cells, resulting in autoimmunity. For example, melanoma treatments can result in vitiligo, an autoimmune response against melanocytes[234].

In this era of the emergence of precision medicine[235], we should instead attempt to understand what lies at the heart of this interplay between effector T cells and Tregs. If

we can elucidate the molecular underpinnings of this important tenet of the immune response, as we have begun to do in the studies detailed in this dissertation, we can offer much more targeted approaches that avoid systemic effects. The development of CAR-T cell technology is an exemplary model of incorporating advances in our understanding of signaling pathways in T cells to strategically enhance their responsiveness to cancer[236]. With this technology, a patient's own T cells can be harnessed to eradicate tumors. CAR-T technology has also been proposed for the development of more potently suppressive Tregs to treat autoimmune disease[237].

Immune cell-based therapies represent the future of healthcare for an enormous number of people. Up to 50 million Americans currently suffer from autoimmune and autoimmune-related diseases (AARDA statistic[238]). Acute GVHD occurs in anywhere from 35-80% of transplant recipients depending on the degree of HLA-mismatch, and severe GVHD can result in transplant rejection and death[239]. Estimates suggest that in 2016 alone, 1.6 million Americans will be newly diagnosed with some type of cancer[240]. In terms of chronic viral infections, 36.7 million people worldwide are infected with HIV (AIDS.gov[241]), 257 million people worldwide have Hepatitis B virus, 71 million people worldwide have chronic Hepatitis C virus (WHO statistics[242,243]), and that does not even include other chronic viral infections like Epstein-Barr virus or cytomegalovirus. The studies undertaken in this dissertation could have a meaningful impact on immunotherapy for this vast array of diseases. Newer bioinformatics techniques, such as RNA-seq, will speed up the ability to identify pathways within immune cells that can be modulated in a given disease setting. Coupled with large-scale drug discovery efforts, we can expect to

see an increase in the modulation of signaling molecules in immune cells for successful immunotherapy.

Furthermore, we may gain insight into the mechanisms of currently used immunotherapeutics, with formerly unappreciated aspects of the immune response coming to the forefront. Our work alludes to the possibility that current cancer immunotherapies act, in part, by inducing T cells to resist Treg suppression. Further studies on the mechanisms by which SHP-1 regulates T cell susceptibility to Treg suppression are needed, to facilitate incorporation of SHP-1 ablation into the next-generation of CAR-T cells for cancer and CAR-Tregs for autoimmune disease.

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Appendix A

Review article:

Mercadante, ER, UM Lorenz. Breaking Free of Control: How Conventional T cells Overcome Regulatory T cell Suppression. 2016. *Front. Immunol.* **7**:193.

Abstract:

Conventional T (Tcon) cells are crucial in shaping the immune response, whether it is protection against a pathogen, a cytotoxic attack on tumor cells, or an unwanted response to self-antigens in the context of autoimmunity. In each of these immune settings, regulatory T cells (Tregs) can potentially exert control over the Tcon cell response, resulting in either suppression or activation of the Tcon cells. Under physiological conditions, Tcon cells are able to transiently overcome Treg-imposed restraints to mount a protective response against an infectious threat, achieving clonal expansion, differentiation, and effector function. However, evidence has accumulated in recent years to suggest that Tcon cell resistance to Treg-mediated suppression centrally contributes to the pathogenesis of autoimmune disease. Tipping the balance too far in the other direction, cancerous tumors utilize Tregs to establish an overly suppressive microenvironment, preventing anti-tumor Tcon cell responses. Given the wide-ranging clinical importance of the Tcon/Treg interaction, this review aims to provide a better understanding of what determines whether a Tcon cell is susceptible to Treg-mediated suppression and how perturbations to this finely-tuned balance play a role in pathological conditions. Here, we focus in detail on the complex array of factors that confer Tcon cells with resistance to Treg suppression, which we have divided into two categories: 1) extracellular factor-mediated signaling and 2) intracellular signaling molecules. Further, we explore the therapeutic implications of manipulating the phosphatidylinositol-3 kinase (PI3K)/Akt signaling pathway, which is proposed to be the convergence point of signaling pathways that mediate Tcon resistance to suppression. Finally, we address

important unresolved questions on the timing and location of acquisition of resistance, and the stability of the "Treg-resistant" phenotype.

Introduction:

It is well known that Tregs can employ a diverse repertoire of suppressive mechanisms, including secretion of suppressive cytokines, cytotoxicity, metabolic disruption, and modulation of antigen-presenting cell (APC) function[1]. Much work has been devoted to delineating how Treg suppressive mechanisms differ *in vitro* versus *in vivo*[2] and how these mechanisms function within specific tissues to shape immune responses[1,3]. Initially, it appeared that most models of autoimmune diseases featured either qualitative or quantitative abnormalities of the Tregs, rendering them inadequate to suppress autoimmune responses (for more detail, see[4]). This conclusion arose from the overwhelming evidence that systemic autoimmunity ensued in the absence of Tregs, as in day 3 thymectomy models[5], *Foxp3* mutation in mice (*scurfy*)[6] and humans (IPEX syndrome)[7], or even in Foxp3 conditional KO models[8,9]. Furthermore, genetic models where key components of Treg function are impaired, such as CTLA-4 KO[10] or IL-10 KO[11] mice, supported the idea that Tregs were necessary for immune tolerance, and were the likely culprits in autoimmune disease. More recently, there have been conflicting reports on whether Treg frequency and/or function is actually reduced in autoimmune disease[12]. Despite these discrepancies, both reduced Treg number and/or function remain as possible pathological mechanisms[12,13]. However, compelling evidence acquired over the past decade now suggests that Tcon cells that are refractory to Treg suppression also act as mediators of autoimmune disease in mice[14–22] and humans (see Table 1). It has

been clearly demonstrated that Tcon cells - including naïve (also called "Th0") T cells, differentiated effector T cells, and memory T cells - can become refractory to Treg-mediated suppression both *in vitro* and *in vivo*[14–20,22–36]. Tcon cells can become insensitive to Treg-mediated suppression when the ratio of Tcon cells to Tregs is skewed in favor of Tcon cells, when intracellular signaling pathways have been modified by mutations, or through extracellular signals, such as strong activation or a specific cytokine milieu, that induce Tcon cell-intrinsic changes[4]. The latter mechanism refers to potentially pathogenic Tcon cells that have become *resistant* to Treg suppression, a phenomenon which has been observed in several autoimmune diseases and is the focus of this review.

The current body of work on this topic predominantly addresses how Tcon cells escape *in vitro* Treg suppression, and how cells that have already become Treg-resistant *in vivo* can continue to resist suppression *in vitro*. The suppressive mechanisms employed by Tregs *in vitro* appear to be distinct from those used *in vivo*[2], complicating the interpretation of results from *in vitro* or *ex vivo* systems with regard to their applicability *in vivo.* For example, IL-2 is needed for Treg survival and homeostasis *in vivo*, but IL-2 signaling is not only dispensable, but counteracts Treg suppressive function *in vitro*[37]. Furthermore, Tregs are anergic and generally non-proliferative *in vitro*, but can expand *in vivo* after antigen encounter[2]. Despite these Treg differences, *in vitro* systems have provided insights into the molecular mechanism(s) of Tcon cell resistance to Treg suppression, mechanisms that may also be relevant *in vivo*.

The standard method for measuring Treg suppression of Tcon cells is an *in vitro* suppression assay, wherein suppression is the reduction of Tcon cell proliferation and/or

cytokine production compared to Tcon cells in the absence of Tregs. Resistance to suppression, therefore, is defined as an increased proliferation and/or cytokine secretion by Tcon cells in the presence of Tregs compared to that of a control Tcon cell (e.g. from a healthy patient, or not treated with a resistance-inducing factor). The use of CFSE or CellTrace proliferation dyes was an important technical advance that allowed investigators to gain more detailed information about Tcon resistance to suppression, which was not initially possible using ^{3}H-thymidine incorporation. By labeling Tregs or Tcon cells with separate proliferation dyes, investigators were able to directly measure the proliferation of Tcon cells independent of any Treg proliferation occurring in co-culture.

Early studies laid the foundation for the standard *in vitro* suppression assay by defining the conditions that allowed Tregs to suppress Tcon cells, as well as conditions that allowed Tcon cells to overcome suppression. Provision of strong TCR stimulation via platebound anti-CD3 allowed Tcon cells to proliferate even in the presence of Tregs, whereas lower concentrations of platebound antibody, or use of soluble anti-CD3 stimulation, allowed Tregs to suppress both proliferation and cytokine production by Tcon cells[38,39]. Additionally, strong costimulatory signals via anti-CD28 allowed Tcon cells to resist Treg suppression *in vitro* [38,40,41]. Physiologically, Tcon cells that only receive signal 1 (TCR stimulation) without concomitant signal 2 (costimulation) will become anergic and/or apoptotic[42]. Likewise, for Tcon cells to overcome Treg-imposed restraints and mount a protective response during infection, APCs must upregulate B7 molecules (CD80, CD86) in order to provide Tcon cells with strong costimulatory signals. This paradigm was demonstrated in a study by Norment and colleagues, who

showed that splenic dendritic cells (DCs), which upon activation express high levels of CD80 and CD86, induced Tcon cells to become refractory to Treg-mediated suppression[43]. In contrast, stimulation of Tcon cells by antigen-pulsed B cells or plasmacytoid DCs could induce Tcon cells proliferation in the absence of Tregs, but could not induce resistance to Treg suppression due to lower expression of costimulatory molecules[43]. The critical nature of costimulation was confirmed by another study, which found that anti-CD28 increased the number of Tcon cells producing IL-2 and accelerated the kinetics of IL-2 production, allowing resistance to Treg suppression[41]. Strong antigen dose alone did not alter IL-2 kinetics, and did not achieve the same level of Tcon cell resistance to Treg suppression. It was therefore suggested that costimulation allows Tcon cells to resist suppression in a manner distinct from strong TCR signaling alone[41]. This is consistent with the concept that costimulatory signals are required for optimal Tcon cell activation during an infectious threat, whereas lack of costimulation may provide a mechanism to maintain peripheral tolerance toward self[44].

These initial *in vitro* studies were the first to demonstrate Tcon resistance to suppression in a situation where Treg suppressive function remained intact. During a pathogenic infection, Tcon cells are provided strong TCR stimulation and costimulation, allowing them to circumvent Treg restraints in order to mount a response. By these rules, a low abundance of self-antigen coupled with weak costimulation favors Treg suppression of self-reactive Tcon cells that escaped negative selection, thereby preventing autoimmune disease. Of course, this ideal balance is not always maintained, and regulatory mechanisms gone awry result in disease.

1. Resistance-inducing mechanisms

1.1 Extracellular factors

1.1.1 Cytokine milieu

Autoimmune diseases are organ- or tissue-specific and characterized by overproduction of inflammatory cytokines. This is in line with the observation that numerous cytokines associated with autoimmune disease have been found to induce Tcon resistance to Treg suppression: IL-6[16,30,33,34,45–48], TNFα [16,32,49], IL-15[50–52], IL-21[18,47,53,54], IL-1β[55,56], and IL-4[57,58] (Figure 1). Beyond pro-inflammatory cytokines, IL-2 has also long been known to overrule Treg suppression *in vitro*[38,40,52]. One of the difficulties with experiments assessing the effect of cytokines on Treg suppression is that simply adding a cytokine to an *in vitro* co-culture system simultaneously affects Tregs and Tcon cells, making it difficult to distinguish whether there is impaired Treg function, Tcon cell resistance to suppression, or both. Many studies have therefore focused on downstream signaling pathways, or used genetic deletion of cytokine receptors, to delineate effects on Tcon cells independent of Tregs. The primary focus of this review is the discussion of factors that induce Tcon cells to resist suppression, with the caveat that many of these factors may also affect Treg function.

1.1.1.1 IL-6

Elevated levels of IL-6 have been found to play a pathological role in rheumatoid and juvenile idiopathic arthritis (RA, JIA respectively), systemic lupus erythematosus (SLE), multiple sclerosis (MS), inflammatory bowel disease (IBD), and allergic asthma[59]. Antibody blockade of IL-6 signaling has proven an effective treatment of RA and JIA, and ongoing clinical trials are investigating its use in SLE and Crohn's disease[59]. By far,

it has been the most frequently implicated cytokine in inducing Tcon cells to become resistant to Treg-mediated suppression[16,33,34,45–48]. Almost all immune cells produce IL-6, and its production is regulated by IL-1, TNFα, interferons, and other stress signals[59]. While toll-like receptor (TLR) signaling on monocytes and macrophages leads to IL-6 production during acute inflammation, T cells are the major producers of IL-6 during chronic inflammation[59]. Acting in concert with TGF-β, IL-6 induces Th17 cells, thereby preventing the induction of Tregs by TGF-β.

In terms of its role in Tcon resistance, Medzhitov and colleagues demonstrated that activation of DCs through TLRs, such as during bacterial infection, could overcome Treg-mediated suppression by producing IL-6. Their results showed that IL-6 alone was necessary but not sufficient to overcome Treg suppression, suggesting that TLR-activated DCs likely produced another cytokine that worked in tandem with IL-6 to induce resistance to Treg suppression[45]. It is likely that the DCs also produced TNFα, which has often been found to act along with IL-6 to induce Tcon resistance to Tregs. IL-6 has also been shown to drive Tcon cells to resist Treg-mediated suppression in a chronic inflammatory environment. Tcon cells isolated from the CNS of mice with experimental autoimmune encephalomyelitis (EAE), a mouse model of MS, produced high levels of IL-6 and TNFα and were resistant to Treg suppression[16]. IL-6 alone accounted for only half of the observed resistance to suppression, with the other half from an additive effect of TNFα[16]. Tcon cells from MS patients, when transferred into NOD/SCID mice, could not be suppressed by healthy donor Tregs *in vivo*[60]. Treatment with IFN-β restored susceptibility of MS Tcon cells to Treg suppression, concomitantly lowering IL-6R expression and IL-6 production[60]. Like EAE/MS, psoriasis is a Th1/Th17 driven

autoimmune disease characterized by a local inflammatory environment with high levels of IL-6[46]. In addition to Th17 cells producing IL-6 in psoriatic lesions, DCs and endothelial cells produce IL-6 as well, dampening Treg suppression[46].

Mechanistically, IL-6-mediated phosphorylation of STAT3 was found to be crucial in conferring Tcon cells with resistance to Treg suppression. Indeed, high pSTAT3 levels correlated with disease severity in MS[34]. Furthermore, IL-6 produced by MS Tcon cells *in vitro* was able to confer "bystander resistance" to Tcon cells from healthy control patients[33]. Treatment with pSTAT3 inhibitors restored Tcon cell susceptibility to suppression in cells from MS patients and in healthy control Tcon cells cultured with rhIL-6[34,47]. On the other hand,, IL-27, which also phosphorylates STAT3, could not induce Tcon cell resistance, suggesting signaling downstream of the IL-6-STAT3 axis specifically induces resistance[47]. In line with these findings, several studies demonstrated that IL-6-STAT3 signaling led to the activation of Akt (see Figure 1), and that Akt inhibition could restore Tcon susceptibility to Treg suppression[29,32,33]. Tcon cells isolated from the synovial fluid (SF) of RA patients have been shown *ex vivo* to resist Treg suppression[36]. Consistently, antibody-mediated neutralization of IL-6 has demonstrated clinical benefit in treating RA and JIA[32], and may also counteract Tcon cell resistance to Treg suppression. Although early studies questioned the ability of IL-6 to induce Tcon cell resistance in RA/JIA[50,52], more recent studies showed that IL-6, in combination with TNFα, allowed Tcon cells to resist Treg suppression. Blockade of both cytokines effectively restored Tcon susceptibility to suppression[29,32,61]. Thus, the current view is that IL-6, especially in combination with TNFα, is capable of inducing Tcon cells

to resist Treg suppression, providing an attractive therapeutic target for reducing inflammation and restoring suppressive balance in autoimmune disease.

1.1.1.2 TNFα

Like IL-6, antibody blockade of TNFα is clinically beneficial for RA and JIA, with it being the first cytokine identified as a therapeutic target in RA[62]. TNFα and IL-6 are often produced together in inflammatory settings like the synovium in RA or the CNS in EAE/MS; IL-17, interferons, or other stress factors can drive the production of both cytokines, and TNFα itself can drive the production of IL-6[16,59]. The complex feedback loops make it harder to dissect the exact role played by a cytokine with regards to Tcon cells acquiring resistance to suppression versus the effects on Tregs themselves. TNFα has been reported to act directly on Tregs to inhibit their suppressive capability[49]. When pre-incubating Tregs and Tcon cells with TNFα, Shevach and colleagues observed that TNFα did not affect Tcon cells' ability to resist suppression but rather inhibited Tregs from subsequently suppressing proliferation and cytokine production of Tcon cells[49]. TNFα signaled through TNFRII on Tregs, thereby downregulating the expression of Foxp3 and inhibiting Treg suppressive function[49]. An inverse correlation was reported between levels of IL-6 and TNFα in synovial fluid from RA patients and the percentage of Foxp3+ CD4+ Treg cells[61]. It is possible that in autoimmune diseases like RA, IL-6 may induce Tcon cells to resist Treg suppression, while TNFα acts on the other side of the equation to further prevent Tregs from suppressing Tcon cells. More recently, however, van Wijk and colleagues demonstrated that TNFα signaling activated Akt in Tcon cells allowing them to resist Treg suppression, as was seen with IL-6[29,32]. TNFα

blockade directly reduced Tcon cell proliferation, and potentiated suppression by Tregs[32]. *In vivo* treatment with a TNFα-blocking antibody did not affect Treg function, but reduced phospho-Akt levels in Tcon cells, thereby reducing their resistance to Treg-mediated suppression[32].

1.1.1.3 Common γ chain cytokines: IL-7 and IL-15, IL-2, IL-21, and IL-4

A role for common γ chain (γ_C) cytokines in Tcon resistance to suppression seems logical, as these cytokines generally promote T cell activation, proliferation, and survival[63]. IL-7 and IL-15 have been found at elevated levels in the synovial fluid from RA and JIA patients[50,52], and in the pancreas of murine models of Type 1 diabetes (T1D)[64,65]. Furthermore, IL-7 has been implicated in the pathogenesis of MS and SLE[65]. There are several reports of IL-7 and IL-15 inducing Tcon resistance to Treg suppression, either alone[50,51] or together[52,53]. It appears that both IL-7 and IL-15 act directly on Tcon cells to induce activation of the PI3K/Akt pathway (Figure 1)[51], possibly the mechanism by which Tcon cells become resistant. Thus, IL-7 and IL-15 represent another pair of cytokines that coincide in disease states and can synergize to induce Tcon cells to resist Treg suppression.

Early *in vitro* suppression assays revealed that IL-2 prevented Treg-mediated suppression, though the exact molecular mechanism remains unclear[38,40]. The effects of IL-2 on Tregs *in vitro* and *in vivo* remain complex and whether IL-2 directly induces Tcon cells to resist Treg suppression is unknown. It is possible that IL-2 signaling induces Treg resistance through activation of the PI3K/Akt pathway[66], but since naïve T cells do not express the IL-2 receptor[67], induction of resistance would occur after Tcon cells have become activated. A more recently characterized γ_C cytokine, IL-21 has been

shown to abrogate Treg suppression of Tcon cells *in vitro* and *in vivo*[18] without impairing Treg function[53]. Importantly, IL-21 did not increase baseline proliferation of Tcon cells, suggesting that resistance to Treg-mediated suppression can occur independently of mechanisms that simply enhance T cell proliferation[47,53]. IL-21 has also been found to promote T cell survival by signaling through the PI3K/Akt pathway[54], likely the mechanism allowing resistance to Treg-mediated suppression. Finally, IL-4 is another common γ_C cytokine with the capacity to induce Treg resistance. IL-4 signaling through STAT6 induced Tcon cells to resist Treg suppression[57,58]. IL-4 can activate the PI3K/Akt pathway in T cells[68], further suggesting that PI3K/Akt is a potential signaling "hub" for Tcon cell acquisition of Treg resistance.

1.1.2 Toll-like receptors

Toll-like receptors (TLRs) are an essential line of defense against microbial and viral pathogens. Various pathogen-derived ligands signal through TLRs, which recruit adaptor molecules such as MyD88 to trigger the production of pro-inflammatory mediators[69]. The goal of TLR signaling is to sense a pathogenic threat and mount innate and adaptive immune responses. TLR ligands can influence T cell responses via direct receptor activation or indirectly, by inducing APCs to produce cytokines that affect T cells[70,71]. For example, stimulation of DCs with LPS or CpG (TLR4 and 9 agonists, respectively) induced their production of IL-6, contributing to Tcon cell resistance to Treg suppression[45]. Studies of the effects of TLR agonists on Treg suppressive function are contradictory (discussed in[72]), with some suggesting that TLR signaling enhances suppressive function[70,73], while others show inhibition[74–77], or no change in suppressive

function but enhanced Treg survival[72,78]. While it is apparent that TLR signaling directly affects Tregs[71,79], there is also evidence that TLR signaling can directly induce Tcon cell resistance to suppression.

Both human and murine T cells express mRNA for TLRs 1-9, but protein expression levels vary and depend on the genetic background (in mice) and activation status of the T cell[71,79,80]. In general, TLR engagement acts as a costimulatory signal to T cells and subsequently activates the PI3K/Akt pathway, consistent with a role in inducing Tcon cells to resist Treg suppression[79,80]. CpG DNA signaling through TLR9 on Tcon cells induced IL-2 production, allowing them to escape suppression from MyD88$^{-/-}$ Tregs, which cannot respond to CpG DNA[75,81]. Similarly, TLR2 agonists induced Tcon cell resistance to suppression by TLR2$^{-/-}$ Tregs[82,83], with concurrent activation of the PI3K/Akt pathway[84,85]. Like cytokines, TLR signaling impacts both Treg and Tcon cells differentially and therefore must be carefully considered in the context of the overall Treg/Tcon balance. Initially, infection by a bacterial or viral pathogen requires temporary abrogation of Treg suppression in order to allow a T effector response. Thus, TLR agonists might transiently inhibit Treg suppressive function while simultaneously inducing resistance in Tcon cells, but as the infection is cleared and agonist levels wane, Tregs regain the ability to suppress. Studies in which Treg function was enhanced by TLR signaling speak to the possibility that Tregs are needed to regulate the inflammatory responses induced by bacteria[70]. It has been proposed that early during infection, TLR signals render Tcon cells resistant to suppression, and Tregs undergo expansion (perhaps due to IL-2 secreted by Tcon cells), so that the increased population of Tregs are later able to restrict and resolve the inflammatory response[73]. Thus, there is likely a complex

spatio-temporal regulation of induction of Tcon cell resistance to Treg suppression versus enhancement of Treg suppression by TLR signaling.

1.1.3 IL-1β

IL-1β is a potent pro-inflammatory cytokine associated with a wide array of inflammatory states, including some autoimmune diseases[86]. Monocytes release IL-1β in response to pathogen or "danger" signals[86]. Like TLRs, the IL-1R also contains a Toll/interleukin-1 receptor domain and utilizes MyD88 in signaling[87]. Tcon cells and Tregs both express the IL-1R, and IL-1β has been found to enhance the expansion and survival of T cells by activating NFκB and PI3K pathways[87,88]. IL-1β was found to inhibit Treg suppression of Tcon cells *in vitro*[55] by acting directly on Tcon cells rather than by impairing Treg function[56]. These data suggest that IL-1β may be another factor that, during pathogenic infection, allows Tcon cells to mount a response despite the presence of Tregs. It is possible that IL-1β also induces Tcon cell resistance to suppression in autoimmune disease settings, but this remains to be investigated. IL-1R antibody blockade is being used successfully to treat RA[89], which, in addition to its inflammation-dampening effects, may also reverse Tcon cell resistance to suppression.

1.1.4 TNF receptors

Engagement of certain tumor necrosis factor receptors (TNFRs) on T cells provides costimulatory signals that lead to activation, proliferation, differentiation, and survival[90]. In particular, the four TRAF-binding TNFRs described below have been found to render Tcon cells resistant to Treg suppression[91–98]. These TNFRs are constitutively expressed on Tregs and become upregulated on activated Tcon cells[96,99–101]. The ligands for these

TNFRs are generally expressed on APCs, but can also be induced on other cell types during infection[91,92,102]. TNFRs, like TLRs, play an important role during an infectious threat by allowing Tcon cells to become efficiently activated in order to mount a response, unrestrained by Tregs. It has therefore been proposed that TNFR ligand expression becomes upregulated during inflammatory conditions and provides costimulatory signals to both Tregs and Tcon cells, with Tcon cells becoming activated, producing IL-2, and resisting Treg suppression. As TNFR ligand levels wane and Tcon cells are no longer able to resist suppression, Tregs can assume control of the immune response[91].

1.1.4.1 GITR

GITR signaling in Tcon cells enhanced their proliferation and allowed them to resist Treg-mediated suppression[91]. In order to translate this into a therapeutically useful model, Gnjatic and colleagues activated tumor-specific CD4+ and CD8+ T cells in the presence of GITR signaling, making them become resistant to Treg suppression and able to control tumor growth[103].

1.1.4.2 4-1BB

Signaling through 4-1BB in Tcon cells has been shown to induce proliferation and enhance survival, especially in CD8+ T cells[104]. Treatment with agonistic 4-1BB antibodies has beneficial effects on CD8+ T cell-mediated viral clearance and anti-tumor immunity[104]. *In vitro* studies of 4-1BB signaling have shown a clear role for its CD28-independent costimulation of Tcon cells[104] as well as its ability to induce resistance to Treg-mediated suppression[93–95]. Likewise, *in vivo* treatment of mice with anti-4-1BB

induced CD8+ T cells to become resistant to Treg-mediated suppression in a chronic viral infection model[95]. 4-1BB regulation of Treg suppressive function remains controversial[93], but 4-1BBL is capable of *ex vivo* expanding Tregs for therapeutic use[94]. Therefore, 4-1BB signaling can induce proliferation of both Tregs and Tcon cells, but directly induces Tcon cells to resist Treg-mediated suppression, likely through costimulatory signaling. Interestingly, 4-1BB signaling has been shown to augment TCR-induced activation of the PI3K/Akt pathway[105], pointing again to a role for PI3K/Akt signaling in Tcon resistance.

1.1.4.3 OX40

OX40 signaling has been reported to both inhibit and enhance Treg suppressive function[96–98,106–108]. In contrast to these conflicting studies, it is clear that OX40 signaling provides costimulation for Tcon cells, promoting their survival and development into memory cells[109]. Several studies are in agreement that OX40 signaling in Tcon cells induces resistance to Treg-mediated suppression[96–98], possibly via PI3K/Akt activation[110,111]. The expression of OX40 is associated with many autoimmune diseases including SLE[111,112], RA[113], IBD[114–116], and GVHD[117]. In fact, Tcon cells from patients with active SLE had higher expression levels of OX40 compared to inactive SLE or healthy controls[111] indicating a possible correlation with resistance to suppression and thus autoimmune disease severity.

1.1.4.4 TNFR2

Originally characterized by its expression on activated/memory Treg cells, TNFR2 marks potently suppressive Tregs present in peripheral lymphoid tissues as well as in

tumors, but can also be induced upon TCR activation on Tcon cells[101]. While studies have shown that TNF signaling can inhibit Treg suppression, long-term exposure to TNF signaling via TNFR2 expanded Tregs and enhanced their suppressive function when given in combination with IL-2[118]. Intriguingly, TNFR2 expression correlated with the suppressive capability of tumor-derived Tregs, with TNFR2-negative Tregs being unable to suppress tumor-derived TNFR2-positive Tcon cells[119]. This suggested that TNFR2 expression marked a subpopulation of Tcon cells, which were more difficult to suppress and could only be controlled by the more potent TNFR2-positive Tregs. These data are reminiscent of the inherent ability of memory T cells to resist Treg suppression[120], although it was not determined whether TNFR2-positive Tcon cells represent memory T cells[119].

1.2 Intracellular signaling molecules linked to Tcon resistance

1.2.1 Cbl-b

Cbl-b is an E3 ubiquitin ligase that catalyzes the ubiquitylation of target proteins, which can result in their degradation by the proteasome, translocation inside the cell, or alteration in function[121]. In T cells, Cbl-b sets the threshold for weak antigen stimulation[122] and enforces the need for costimulation, or "signal 2", by regulating CD28 signaling[123]. Cbl-b negatively regulates the recruitment of the p85 subunit of PI3K to CD28, thereby enforcing T cell anergy and tolerance when signal 2 is lacking[124]. Upon CD28 signaling, Cbl-b itself becomes ubiquitylated and degraded, allowing PI3K recruitment and other downstream signaling required for full T cell activation[125]. Consistent with its negative regulatory functions, Cbl-b knockout (KO) mice develop

systemic autoimmunity due to hyper-proliferation and increased activation of lymphocytes, with T cells that can be activated in the absence of CD28 costimulation[126]. Cbl-b KO Tregs were found to be normal, whereas Tcon cells were found to resist suppression by both wild type and Cbl-b KO Tregs, *in vitro*[127] and *in vivo* in a graft-versus-host disease (GVHD) model[128]. In addition to CD4+ T cells, Cbl-b KO CD8+ T cells also resisted Treg-mediated suppression, providing a mechanism by which Cbl-b KO mice were able to spontaneously reject different types of xenograft tumors as well as ultraviolet-B light-induced skin cancer[129,130]. While the exact downstream mechanism of resistance in Cbl-b KO Tcon cells remains unclear, it is notable that Cbl-b KO T cells showed enhanced PI3K/Akt activation[124] (see below).

1.2.2 TRAF6

TRAF6 belongs to the E3 ubiquitin ligase family and transduces signals downstream of members of the TNFR superfamily, including IL-1R/TLRs[131], thereby activating NFκB, NFAT, MAP kinases, and Akt signaling pathways[131]. A role for TRAF6 in the negative regulation of T cell signaling was discovered by Choi and colleagues in 2006[132]. Their study demonstrated that TRAF6 KO mice developed multi-organ inflammatory disease characterized by hyper-activated T cells. Using mice in which TRAF6 was specifically deleted in T cells, the group showed that while TRAF6 KO Tregs were normal, the Tcon cells resisted Treg suppression both *in vitro* and *in vivo*[132]. Re-expression of TRAF6 via retroviral transduction restored susceptibility of Tcon cells to Treg-mediated suppression[132]. Like Cbl-b KO T cells, TRAF6 KO T cells could also be activated independently of CD28 costimulation, and showed enhanced Akt activation

upon TCR signaling. Importantly, sensitivity to Tregs could by restored by overexpression of PTEN, an inhibitor of PI3K/Akt[132]. These findings were also supported by human studies indicating that T cells from SLE patients had reduced induction of TRAF6 mRNA upon TCR stimulation, which correlated with increased levels of phospho-Akt and resistance to Treg suppression[111].

1.2.3 SHP-1

SHP-1, a protein tyrosine phosphatase, negatively regulates TCR signaling by dephosphorylating signaling mediators such as Zap70, Vav, Lck, and SLP76[133]. Many studies have demonstrated the ability of SHP-1 to regulate the threshold for TCR signaling (reviewed in[133]) and influence peripheral T cell activation and differentiation[134–137]. SHP-1 KO mice develop inflammation in skin and lungs due to myeloid hyper-proliferation[138,139]. These mice also accumulate memory T cells, and T cells are hyper-responsive to TCR stimulation[136,140–142]. We have previously reported that SHP-1 KO Tregs have an increased suppressive capacity[143]. Recently, we found that Tcon cells deficient in SHP-1 via genetic deletion or pharmacological inhibition, can resist Treg suppression *in vitro*[144]. SHP-1 has been described as a negative regulator of PI3K/Akt signaling[145], providing a possible mechanism for increased activation and resistance to Treg suppression. SHP-1 also negatively regulates activation of STAT3 in response to IL-6 signaling, with SHP-1-deficient cells being hyper-sensitive to IL-6[137]. Therefore, SHP-1 deficient Tcon cells may be more responsive to IL-6, resulting in activation of STAT3 and subsequent activation of PI3K/Akt. Like Cbl-b KO Tcon cells, SHP-1-deficient CD8+ T cells proved an effective method for improving anti-cancer

cytotoxicity[146,147] (see Cancer Immunotherapy section). Whether the enhanced anti-tumor activity was attributable to Tcon cells resisting Treg suppression remains to be addressed.

Tcon cells from the three aforementioned genetic KO models share the ability to become activated and proliferate with decreased dependence on CD28 costimulation[126,132,148]. This suggests that the perturbed signaling allows the cells to bypass the need for costimulatory signals that would ultimately activate PI3K/Akt and allow subsequent proliferation. Not only does this provide a means of identifying potentially Treg-resistant Tcon cells as those that do not require costimulation, but also reinforces the concept that the PI3K/Akt pathway is hyper-active in Treg-resistant Tcon cells.

1.3 PI3K/Akt: Node of convergence

Many of the above discussed studies directly demonstrated hyper-activation of the PI3K/Akt pathway in Tcon cells that resist Treg suppression. Evidence is accumulating to suggest that increased PI3K/Akt signaling may be at the heart of Tcon resistance. Wohlfert[149] was the first to propose that the PI3K/Akt pathway was central in allowing Tcon cells to resist suppression. Furthermore, murine models with genetic deficiencies in molecules that negatively regulate the PI3K pathway exhibit Tcon cells resistant to suppression[127,132,144,]. Most compelling is the finding that inhibitors of PI3K and/or Akt can reverse Tcon cell resistance to Treg suppression, making Tcon cells once again susceptible to suppression. This has been accomplished in several ways: by overexpressing the phosphatase PTEN (which antagonizes the activity of PI3K)[132], by using pharmacological PI3K inhibitors wortmannin and Ly294002[51], by using Akt

inhibitors (Akt inhibitor VIII)[29,33,111], or by inhibiting cytokine signaling thereby decreasing Akt activation[32]. Importantly, carefully titrated inhibition of PI3K and/or Akt did not affect the baseline proliferation of resistant Tcon cells, but instead returned their full susceptibility to suppression by Tregs[29,32,51,132].

It is unknown how increased activation of the PI3K/Akt pathway allows Tcon cells to overcome suppression, especially because the specific mechanisms of suppression employed by Tregs in a given setting vary. In T cells, signaling through the TCR and CD28 rapidly recruits and activates PI3K, but cytokines and other costimulatory receptors can similarly activate PI3K[150]. Lipid second messengers produced by activated PI3K bind to Akt and relocate it to the plasma membrane, where it becomes primed for activation[151]. Upon activation, Akt promotes proliferation by increasing cell size, inactivating cell cycle inhibitors, and increasing glucose metabolism, as well as enhancing cell survival and allowing cytokine production[152]. Mice in which T cells overexpress constitutively active PI3K or Akt develop lymphadenopathy and autoimmunity, underscoring the importance of regulated PI3K/Akt signaling in T cells[152,153]. Inhibition of pro-apoptotic factors such as Bim and the expression of anti-apoptotic factors such as Bcl-xL or Bcl-2 are downstream consequences of Akt activation, and a possible mechanism by which Tcon cells escape Treg suppression[54,154,155]. However, there is little evidence of Tcon cell apoptosis observed under *in vitro* suppression assay conditions, suggesting that alternative suppression mechanisms are overcome by PI3K/Akt activation[51]. Both Cbl-b KO and TRAF6 KO Tcon cells, which resist suppression, were still susceptible to Fas-mediated apoptosis[126,132]. Taking these studies into account, although PI3K/Akt activation

enhances Tcon cell survival, it does not seem to be the main mechanism by which Tcon cells resist Treg suppression.

Bypassing the need for costimulation is a likely candidate mechanism by which Tcon cells with hyper-activated PI3K/Akt can overcome Treg suppression. Tregs employ various molecules to effectively inhibit APC costimulation of Tcon cells[2]. For example, Tregs express CTLA-4, which binds to costimulatory B7 molecules (CD80, CD86) on APCs, leading to their downregulation and preventing Tcon cell costimulation[156]. Similarly, LAG3 on Tregs inhibits maturation of DCs to prevent them from activating Tcon cells[157]. Thus, engagement of CD28 with CD80 is inhibited, and Tcon cells fail to receive costimulation and subsequent PI3K/Akt activation[13]. Treg deprivation of costimulatory signaling would not affect genetically modified Tcon cells that do not require costimulation for full activation, such as Cbl-b, SHP-1, or TRAF6 KO Tcon cells. Furthermore, Treg-resistant Tcon cells from autoimmune diseases may receive adequate stimulation of the PI3K/Akt pathway through other means, such as cytokine, TLR, or TNFR signaling, eliminating the need for costimulation. In this way, any dysregulation of signaling events that lead to hyper-activation of PI3K/Akt can bypass those types of Treg suppression that are mediated by interference of costimulation. While this may not be the only suppressive mechanism overcome by PI3K/Akt hyper-activation, it is certainly a relevant suppressive mechanism both *in vitro* and *in vivo*[13,157]. Akt inactivates FOXO transcription factors, thereby allowing increased cellular metabolism and concomitant entry into cell cycle[152]. Thus, another possible mechanism to interrogate is whether enhanced PI3K/Akt signaling results in metabolic changes in Tcon cells that might allow resistance to Treg suppression.

It is important to note that resistance to suppression occurs in both naïve and memory Tcon cells[29,51,128] and that hyper-activation of PI3K/Akt induces resistance in both subsets[51]. Future studies should investigate which suppressive mechanism(s) Tcon cells are able to overcome when PI3K/Akt is hyper-activated, and whether these differ depending on the subset of Tcon cell. Interestingly, Tcon cells rendered hyper-responsive by NFATc2/NFATc3 double KO were also able to resist Treg suppression and become activated independently of CD28 costimulation[158]. NFAT proteins are regulators of T cell activation, inducing transcription of genes necessary for T cell responses[158]. However, the findings of this study suggest that NFATc2/NFATc3 also play a regulatory role in T cell activation, representing a signaling pathway aside from PI3K/Akt that can render Tcon cells resistant to suppression. This finding warrants further investigation into the signaling events that allow Tcon cells to become Treg-resistant, and whether there is a common molecular mediator downstream of both the PI3K/Akt and NFAT pathways.

2. Employing Tcon resistance for cancer immunotherapy

Many cancers develop within an immunosuppressive tumor microenvironment, which is detrimental to anti-tumor immunity. Thus, the ability to induce Tcon cells to resist Treg-mediated suppression would be a desired outcome for immunotherapy. There are several barriers to successful control and/or eradication of tumors, owing to the complex mechanisms that tumors employ to evade the immune system. First, the ability of T cells, namely CD8+ CTLs, to recognize antigen on tumors is impaired because tumor cells can decreased expression of MHC I, and because ongoing immune surveillance leads to tumor immunoediting[159]. Furthermore, many tumor-associated

antigens are in fact self-antigens, to which T cells remain tolerant through peripheral tolerance mechanisms such as Treg suppression[159]. Even when a T cell recognizes a tumor-associated antigen, lack of costimulatory signals prevents effective priming of the T cell. The preponderance of TGF-β secreted by many tumors not only suppresses T cell activation but can also convert T effector cells into Tregs[160]. Tregs are enriched in tumors, through chemokine-mediated trafficking to tumors, *de novo* generation, and preferential expansion due to the cytokine environment[160]. In many cases, the ratio of Treg/Teff cells is a prognostic indicator, with greater numbers of Tregs indicating a poorer prognosis[160].

Given these obstacles, treatment strategies have attempted to overcome Treg suppression and increase the activation and number of CTLs in the tumor. Treg depletion via anti-CD25 antibodies or inhibition of Treg function (through antibodies against molecules like CTLA-4), have had some success in boosting anti-tumor immunity, but typically require combination with tumor vaccines to be highly effective[160,161]. Problematic to these treatments is that Treg depletion is transient and Tregs recover quickly, and some depletion agents can also destroy T effector cells[160]. Adoptive cell transfer (ACT)[159] is another current treatment strategy, using patient-isolated tumor-specific CD8+ T cells and expanding them *ex vivo* typically with IL-2 or other cytokines. However, ACT is not always effective because transferred T cells do not persist well *in vivo* without the addition of exogenous cytokines, which can have adverse effects[162]. Tregs and the immunosuppressive tumor environment also impact the sustained function of the transferred CTLs[163]. Thus, investigators have begun to take advantage of the ability to enhance T cell signaling pathways to increase Tcon cell responsiveness (and,

potentially, induce resistance to Treg suppression) for use in cancer immunotherapy. To create more potent tumor-specific T cells that can be activated even in a suppressive microenvironment, chimeric antigen receptor (CAR) T cells are being utilized[164]. This approach has made use of intracellular signaling domains of costimulatory molecules in order to make the modified T cells hyper-responsive. One strategy was to fuse the intracellular domains of CD28 and the CD3ζ chain to an extracellular, CD19-targeting Ab (to recognize leukemic B cells), resulting in CAR T cells with enhanced proliferation, resistance to suppression by Treg cells *in vitro*, and acquisition of cytotoxic activity[165]. The previous generation of CAR contained only the CD3ζ fused to the CD19-recognizing Ab and also exhibited cytotoxic activity, but could not resist Treg suppression. An increase in NFκB activity was observed in CAR T cells containing the CD28 signaling domain. Although not assessed, it is likely that other signaling events downstream of CD28 were enhanced, such as PI3K/Akt, which may have conferred Treg resistance. Therefore, the possibility of inducing T cells to become resistant to Treg suppression and combining this with ACT or other immunotherapies is an attractive solution.

Many of the molecules discussed above that regulate Tcon cell resistance to Treg suppression have also been investigated for their role in anti-tumor immunity. One way to overcome the need for costimulation is by eliminating Cbl-b. Cbl-b KO mice spontaneously rejected TC-1 tumors and UVB-induced skin tumors[130], as well as thymomas[129], due to increased CD8+ T cell tumor infiltration and enhanced cytotoxicity. Importantly, despite there being a greater number of Tregs present in these tumors compared to wild type, the CD8+ T cells were resistant to Treg suppression[129,130]. Cbl-b

KO CD8+ T cells also inhibited the growth of disseminated leukemia[166] and melanoma[167] in mice. These studies clearly demonstrated the advantages to using T cells that have a lower threshold for activation, increased survival, and resistance to Treg- and TGF-β-mediated suppression in order to control tumor growth. It remains to be elucidated how T cell resistance to Treg suppression contributes to tumor control compared to simple hyper-responsiveness of the T cells, and whether or not resistance and hyper-responsiveness are two distinct characteristics of the T cells or represent an overall phenotype.

Similar to Cbl-b KO CD8+ T cells, SHP-1 KO CD8+ T cells also showed enhanced proliferation without the need for IL-2 supplementation[146]. In a model of disseminated leukemia, adoptively transferred SHP-1 KO CD8+ T cells decreased tumor size and increased survival rate, with the T cells demonstrating increased cytotoxicity and enhanced survival[146]. These results were recapitulated by adoptive transfer of tumor-specific T cells that underwent shRNA knockdown of SHP-1[146]. Similarly, a pharmacological inhibitor of SHP-1, sodium stibogluconate (SSG), showed improved anti-tumor immunity in a T cell-dependent manner[168]. While these studies did not directly assess the influence of Tcon resistance to Treg suppression on tumor control, our studies[144] suggest that SHP-1 KO T cells and Tcon cells from mice treated with SSG do in fact resist Treg suppression and would likely provide an additional advantage for enhanced tumor control.

As discussed above, TLR2 signaling inhibits Treg suppression and also confers Tcon cells with resistance to suppression. Not surprisingly, administration of a TLR2 ligand with an oncoprotein vaccine expanded T effector cells in the presence of Tregs and

increased median survival[78]. T effector cells became resistant to Treg suppression, upregulated Bcl-xL, and produced increased cytokines[78]. The effect was only elicited by the combination of a TLR2 ligand and the oncoprotein vaccine, but not by either alone. Similarly, in mice immunized with the tumor antigen mERK2 along with plasmids encoding GITR-L, antigen-specific CD8+ T cells were capable of inhibiting tumor growth and resisted Treg suppression[103]. In a CT26 tumor model, GITR agonist rendered CD4+ T cells resistant to suppression and capable of tumor control, as well[169]. OX40 signaling prior to tumor challenge also provided tumor control, but in a Treg-dependent manner[97]. In this model, OX40 signaling inhibited Treg suppressive function, while also boosting CD8+ T cell effector function[97]. This provides yet another example of the superior efficacy of treatments that not only inhibit Treg suppressive function but simultaneously boost T effector function.

PD-1 signaling in T cells is an inhibitory pathway meant to maintain tolerance by blocking T cell activation and downregulating PI3K/Akt signaling[170]. PD-1 blocking antibodies have shown success in the treatment of metastatic melanoma and non-small cell lung cancer[171]. Inhibition of this pathway resulted in greater CD8+ T cell differentiation into melanoma-specific CTLs even in the presence of Tregs, conferring them with resistance to Treg suppression, while also inhibiting Treg function[172]. These studies are consistent with the idea that increased activation of the Akt pathway allows T cells to resist Treg suppression, and that T cells resistant to suppression are better able to control tumor growth. Indeed, transducing CD8+ T cells with constitutively active Akt (caAkt) enhanced their cytotoxicity toward neuroblastoma[173]. The caAkt T cells showed increased proliferation and survival, and were resistant to Treg suppression, and had

reduced susceptibility to TGF-β-induced conversion into Tregs[173]. Future strategies for cancer immunotherapy should take into consideration the importance of inducing T cells to resist suppressive mechanisms and strive to better understand how Treg resistance re-shapes the immune response. Furthermore, current therapies may actually, in part, act by inducing Tcon resistance to Treg suppression, which is worth examining. Suited to the era of personalized medicine, therapies that induce Tcon resistance would be most beneficial in patients whose tumors have a high degree of Treg infiltration or a highly suppressive tumor microenvironment.

3. Remaining questions

While the characterization of the phenomenon of Tcon cells resisting Treg-mediated suppression has come a long way in the past decade, there are still several important questions left unanswered.

3.1 Where does the acquisition of resistance occur?

In autoimmune diseases, the local inflammatory environment enables Tcon cells to become resistant to suppression. However, there are also examples of Tcon cells acquiring resistance to suppression in the absence of inflammation, when TCR signaling is dysregulated (see Table 1). For example, Tcon cells isolated from the spleen or lymph nodes of mice with a T cell-specific SHP-1 deletion are resistant to Treg suppression *in vitro*[144]. Furthermore, CD8+ T cells targeted with siRNA to knockdown either Cbl-b or SHP-1 acquire resistance to Treg suppression[146,166], suggesting that at least under conditions of deficient regulatory molecules, T cells do not require an inflammatory

environment to become Treg-resistant. While not necessarily physiological, genetic deficiencies of intracellular signaling molecules have provided information about the mechanism of Tcon resistance and the pathways involved. It is possible that as a result of strong inflammatory signals received by a Tcon cell during autoimmune disease, molecules such as Cbl-b or SHP-1 are sequestered or degraded, so that they no longer regulate T cell signaling. Although this remains to be seen, the fact that Tcon cells can acquire resistance in a TCR-signaling-dependent manner in genetic knockout models suggests that acquisition of resistance might occur in secondary lymphoid organs (SLOs).

Studies of autoimmune disease in mice have demonstrated that Tcon cells isolated from sites of inflammation, as well as those from SLOs, are resistant to suppression. Similarly, Tcon cells from peripheral blood of autoimmune disease patients have been found to be resistant to Treg suppression. It is therefore difficult to determine whether Tcon cells became resistant in the inflamed tissue (e.g. synovium, pancreatic islets, CNS) and are re-circulating, or whether they acquired resistance in an SLO upon antigen and/or cytokine encounter. It appears that when certain conditions are met during TCR stimulation, such that the PI3K/Akt pathway becomes hyper-activated, a Tcon cell can become resistant to suppression. Given the number of documented pathways by which a Tcon cell can become resistant to suppression, it would seem that there is opportunity for naïve T cells, as well as differentiated effector and memory T cells, to acquire resistance, albeit possibly in different locations. It is likely that naïve Tcon cells acquire resistance in SLOs, as they would be primed in the SLO and have yet to traffic to a site of inflammation. Resistant T effector cells that are isolated from active disease settings may represent naïve Tcon cells that acquired resistance in an SLO, became activated, and

subsequently trafficked to a particular tissue, or may represent cells that became resistant in the inflamed tissue. It will be difficult to determine the location of acquisition of resistance in particular, but use of more sophisticated animal models in conjunction with *in vivo* imaging of Tcon cell activation status should help gain further insights. It is clinically relevant to pinpoint the location of acquisition of resistance in order to employ targeted therapeutic approaches, such as nanoparticle-directed delivery[174] of a compound that could reverse resistance in autoimmunity, or intratumoral injection of a compound to induce resistance in cancer[175].

3.2 How stable is the Treg-resistant phenotype?

When Tcon cells become resistant to Treg suppression, they undergo cell-intrinsic changes that mediate their resistance. Because of the limitations of *in vitro* suppression assays, many studies have assessed Tcon cell resistance *in vivo*. T cell transfer-induced colitis[132] and GVHD[176] mouse models provided insight as to how Treg-resistant Tcon cells function once transferred to an *in vivo* setting. Tcon cells deficient in TRAF6 or Cbl-b maintain Treg resistance when transferred into a host mouse, as demonstrated by induction of colitis[132] and GVHD[176] in the presence of otherwise protective Tregs. Perhaps not surprisingly, this suggests that despite removal from the inflammatory environment in which they developed, Tcon cells genetically deficient in specific molecules maintain resistance to Treg suppression. Likewise, CD8+ T cells lacking Cbl-b or SHP-1 maintain resistance *in vivo* despite their accumulation in a highly suppressive tumor microenvironment, and can successfully control tumor outgrowth[146,166].

There may be qualitative differences in just how stable the Tcon cell resistance program is, depending upon the circumstances of acquisition. Ideally, for a Tcon cell to respond to a pathogenic threat, it would transiently need to resist Treg suppression. Thus, an abundance of pro-inflammatory cytokines would drive the Tcon cell to resist suppression, perhaps through activation of PI3K/Akt signaling. When the cytokine concentration is reduced as the threat is cleared, signaling would wane and Tcon cells would once again be suppressible. Based on this paradigm, Tcon cells that become resistant in autoimmune disease likely stay that way because of aberrant and chronic cytokine production, the presence of self-antigen, and feed-forward autocrine loops. Tcon cells isolated from JIA patients maintained *in vitro* resistance to Treg suppression, producing high amounts of pro-inflammatory cytokines after 4 days in culture, likely reinforcing their own resistance through PI3K/Akt signaling[29,32]. However, blockade of IL-6 or TNFα signaling, or inhibition of Akt, could restore susceptibility to suppression[29,32]. Interestingly, Tcon cells isolated from MS patients have an accelerated kinetics of IL-6 production and resist Treg suppression and maintained resistance even after being cultured for 24 hours in the absence of any cytokines[33]. This is consistent with the idea that the cells may continue to produce excess cytokines to maintain a state of resistance, unless their ability to receive those signals is blocked, or PI3K/Akt is inhibited. Indeed, it was recently found that CD8+ T cells from the synovial fluid of JIA patients were able to self-sustain resistance to suppression by secreting large amounts of IFNγ, and only antibody blockade of IFNγ could restore susceptibility to suppression[177]. Overall, the Treg-resistant phenotype of Tcon cells appears to be relatively stable, able to persist in the absence of pro-inflammatory cytokines or other resistance-inducing factors.

Future studies will need to assess the ability of Tcon cells to maintain Treg resistance, especially in light of efforts to use adoptive Treg therapy for treatment of autoimmune diseases[178]. Infusion of Tregs into patients with Tcon cells resistant to suppression might prove to be ineffective, and should be examined further. Additionally, the stability of induction of Tcon cell resistance to suppression *ex vivo* should be investigated to determine if Tcon cells can maintain resistance in a suppressive tumor microenvironment for cancer immunotherapy.

3.3 What is the time window for a Tcon cell to become resistant?

In vitro, there seems to be a limited window of time during which a Tcon cell can resist Treg suppression. Whether a Tcon cell will become successfully activated and be able to proliferate or instead be suppressed by a Treg occurs early on in co-culture, within the first 6-12 hours[41]. Addition of pre-activated Tregs to culture with Tcon cells after 12 hours could not induce suppression of Tcon proliferation, which correlated with the peak of IL-2 production by Tcon cells[41]. These findings are consistent with the kinetics of cytokine-induced resistance to suppression. For example, IL-6 is able to induce Tcon cells to resist Treg suppression only if given within the first 16 hours of co-culture. Although there was a modest reduction of suppression if given at 24 hours, it was only half as effective as when given at 4 or 16 hours of culture[33]. Likewise, incubation of Tcon cells with IL-15 *in vitro* rendered them refractory to suppression owing to increased PI3K/Akt activation[51]. In this setting, PI3K inhibitors had to be added to culture within the first 24 hours or resistance could not be reversed[51]. *In vitro* studies of Treg suppression have provided valuable information regarding the window in which a Tcon

cell can become resistant, but the acquisition of resistance *in vivo* is likely a much more complex process. The mechanisms employed by Tregs to suppress Tcon cells *in vivo* are most likely different than *in vitro*, and depend on the anatomical location of the Treg[179]. *In vitro*, if a quorum of Tcon cells resist suppression and quickly produce cytokines, this might trigger nearby Tcon cells to also resist suppression as they are concentrated (in a well of a tissue culture dish). This is in contrast to a physiological setting, where only a small subset of T cells might be in close enough proximity to spread resistance via cytokine secretion. In the context of autoimmune disease, this begs the question, at what stage do Tcon cells become resistant to Treg suppression, and is it a causative factor of the disease or a consequence? If Tcon cells in autoimmune disease settings become resistant due to a preponderance of inflammatory cytokines, this would suggest that the disease must already be underway before resistance is induced. Indeed, Tcon cells from patients with inactive lupus nephritis showed a higher level of activated Akt compared to healthy control cells, but not as high as that from patients with active lupus, suggesting that the degree of resistance corresponds to severity of disease[111]. Therefore, a break in tolerance may be responsible for autoimmune disease initiation, but as the disease progresses, Tcon cells become Treg-resistant, exacerbating disease severity. It is yet to be determined whether *in vivo* treatment with PI3K and/or Akt inhibitors could reverse Treg resistance in established autoimmune disease, or whether there is only a short window during disease progression in which Tcon cell resistance can be blocked. This is not easily answered, as therapeutic PI3K/Akt inhibitors are currently unavailable. However, successful treatment of MS and RA/JIA symptoms using anti-IL-6 or anti-TNF therapy suggests that the cycle of Tcon cell resistance *in vivo* can be broken during ongoing

disease[59,62], and T cell-specific manipulation of PI3K/Akt pathway might be a future option for the treatment of autoimmune diseases and/or tumor immunotherapy.

Concluding remarks

Deepening our understanding of what determines the susceptibility of a Tcon cell to Treg-mediated suppression will prove extremely useful in advancing therapies for both autoimmunity and cancer. Although there are various mechanisms employed by Tregs to suppress Tcon cells, the PI3K/Akt pathway is a downstream point of convergence, representing an ideal therapeutic target. Already, efforts have been made to utilize Tcon cells resistant to suppression in controlling tumor outgrowth, and have shown promise as part of a combinatorial therapy. Further improvements upon autoimmune disease treatments could be made if the PI3K/Akt pathway could be specifically inhibited in out-of-control Tcon cells in order to rein them in. Finding the appropriate balance between Tregs and Tcon cells in different settings remains elusive, but further studies addressing the questions posed in this review will allow better manipulation of the delicate balance between Tregs and Tcon cells.

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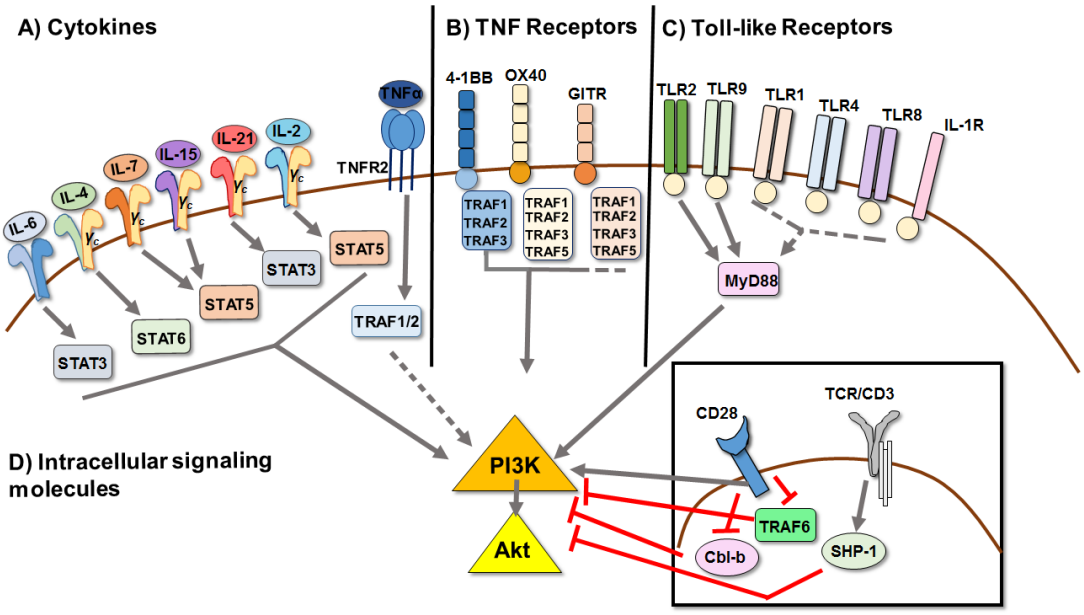

Figure 1. Convergence of Treg-resistance factors on the PI3K/Akt pathway.

(A) Cytokines IL-6, IL-4, IL-7, IL-15, IL-21, IL-2, and TNFα have been shown to induce Tcon cells to resist Treg suppression. The STAT molecule through which they predominantly signal is depicted. IL-6 signaling through Jak3/STAT3 induces activation of PI3K/Akt[33]. IL-4 signaling through Jak1/3 phosphorylates IRS-2 adapter molecule which then activates PI3K[68]. IL-7 similarly activates Jak1/3, which recruit and phosphorylate IRS-1/2, leading to recruitment and activation of PI3K[180]. IL-15 induces PI3K/Akt activation via recruitment of Shc, which recruits Grb2-Gab2 proteins to form a complex that then recruits and activates PI3K[155]. IL-21 can also induce PI3K activation, although the exact mechanism is unclear[54]. IL-2, like IL-15, activates Jak1/3, which recruit Shc, leading to formation of Grb-2/Gab-2 complex to recruit and activate PI3K[181]. TNFα signaling through TNFR2 recruits adaptor proteins TRAF1 and TRAF2, which may be able to recruit/activate PI3K. Signaling through TNFR2 was shown to sustain Akt activity induced by TCR/CD28 signaling[182], and TNFα induced Akt activation in T

cells[29]. (**B)** Signaling through TNF receptors 4-1BB, OX40, and GITR can induce Tcon cell resistance to Treg suppression, as they provide costimulatory signals similar to CD28 ligation. These TNF receptors recruit various TRAF adaptor proteins: TRAFS 1, 2, 3, and 5. Signaling through OX40 has been shown to recruit and activate PI3K via TRAF2[110]. 4-1BB signaling has been shown to augment TCR/CD3 induced PI3K/Akt activation[105], and promote phosphorylation of Akt[182]. GITR ligation has not been directly demonstrated to activate PI3K/Akt. Since TNFRs do not contain PI3K-binding motifs, it is likely that activation of PI3K is mediated by TRAFs. Evidence supports a role for TRAF2 in activating PI3K/Akt downstream of TNFRs[182]. (**C)** Toll-like receptors 1, 2, 4, 8, and 9, as well as IL-1R, also a member of the TLR family, have been shown to induce Treg resistance. TLR9 signaling recruits adaptor protein MyD88, which in turn recruits and activates PI3K via its Toll/interleukin-1 receptor domain[81]. Likewise, TLR2 engagement recruits MyD88 and subsequently activates PI3K/Akt[84,85]. TLR1 can induce resistance to Treg suppression[183], and TLR4 has been shown to indirectly induce resistance by stimulating DC production of IL-6[184]. TLR8 has not been shown to have direct effects on Tcon cells, but can inhibit the suppressive capacity of Tregs[74]. IL-1R signaling through MyD88 has also been shown to induce Tcon cells to resist Treg suppression, but whether this involves PI3K signaling was not investigated[56]. It is, however, possible that TLR1, 4, and 8, as well as IL-1R signal through MyD88 to activate PI3K in an as yet undescribed pathway. (**D)** Intracellular signaling molecules Cbl-b and SHP-1 act as negative regulators downstream of TCR signaling. Cbl-b enforces the requirement for CD28 costimulatory signaling by inhibiting the recruitment of PI3K to CD28. Upon CD28 signaling, PKCθ mediates the degradation of Cbl-b such that PI3K is recruited to CD28

and becomes activated[125,185]. SHP-1 dephosphorylates the p85 regulatory subunit of PI3K, preventing its recruitment and activation downstream of TCR signaling[145]. TRAF6 acts as an adaptor protein, transducing signals downstream of TNFRs and TLRs, resulting in the activation of multiple pathways including PI3K/Akt[186]. However, TRAF6 also negatively regulates activation of PI3K downstream of CD28 costimulation by an undefined mechanism[154,187]. Dashed lines indicate possible, but unconfirmed, links between receptors and/or signaling molecules and the PI3K/Akt pathway.

Table 1. Diseases in which Tcon cells resist Treg-mediated suppression.

Abbreviation: ND – not determined
CNS – central nervous system
[a]Teff – total synovial fluid or peripheral blood mononuclear cells (as indicated) isolated as CD4+ or CD8+
[b] Teff – contains both CD4+ and CD8+ Teff cells, isolated as CD3+

Disease	Subject	Type of effector cell	Suggested mechanism
Juvenile idiopathic arthritis (JIA)	Human	Synovial fluid CD4+CD25-	Enhanced activation[69]
		Synovial fluid CD4+ and CD8+ Teff[a]	Akt hyperactivation in response to IL-6/TNFα[70,73]
Rheumatoid arthritis (RA)	Human	Peripheral blood CD4+CD25-	Increased TRAIL expression on Teff leading to Treg apoptosis[77]
Type 1 diabetes (T1D)	NOD mice	Splenic CD4+CD25-	ND[56]
	DO11.10 RIP-mOVA mice	Lymph node CD4+CD25-	Increased IL-21[59]
	NOD mice	Splenic CD4+CD25-	ND[61]
	NOD mice	Splenic CD4+ and CD8+ Teff	Reduced ganglioside M1 expression on Teff[62]
	Human	Peripheral blood CD4+CD25-	ND[66]
		Peripheral blood CD4+CD25-	ND[65]
Systemic lupus erythematosus (SLE)	MRL/lpr and NZB/WF1 mice	Splenic and lymph node CD4+CD25-	ND[55]

	MRL/lpr mice	Lymph node CD4+CD25-	ND[60]
	Human	Peripheral blood CD4+CD25-	ND[68]
		Peripheral blood CD4+CD25-	ND[67]
		Peripheral blood CD4+CD45RA- FoxP3-	Akt hyperactivation, upregulation of OX40 and impaired TRAF6 in Teff [78]
	FoxP3.gfp KI mice	CNS CD4+GFP-	High IL-6 and TNFα[57]
	C57BL/6 mice	CNS CD4+CD25-	ND[58]
	B6.SLE mice	Splenic CD4+CD25-	ND[63]
	Human	Peripheral blood CD3+ Teff[b]	Accelerated production of IL-6 and higher expression of IL-6R on Teff leads to Akt hyperactivation[74]
		Peripheral blood CD4+CD25-	Increased IL-6 induction of pSTAT3[75]
		Peripheral blood CD4+CD25-	Increased Granzyme B production by Teff w/ TCR activation/IL-6 stimulation, inactivating Tregs[72]
Inflammatory bowel disease (IBD)	Human	Lamina propria CD4+CD25-	Higher expression of Smad7 interfering with TGF-β signaling[64]
		Lamina propria CD4+CD25-	Increased IL-15 in lamina propria[76]

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