1-1-2012

Signals Controlling Regulatory T Cell Differentiation and Homeostasis

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Abstract
CD4+Foxp3+ regulatory T cells (Treg)s are essential for the prevention of autoimmunity. Treg lineage commitment requires T cell receptor (TCR) interactions that induce expression of foxp3, whose protein product enforces Treg fate. Treg homeostasis is critical for self-tolerance and is achieved through both Treg generation and maintenance. Treg maintenance occurs in part through a process of self-renewing cell division of existing Tregs. This self-renewing Treg division has been shown to be TCR dependent. Despite the crucial role of the TCR in Treg generation and maintenance, neither the specific signaling pathways that control Treg generation nor the nature of the TCR signals required for their division in the periphery are well understood.

Here, we demonstrated that dendritic cells (DC)s coordinate Treg division in vitro. DCs elicit interleukin-2 (IL-2) production from conventional CD4+ T cells (Tconv)s in a major histocompatibility complex class II (MHCII)-dependent fashion. Tconv-derived IL-2 cooperates with contact-dependent signals from DCs to induce Treg division. Contrary to prior studies, we showed that in the presence of exogenous IL-2, Treg division becomes MHCII-independent in vitro. Treg division required only MHCII-independent DC-derived signals and a source of IL-2. Next, we found that peripheral Tregs can divide in the absence of TCR signaling in vivo if exogenous IL-2 receptor (IL-2R) agonists are administered. Furthermore, activation of the IL-2-induced STAT5 pathway is minimally sufficient to support Treg division independent of TCR signaling. These data suggest that depending on the degree of concomitant IL-2 receptor/STAT5 activation, a range of TCR signals can sustain Treg division.

In addition to Treg division, we also investigated the TCR signals that promote Treg development. Our preliminary experiments showed that diacylglycerol (DAG) signaling promotes Treg differentiation. Deletion of diacylglycerol kinase ζ, a negative regulator of this pathway, resulted in augmented DAG signaling and enhanced Treg generation. We hypothesize that DAG signaling enhances NF-κB activation through c-Rel, a transcription factor known to promote Treg differentiation directly. Future studies are necessary to establish the DAG pathway as a link between TCR signaling and Treg differentiation.

Degree Type
Dissertation

Degree Name
Doctor of Philosophy (PhD)

Graduate Group
Immunology

First Advisor
Gary A. Koretzky

Second Advisor
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SIGNALS CONTROLLING REGULATORY T CELL DIFFERENTIATION AND HOMEOSTASIS

Tao Zou

A DISSERTATION

in

Immunology

Presented to the Faculties of the University of Pennsylvania

in

Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy

2012

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DEDICATION

To my parents Mingteh Zou and Sueping Zhang

ACKNOWLEDGEMENTS

I am indebted to all the past and current members of the Koretzky and Kambayashi laboratories for their help, support, advice, and friendship throughout the past four years. In particular, I am grateful to Evann Corbo, Rohan Joshi, Jiyeon Kim, Matthew Pimentel, Matthew Riese, Atsushi Satake, Amanda Schmidt, Nikos Svoronos, and Enjun Yang for helping with experiments presented in this thesis. Ed Behrens, Martha Jordan, and Jonathan Maltzman have provided valuable mentorship. I would like to thank Mercy Gohil, Mariko Okumura, and Justina Standalick, without whom none of this work would be possible. I would also like to thank the members of the Behrens, Nichols, and Maltzman laboratories for discussions and collaborations. I would like to acknowledge the support and advice provided by Amar Majmundar and Waihay Wong. Additionally, I am appreciative of the help provided by Avinash Bhandoola and the members of his laboratory, especially Ellen De Obaldia and Dan Zlotoff. In particular, I am grateful to Dan Zlotoff for his friendship and our scientific interactions, which have influenced my thesis work immeasurably. I thank my thesis committee for their attention and critical analysis, which has improved the quality of this thesis. The Immunology Graduate Group and the MSTP Program have supplied continuous guidance. Gabrielle Wong has provided unwavering support, continuous encouragement, and advice throughout my graduate school career. I am appreciative of the friendship, support, and brilliance of the classmates I have been so fortunate to have during my education at Penn. A portion of my stipend was funded by a pre-doctoral training grant from the Cancer Research Institute, for which I am indebted to Warren Pear and Yvonne Paterson. Finally, I would like to offer my sincerest thanks to my co-mentors Taku Kambayashi and Gary Koretzky for all that they have taught me throughout graduate school. Their inexhaustible enthusiasm, unwavering optimism, endless patience, and dedication to teaching created a truly comforting and educational atmosphere. The guidance and mentorship Taku and Gary offered were invaluable and I owe them for all the time and effort they invested toward my development as a scientist.

ABSTRACT

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$CD4^+Foxp3^+$ regulatory T cells (Treg)s are essential for the prevention of autoimmunity. Treg lineage commitment requires T cell receptor (TCR) interactions that induce expression of *foxp3*, whose protein product enforces Treg fate. Treg homeostasis is critical for self-tolerance and is achieved through both Treg generation and maintenance. Treg maintenance occurs in part through a process of self-renewing cell division of existing Tregs. This self-renewing Treg division has been shown to be TCR dependent. Despite the crucial role of the TCR in Treg generation and maintenance, neither the specific signaling pathways that control Treg generation nor the nature of the TCR signals required for their division in the periphery are well understood.

Here, we demonstrated that dendritic cells (DC)s coordinate Treg division *in vitro*. DCs elicit interleukin-2 (IL-2) production from conventional $CD4^+$ T cells (Tconv)s in a major histocompatibility complex class II (MHCII)-dependent fashion. Tconv-derived IL-2 cooperates with contact-dependent signals from DCs to induce Treg division. Contrary to prior studies, we showed that in the presence of exogenous IL-2, Treg division becomes MHCII-independent *in vitro*. Treg division required only MHCII-independent DC-derived signals and a source of IL-2. Next, we found that peripheral Tregs can divide in the absence of TCR signaling *in vivo* if exogenous IL-2 receptor (IL-2R) agonists are administered. Furthermore, activation of the IL-2-induced STAT5 pathway is minimally sufficient to support Treg division independent of TCR signaling. These data suggest that depending on the degree of concomitant IL-2 receptor/STAT5 activation, a range of TCR signals can sustain Treg division.

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resulted in augmented DAG signaling and enhanced Treg generation. We hypothesize that DAG signaling enhances NF-κB activation through c-Rel, a transcription factor known to promote Treg differentiation directly. Future studies are necessary to establish the DAG pathway as a link between TCR signaling and Treg differentiation.

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Chapter I

Introduction

Cell-mediated immunity

The adaptive immune system is critical for the elimination of infectious pathogens. T lymphocytes, or T cells, constitute one arm of the adaptive immune response, termed cell-mediated immunity. These lymphocytes originate from the hematopoietic system and develop in a specialized organ called the thymus. T cells are responsible for the clearance of infected or otherwise damaged host cells and surveillance against tumor cells. However, cell-mediated immunity is not constitutive, as T cells must become activated upon the detection of pathogenic insults. Antigen presenting cells (APC)s, such as dendritic cells (DC)s, first recognize infectious pathogens and subsequently activate T cells by presenting a microbial-derived peptide fragment derived in the context of major histocompatibility complex (MHC) molecules to the T cell receptor (TCR). Upon engagement of the TCR, the activation of intracellular signal transduction pathways induce T cells to proliferate and differentiate into effector cells capable of carrying out protective functions. As invading pathogens are cleared from the host, the majority of activated effector T cells are culled through apoptosis, leaving populations of memory T cells to guard against future re-infection.

The health of the host depends not only on the successful activation of cell-mediated immunity, but also on the efficient contraction of the adaptive immune response after elimination of the pathogen. This suppression of T cell immunity is particularly important because of the presence of T cells that can recognize and become activated by host-derived antigens. Despite the existence of central tolerance, a mechanism that eliminates developing T cells recognizing host-derived antigens, studies describing the syngeneic mixed lymphocyte reaction (MLR) demonstrate that self-reactive T cell clones are readily produced and circulate throughout peripheral tissues (Von Boehmer and Adams, 1973; Von Boehmer et al., 1972). Despite the presence of these self-reactive T cell clones, autoimmune disease remains relatively rare. Although the mechanisms that restrain T cell responses and prevent the development of

autoimmunity were not known initially, the concept that a dedicated immune cell with suppressive function existed gradually emerged.

Regulatory T cells mediate self-tolerance: A historical perspective

In 1969, Nishizuka and Sakakura described a phenomenon in which thymectomy of mice between postnatal days 2 and 4 resulted in severe damage to its ovaries (Nishizuka and Sakakura, 1969). Although the reason for this observation was a mystery, the authors hypothesized that ovarian development required a growth factor produced by the thymus (Nishizuka and Sakakura, 1969). Almost concurrently, Gershon and Kondo described experiments showing that T cells could mediate tolerance, in addition to immunity, to antigenic challenge (Gershon and Kondo, 1970). Researchers soon established a link between the two phenomena and the mechanism of ovarian destruction in thymectomized mice was shown to be autoimmune (Miyara and Sakaguchi, 2007), supporting the existence of a T cell-mediated form of self-tolerance. Subsequently, the study of these suppressor T cells proliferated and complex models describing the their modes of function were constructed (Sakaguchi et al., 2007). Importantly, it was believed that a subset of these suppressor T cells expressed a protein called the I-J molecule, which was hypothesized to exist within the MHC gene region and to be critical to their suppressive function (Green et al., 1983). However, in the mid-1980's, molecular cloning of the MHC region revealed no such I-J genes (Kronenberg et al., 1983). This finding discredited the entire field of suppressor T cell biology, rendering further research on T cell-mediated suppression dubious. The term "suppressor T cell" became taboo within immunology, as investigative inquiry on self-tolerance shifted toward other mechanisms to dampen immunity.

In spite of these setbacks, the notion of a T cell population with suppressive function continued to intrigue several research groups around the world. One of the major issues that plagued the field of suppressor T cells was the lack of a reliable marker to distinguish these cells. In 1985, Sakaguchi and Masuda made the first inroads into this problem when they identified the CD5 molecule as a marker for T cells with suppressive function. They demonstrated that depletion of cells expressing high levels of CD5 from splenic $CD4^+$ T cells prior to transfer into

athymic mice caused a multi-organ autoimmune syndrome (Sakaguchi et al., 1985). Subsequently, in a 1990 study, Powrie and Mason utilized similar adoptive transfer experiments in rats to show that a subset of $CD4^+$ T cells expressing low levels of CD45RC conferred tolerance against an immune-mediated wasting disease (Powrie and Mason, 1990). Despite these advances, CD5 and CD45RB expression were relatively nonspecific markers for these suppressor T cells. In fact, a high frequency of all $CD4^+$ T cells expresses high levels of CD5 or low levels of CD45RB. The phenotypic characterization of suppressor T cells cumulated in 1995 with Sakaguchi and Toda's demonstration that immune tolerance in adoptive transfer models is mediated by a subset of $CD4^+$ T cells that constitutively express the α chain of the IL-2 receptor (IL-2R), CD25 (Sakaguchi et al., 1995). Since only 5-15% of all $CD4^+$ T cells expressed CD25, it proved to be the most specific marker of suppressor T cells to date.

The identification of CD25 as a reliable and specific marker for suppressor T cells, which have subsequently become known as regulatory T cells (Treg)s, represented a major advance for the field. Using CD25 as a marker, various groups confirmed Sakaguchi's initial findings that $CD4^+CD25^+$ Tregs were capable of mediating organ-specific self-tolerance. In a spontaneous model of experimental autoimmune encelphalomyelitis (EAE), Lafaille and colleagues demonstrated that Tregs were necessary to prevent disease (Lafaille et al., 1994; Olivares-Villagómez et al., 1998). Sakaguchi's group provided a mechanistic explanation for the autoimmune syndrome observed in neonatal thymectomy. They showed that adoptive transfer of Tregs after thymectomy at postnatal day 3 prevented multi-organ autoimmunity, implying that Tregs are not exported from the thymus in sufficient numbers in early neonatal life (Asano et al., 1996). Furthermore, Shevach's group demonstrated that the presence of Tregs prevented the development of autoimmune gastritis upon adoptive transfer of pathogenic gastritis-inducing TCR clones (Suri-Payer et al., 1998). These studies solidified the importance of Tregs for self-tolerance and increased interest in this once forsaken cell population. Multiple groups began to characterize further the markers that $CD4^+CD25^+$ Tregs express and the mechanism of Treg suppression. However, even after years of investigation, the mechanisms of Treg suppression remain controversial and incompletely understood (Miyara and Sakaguchi, 2007).

Despite the progress in identifying bona fide Tregs, CD25 expression was not a perfect measure for these cells since all activated T cells must upregulate CD25 to gain responsiveness to the growth factor interleukin-2 (IL-2) prior to proliferation. In 2001, a major finding in genetics revolutionized the study of Tregs. The causative mutation in the Scurfy mouse, which spontaneously develops a lethal autoimmune syndrome, was mapped to the *foxp3* gene locus (Brunkow et al., 2001). This locus encodes a forkhead box transcription factor, Foxp3. Autoimmunity in Scrufy mice is characterized by massive lympho-proliferation, immune cell infiltration of the skin, gastrointestinal tract, and multiple internals organs, and lethality by an average of three to four weeks of age (Godfrey et al., 1991). Notably, this disease was similar to a human syndrome termed immune dysregulation, polyendocrinopathy, enteropathy, X-linked, or IPEX. At the same time, the causative mutation in IPEX syndrome was found to localize to the human *foxp3* gene (Bennett et al., 2001; Wildin et al., 2001).

The potential involvement of the *foxp3* gene in human autoimmune disease created intense interest in determining its function. The loss of self-tolerance in both Scurfy mice and IPEX patients resembled the autoimmune syndromes observed in adoptive transfer models utilizing $CD4^+$ T cells depleted of Treg. This connection led Treg biologists to examine the potential link between the *foxp3* gene and $CD4^+CD25^+$ Tregs. Two years after the identification of the *foxp3* mutation in Scurfy mice, three groups described a critical role for Foxp3 in Treg generation and suppressive function (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003). It was determined that *foxp3* is specifically expressed in mouse $CD4^+CD25^+$or $CD4^+CD45RB^{low}$ Tregs (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003). Retroviral gene transfer of *foxp3* endowed T cells with both phenotypic and functional characteristics of Tregs (Hori et al., 2003). Generation of *foxp3* conditional knockout (KO) mice demonstrated that Foxp3 protein is necessary in a cell-autonomous fashion for Treg development (Fontenot et al., 2003). Furthermore, *foxp3* deficient mice recapitulated the lethal autoimmunity observed in Scurfy mice (Fontenot et al., 2003). Collectively, these data established *foxp3* expression as a potential master regulator of the Treg lineage. Consequently, Tregs are now defined by the expression of Foxp3 protein.

Importance of regulatory T cells in disease

As illustrated by Scurfy mice and IPEX patients, Treg suppression of immune responses is an essential mechanism of self-tolerance. A defect in Treg numbers results in autoimmune disease even when thymic central tolerance and peripheral mechanisms of tolerance remain intact. In addition to their tolerogenic role in induced-models of autoimmunity, subsequent studies have also implicated Tregs in the pathogenesis of mouse models of spontaneous autoimmunity, such as type I diabetes in the non-obsese diabetic (NOD) mouse (Salomon et al., 2000). Tregs have also been identified and studied in human autoimmune disease. Consistent with a breakdown of tolerance, Tregs from patients with a range of disorders from multiple sclerosis, rheumatoid arthritis, type I diabetes, and asthma display decreased suppressive function as compared to those from healthy donors (Cools et al., 2007). Despite the limitations of human studies, such consistent correlations in a wide variety of clinically significant conditions emphasize the importance of Tregs in human health.

Although many groups have demonstrated a role for Tregs in promoting self-tolerance, parallel studies have uncovered critical roles for Tregs in a variety of disease states. Indeed, the involvement of T cells with suppressive function in skin transplantation was recognized during the study of suppressor T cells in the 1970s (Kilshaw et al., 1975). In fact, prior to the identification of CD25 as a marker for Tregs, a study of cardiac transplantation in rats identified a population of suppressor $CD4^+$ T cells that express low levels of CD45RC and high levels of CD25 (Hall et al., 1990). Studies in mouse models show that expansion of alloantigen-specific Tregs can be critical in maintaining tolerance for both solid organ and bone marrow (BM) transplantation (Lechler et al., 2003; Wood and Sakaguchi, 2003). Data from human transplantation patients show an enhanced T cell response *in vitro* to graft-derived alloantigens, but not unrelated antigens, when the patient's peripheral blood is depleted of Tregs (Zhai and Kupiec-Weglinski, 2003). Collectively, these data suggest that an environment in which Treg suppressive function overwhelms conventional $CD4^+$ T cell (Tconv) effector activity favors long-term graft tolerance (Lechler et al., 2003; Li and Turka, 2010).

Just as the presence of too few Tregs is detrimental to the host, an excessive number of Tregs can be equally harmful. Indeed, mounting experimental evidence in mouse models and from human patients suggest that over-activity of Tregs can have negative consequences when strong immune responses are desirable, such as in malignancy and during infection. The presence of Tregs within solid tumors is well-documented in both mouse models and samples from human patients. In fact, the recruitment of Tregs to ovarian tumors in human patients is likely counterproductive as the presence of high numbers of Tregs predicts decreased survival in patients (Curiel et al., 2004). Tregs also inhibit immune responses to viral infections in mice, particularly those viruses that induce a state of chronic infection (Cools et al., 2007; Suvas et al., 2003). Similar to studies in transplantation patients, depletion of Tregs from human peripheral blood enhances the T cell response to viral antigens *in vitro* (Cools et al., 2007).

Due to the importance of Tregs in a multitude of diseases in mouse and man, there is much interest in the modulation of Treg numbers or suppressive function in human patients. Thus, many groups have devoted much attention and effort to understanding the mechanisms that control the production and maintenance of Tregs.

T cell development

Tregs develop and mature within the thymus along with all other T cell subsets. Therefore, a discussion of thymopoiesis is required to understand how Tregs are generated. T cell development proceeds in the thymus in discrete developmental stages. As T cell progenitors enter the thymus, they first progress through a set of four stages as double negative (DN) cells that express neither the CD4 nor CD8 co-receptors. In the first two DN stages, T cell precursors actively re-arrange the TCRβ chain in the thymic cortex. At the DN3 stage, the pre-TCR is formed from the pairing of TCRβ with pre-Tα. DN3 cells must pass a developmental checkpoint known as β-selection, which tests for the re-arrangement of a functional TCRβ chain. Those DN3 cells with a pre-TCR capable of signal transduction progress through β-selection and enter a state of extensive cellular proliferation. The thymocytes that emerge from this proliferative burst express both CD4 and CD8 co-receptors and are known as double positive (DP) cells. DP thymocytes re-

arrange the TCRα chain that pairs with the TCRβ chain to complete a bona fide TCR. DP thymocytes must then test their TCRs against antigens presented by MHC molecules. In a classic model, developing thymocytes must pass through a positive selection checkpoint in the thymic cortex, in which self-antigen/MHC complexes on cortical medullary epithelial cells (cTEC)s must induce weak TCR signaling to ensure thymocyte survival. Positively selected thymocytes begin migrating toward the thymic medulla, where negative selection occurs. In this process, thymocytes bearing TCRs that bind too strongly to self-antigen/MHC complexes presented on medullary thymic epithelial cells (mTEC)s or hematopoietically-derived DCs are eliminated by apoptosis. At some point during this process, the developing thymocytes also lose expression of one of its co-receptors to become either single positive (SP) $CD4^+$ helper or $CD8^+$ cytotoxic T cells. These mature SP T cells rapidly egress from the thymus to populate the peripheral lymphoid tissues.

Generation of natural regulatory T cells

The thymus is a critical source of Tregs as demonstrated by the neonatal thymectomy experiments in which removal of this organ results in a relative deficiency of peripheral Tregs, leading to widespread autoimmunity. Subsequent studies characterized the kinetics of early Treg ontogeny and showed that thymic ouput of Tregs is delayed by several days as compared to Tconvs output (Asano et al., 1996; Fontenot et al., 2005). These “natural” thymically-derived Tregs (nTreg)s are believed to constitute a majority of peripheral $CD4^+Foxp3^+$ Tregs. However, the developmental stage from which these cells arise and the signals that promote their generation within the thymus were not known.

A seminal study from Jordan and Caton, later confirmed by other groups, implicated a role for the affinity of the TCR for self-antigen in Treg lineage choice (Apostolou et al., 2002; Jordan et al., 2001; Kawahata et al., 2002; Walker et al., 2003). A complex mouse that expresses both a TCR specific for the S1 peptide of the influenza hemagglutinin (HA) protein (TS1 TCR) and the HA protein itself as a neo-self-antigen was generated (Jordan et al., 2001). In this double transgenic mouse, TS1 TCR-expressing $CD4^+$ T cells preferentially formed $CD4^+CD25^+$ Tregs

compared to control mice (Jordan et al., 2001). However, when the TS1 TCR was replaced with a second TCR with reduced affinity for HA (TS1(SW) TCR) in the double transgenic mouse, the resulting TS1(SW) TCR-expressing $CD4^+$ T cells contained the same frequency of $CD4^+CD25^+$ Tregs as control mice (Jordan et al., 2001). These data led to the conclusion that thymocytes bearing high-affinity TCRs for selecting antigens preferentially differentiate into Tregs, while those expressing low-affinity TCRs instead become Tconvs. Based on these data, several groups proposed a model for thymic selection in which the TCR affinity threshold required for Treg differentiation is above the threshold required for positive selection, but below the affinity necessary for negative selection. In this TCR affinity model, positively selected thymocytes with TCR affinities lower than the threshold for Treg lineage commitment differentiate into naïve Tconvs. Recent work from Caton's group has demonstrated that the TCRs capable of inducing both Treg differentiation and negative selection actually possess a higher affinity for selecting antigens than those TCRs that induce only negative selection (Cozzo Picca et al., 2011). These data argue for a reconfiguration of the prevailing model of Treg differentiation, suggesting that the TCR affinity threshold for Treg lineage commitment is higher than the threshold needed for negative selection (Cozzo Picca et al., 2011).

In contrast to these findings implicating an instructional role for the TCR in Treg differentiation, the absolute number of Tregs is not enhanced in double transgenic mouse models expressing either hen egg lysozyme or pigeon cytochrome c as neo-self-antigens and transgenic TCRs specific for peptides contained within these proteins (Liston and Rudensky, 2007; van Santen et al., 2004). Data from these alternative model systems suggest that rather than augmenting Treg differentiation, the co-expression of transgenic TCRs along with their cognate antigens merely induces enhanced negative selection of Tconvs while preserving Treg populations (Liston and Rudensky, 2007; van Santen et al., 2004). These studies argue that since Tregs are more resistant to negative selection, they do not increase in absolute number, but do increase in proportion to Tconvs (Liston and Rudensky, 2007; van Santen et al., 2004). Despite these alternative interpretations, the instructional role of the TCR in Treg differentiation has gained wide acceptance.

In support of the TCR affinity model of Treg differentiation, recent work has demonstrated the importance of precursor frequency in Treg lineage commitment. These studies utilized TCR transgenic mice expressing TCRs cloned from a polyclonal population of Tregs. Unexpectedly, these Treg-derived TCR transgenic mice contained either very few or no Tregs at all (Bautista et al., 2009; DiPaolo and Shevach, 2009; Leung et al., 2009). However, using mixed BM chimeras and intrathymic injections to achieve stepwise reductions in the frequency of T cell precursors from Treg-derived TCR transgenic mice resulted in the progressive augmentation of antigen-specific Treg generation (Bautista et al., 2009; Leung et al., 2009). These studies demonstrate that while TCRs are instructive for Treg differentiation, a separate Treg niche exists for each unique TCR clone (Bautista et al., 2009; Leung et al., 2009). These clonal Treg niches are rapidly filled at low precursor frequencies and appear important for the diversification of the Treg TCR repertoire. Although no mechanistic explanations for these findings have been identified, one possibility is that intraclonal competition for antigen/MHC class II (MHCII) molecules governs Treg differentiation for each TCR clone. Incorporating this notion into the TCR affinity model for $CD4^+$ T cell lineage commitment, one may speculate that the collective strength of each thymocyte's TCR interactions with antigen/MHCII determines its fate.

In addition to TCR signals, signaling from the common γ chain (γ_c) cytokines IL-2 and IL-15 is also necessary for thymic Treg differentiation (Burchill et al., 2007b; Cheng et al., 2011). IL-2 was initially identified as a T cell mitogen in 1965 (Cheng et al., 2011). Because of its role in stimulating T cell growth and activation, it was surprising that mice deficient in IL-2 or the components of the IL-2R exhibited T cell lymphoproliferation and autoimmunity (Cheng et al., 2011). Subsequent studies demonstrated that adoptive transfer of $CD4^+$ T cells from wild-type (WT) mice prevented disease in mice deficient in IL-2 signaling (Malek et al., 2002; Wolf et al., 2001). These data were reminiscent of Sakaguchi's early adoptive transfer experiments with $CD4^+CD25^+$ Tregs. Indeed, mice deficient in IL-2 signaling contained many fewer Tregs than WT mice (Cheng et al., 2011), leading to their autoimmune syndrome. The source of IL-2 in the thymus appears to be other developing thymocytes, as the Treg frequency in IL-2 KO $CD4^+$ SPs is normalized when WT thymocytes are present in a mixed BM chimera setting.

Although IL-2 is the critical cytokine for Treg development, mice deficient in IL-2 signaling still contain half the number of Tregs as WT mice (Cheng et al., 2011). In this case, IL-15 can compensate for the loss of IL-2 (Burchill et al., 2007b). Indeed, combined deficiency of both IL-2 and IL-15 results in a dramatic loss of Treg numbers (Burchill et al., 2007b). Signal transducer and activation of transcription 5 (STAT5) signaling mediates the effects of IL-2 signaling in Treg development. Mice lacking STAT5 recapitulate the phenotype observed in mice lacking IL-2 signaling (Burchill et al., 2007b). Furthermore, expression of a constitutively active STAT5 protein (STAT5b-CA) or a truncated IL-2Rβ chain capable only of STAT5 activation restores Treg development, peripheral Treg homeostasis, and self-tolerance in IL-2Rβ KO mice (Burchill et al., 2007b; Yu et al., 2009). Finally, STAT5 directly controls commitment to the Treg lineage by binding to the *foxp3* gene to promote its transcriptional activation (Burchill et al., 2007b).

Although instruction from TCR and IL-2R signals are both critical for Treg differentiation, the relative order in which these signals act on precursor thymocytes to induce their differentiation into Tregs was not clear. CD25 expression is first found in DN2 and DN3 thymocytes whereas the mature TCR is not expressed until the DP stage. A related question is whether there is a dedicated Treg precursor cell in the thymus or whether multiple subsets of thymocytes have the capacity to differentiate into Tregs. Recent work has begun to elucidate these questions. Parallel studies identified a $CD4^{+}Foxp3^{-}CD25^{+}$glucocorticoid-induced tumor necrosis factor receptor-related protein $(GITR)^{high}$ thymocyte population (direct Treg precursor cells) that has enhanced potential to develop into Tregs. These Treg precursor cells preferentially differentiate into Tregs upon exposure to either IL-2 or IL-15 (Burchill et al., 2008; Lio and Hsieh, 2008). This cytokine-induced Treg differentiation occurs independent of MHCII and cell division (Lio and Hsieh, 2008). Based on these data, a two-step model for thymic Treg differentiation has been proposed. In the first step, an upstream T cell precursor receives the appropriate TCR stimulation to instruct it to differentiate into a direct Treg precursor cell. This direct Treg precursor, which has upregulated CD25, then receives an IL-2 or IL-15 signal to differentiate into a bona fide Treg (Burchill et al., 2008; Lio and Hsieh, 2008). Despite this advance, the identity of the upstream T cell precursor that gives rise to the direct Treg precursor is still unknown. Although Foxp3 protein can be

detected in DP thymocytes, the significance of Foxp3$^+$ DPs remains unclear (Liston and Rudensky, 2007). Conversely, a recent study has suggested that multiple immature thymocyte subsets have the potential to differentiate into Tregs. It was shown that the potential of thymocyte precursors to differentiate into Tregs varies inversely with their developmental stage and maturity (Wirnsberger et al., 2009). Future investigation is required to clarify the physiologic upstream progenitors of Tregs.

Unlike the straightforward role of IL-2 in Treg development, the requirement for transforming growth factor β (TGF-β1) is more complicated. Initial characterization of TGF-β1 KO or TGF-β receptor I KO (TGF-βRI) mice found normal frequencies of thymic Tregs, suggesting that TGF-β signals are dispensable for nTreg generation (Fahlén et al., 2005; Li et al., 2006; Marie et al., 2005). Subsequent analysis of TGF-βRI KO mice revealed a very early defect in thymic Treg generation between postnatal days three and five (Liu et al., 2008). Despite this early defect, compensatory proliferation of thymic Tregs beginning one week after birth restored the frequency of Tregs in the thymus to normal (Liu et al., 2008). Both enhanced production of IL-2 by Tconvs and heightened Treg sensitivity to IL-2 mediated the compensatory recovery of thymic Tregs in the absence of TGF-β signaling (Liu et al., 2008). Subsequent biochemical studies demonstrated that TGF-β signaling induces the nuclear translocation of Smad3, allowing Smad3 to bind to the *foxp3* gene enhancer to promote *foxp3* expression (Tone et al., 2008).

Along with cytokine signals, costimulatory signals through CD28 are also required for optimal Treg generation. As was the case with IL-2, CD28 stimulation was first documented to be important for T cell growth by providing costimulation that is necessary for optimal IL-2 production. As was the case with mice deficient in IL-2 signaling, it was discovered that CD28 KO mice possess significantly fewer Tregs than WT mice (Salomon et al., 2000; Tang et al., 2003). Although CD28 KO Tconvs are defective in IL-2 production, CD28 signaling also has a cell-intrinsic role in Treg differentiation as shown by mixed bone marrow chimera studies (Tai et al., 2005). Generation of transgenic mice harboring mutations in the CD28 cytoplasmic chain revealed a critical role for the Lck-binding motif in supporting Treg development (Tai et al., 2005).

Detailed analysis of signaling downstream of CD28 is necessary to determine which intracellular pathways mediate Treg generation.

Despite the extensive characterization of the molecular signals required for Treg generation, there is conflicting evidence regarding the identity of the thymic accessory cells that provide these inductive signals to Treg precursors. The majority of Tregs are found in the thymic medulla, suggesting that Treg induction occurs in this region. Additionally, CD28 ligands are found at the highest concentration in the medulla (Liston and Rudensky, 2007). However, mice in which MHCII is expressed solely in the thymic cortex still generate thymic Tregs, suggesting that cTECs are sufficient to induce Treg differentiation (Bensinger et al., 2001). Conversely, other studies indicate that mTECs and thymic DCs together induce Treg differentiation rather than cTECs (Aschenbrenner et al., 2007; Liston et al., 2008; Proietto et al., 2008). Evidence from both sides depends on complicated transgenic mouse models that may not recapitulate thymic physiology. Thus, until the upstream precursors of Tregs are determined, the definitive lineage tracing experiments will be lacking.

Much has been learned in the past 15 years about the requirements for Treg generation in the thymus. Despite a great deal of progress, questions regarding this complex process remain. Future investigation into these questions is critical, since nTregs are thought to represent the majority of peripheral Tregs.

Generation of inducible regulatory T cells

Around the same time that Sakaguchi initially identified $CD4^+CD25^+$ Tregs, a group led by Weiner had been studying a phenomenon called oral tolerance. Oral tolerance refers to a state of systemic hyporesponsiveness to antigen that is induced with oral feeding of that antigen. The mechanisms underlying oral tolerance were not clearly defined, although TGF-β isolated from the intestines of orally tolerized mice appeared to be important. Using a TCR transgenic model of EAE, Weiner's group demonstrated that a population of regulatory $CD4^+$ T cells capable of producing TGF-β mediated oral tolerance and resistance to EAE induction (Chen et al., 1994). Subsequent experiments demonstrated that introduction of low doses of antigen through the oral

or subcutaneous routes resulted in the induction of Tregs through the conversion of mature peripheral Tconvs (Apostolou and von Boehmer, 2004; Thorstenson and Khoruts, 2001; Zhang et al., 2001). It is now known that Tregs can be induced from naïve Tconvs through a number of different methods both *in vitro* and *in vivo* (Curotto de Lafaille and Lafaille, 2009). Optimal generation of these inducible Tregs (iTregs) requires low doses of high-affinity cognate antigen, TGF-β, IL-2 or CD28 co-stimulation, and specialized migratory populations of skin-derived or mucosal DCs that secrete retinoic acid (Curotto de Lafaille and Lafaille, 2009; Gottschalk et al., 2010). These iTregs are thought to exist predominantly at interfaces between the host barrier surfaces and the environment, such as the lungs, skin, and intestines (Curotto de Lafaille and Lafaille, 2009).

At the current time, iTregs and nTregs are largely indistinguishable at the transcriptional, phenotypic, and functional levels (Curotto de Lafaille and Lafaille, 2009). The most reliable distinguishing feature observed between iTregs and nTregs is a different pattern of epigenetic marks at the *foxp3* gene locus (Curotto de Lafaille and Lafaille, 2009). Recently, Shevach's group has suggested that the transcription factor Helios is specifically expressed in nTregs, but not iTregs (Thornton et al., 2010), while others have argued that Helios is not a reliable marker of nTregs (Verhagen and Wraith, 2010). Therefore, no reliable marker currently exists to distinguish nTregs from iTregs. The identification of a marker that is specific to nTregs or iTregs remains a major source of inquiry. Due to the similarities between these two subsets of Tregs and the lack of markers to differentiate between them, the fraction of total Tregs that derive from either the thymus or peripheral conversion of Tconvs is unclear (Curotto de Lafaille and Lafaille, 2009). For the same reason, it is difficult to ascribe differential functions to nTregs or iTregs. However, accumulating evidence suggests that iTregs may be important for suppressing immune responses to a range of stimuli such as allergens, food antigens, commensal bacteria, chronic inflammation, tumor neo-antigens, and infectious agents (Curotto de Lafaille and Lafaille, 2009). Regardless of these difficulties, it is important to remember that a proportion of peripheral Tregs are iTregs derived from conversion of naïve Tconvs.

T cell receptor signaling in regulatory T cell development

Although the TCR plays a central role in Treg generation, the particular downstream signaling pathways that govern Treg differentiation are incompletely understood. There is precedent to indicate that the activation of specific TCR-induced signaling pathways mediate cell fate decisions during T cell development. Studies examining positive and negative selection during thymic development have implicated essential roles for the Ras/extracellular signal-regulated kinase (ERK) and Jun amino-terminal kinase (JNK) pathways (Alberola-Ila et al., 1995; Daniels et al., 2006; Swan et al., 1995). It is known that weak TCR interactions with peptide/MHC molecules induce the activation of ERK and direct the its subcellular localization, allowing ERK to mediate positive selection (Daniels et al., 2006). Developing thymocytes from mice lacking both ERK1 and ERK2 fail positive selection and consequently these mice contain few mature thymic SP T cells (Fischer et al., 2005). Meanwhile, strong TCR interactions with peptide/MHC molecules results in a different pattern of subcellular ERK localization that may allow activated JNK to mediate negative selection (Daniels et al., 2006). Thus, activation of the Ras/ERK pathway may translate the strength of TCR stimulation to intracellular signals that determine cell fate decisions during thymic selection. As with positive and negative selection, it is likely that specific TCR-induced signaling pathways control differentiation down the Treg lineage. To evaluate which TCR-induced signals may promote Treg development, an understanding of signal transduction downstream of TCR activation is necessary.

Upon ligand engagement of the TCR, Src-family protein tyrosine kinases (PTKs) phosphorylate tyrosine residues in immunoreceptor tyrosine-based activation motifs within the TCR complex. Subsequently, the Syk family PTK ζ-chain-associated protein kinase 70 (ZAP-70) is recruited to the TCR complex through its Src homology 2 domains. ZAP-70 then phosphorylates tyrosine residues on the protein adaptor LAT. Phosphorylated LAT behaves as a scaffold for the formation of a multimolecular signaling complex that also includes the adaptor Src homology 2 domain-containing leukocyte protein of 76 kilodaltons (SLP-76). The activated LAT/SLP-76 adaptor complex recruits phospholipase C-γ1 (PLC-γ1), which cleaves the membrane lipid phosphatidulinositol 4,5-bisphosphate (PIP_2) into the second messengers inositol

1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). These second messengers mediate downstream TCR signal transduction through a variety of pathways.

A major outcome of IP_3 signaling is the mobilization of intracellular Ca^{2+} stores within the endoplasmic reticulum (ER). Release of ER-stored Ca^{2+} initiates a sustained influx of extracellular Ca^{2+}. This signal activates the Ca^{2+}-regulated phosphatase calcineurin, which in turn dephosphorylates nuclear factor of activated T cells (NFAT) family members. This protein modification allows NFAT proteins to translocate into the nucleus where they function as transcription factors. Notably, NFAT has been shown to bind to an enhancer element in the *foxp3* locus to promote its transcription (Tone et al., 2008). Thus, Ca^{2+}-mediated TCR signals are likely important for Treg development.

Meanwhile, the generation of DAG results in the activation of a number of downstream pathways, including the Ras/ERK and protein kinase C θ (PKCθ) pathways. The Ras/ERK pathway leads to the nuclear localization of the activator protein-1 transcription factor. As mentioned previously, signaling through this pathway may serve as a gauge for the strength of TCR stimulation. Given the TCR affinity model of thymic T cell development, it is tempting to speculate that high affinity TCR signals may instruct Treg differentiation through activation of Ras/ERK signaling. A second critical pathway regulated by PKCθ is nuclear factor of k-light-chain-enhancer of activated B cells (NF-κB) signaling. Although the classical and non-classical pathways for NF-κB activation are well understood in many cell types, recent studies have identified a mode of NF-κB signaling unique to lymphocytes (Smith-Garvin et al., 2009). Engagement of the TCR induces the assembly of a complex consisting of the proteins caspase recruitment domain and membrane-associated guanlyate kinase-containing scaffold protein (CARMA1), B-cell lymphoma/leukemia 10 (Bcl10), and mucosa-associated lymphoid tissue lymphoma translocation gene 1 (MALT1) or the CBM complex. This CBM complex has been shown to stimulate NF-κB signaling through activation of the IκB kinase (IKK) complex. The CBM complex initially promotes IKKγ ubiquitylation through activation of tumor necrosis factor receptor-associated factor 6 (TRAF6). IKKγ ubiquitylation results in IKK activation, degradation of the inhibitory IκB proteins, and subsequent release and nuclear translocation of NF-κB family

transcription factors. Importantly, a number of signaling molecules in the NF-κB pathway have been implicated in Treg differentiation (Barnes et al., 2009; Gupta et al., 2008; Isomura et al., 2009; Long et al., 2009; Ruan et al., 2009; Schmidt-Supprian et al., 2003; Shimo et al., 2011; Vang et al., 2010; Wan et al., 2006; Zheng et al., 2003). In particular, recent studies have demonstrated a central role for the NF-κB family member c-Rel in promoting Treg differentiation through direct transactivation of the *foxp3* promoter (Long et al., 2009; Ruan et al., 2009).

Although signaling pathways activated by both Ca^{2+} and DAG have been implicated in Treg development, it is not clear how these second messengers link TCR stimulation to Treg lineage commitment. Given the critical roles of the Ras/ERK pathway in translating TCR signal strength into cell fate decisions and the NF-κB pathway in Treg development, it is likely that upstream signaling through DAG may also affect Treg differentiation. While it is difficult to alter DAG levels directly, modifying the processes that initiate or terminate DAG signaling may be a useful approach to study the role of this pathway in Treg development.

Peripheral T cell homeostasis

The homeostasis of peripheral T cell populations is maintained not only through the generation of new T cells, but also through the survival and self-renewing proliferation of existing T cells. The survival of peripheral naïve $CD4^+$ and $CD8^+$ T cells is maintained by the combination of tonic TCR signals provided by interactions with MHCII and MHCI molecules respectively and signaling by the γ_c cytokine IL-7 (Surh and Sprent, 2008). Indeed, adoptive transfer of naïve T cells into MHC or IL-7 deficient environments results in their numerical decline over time (Surh and Sprent, 2008). Likewise, deletion of TCR signaling components or neutralization of IL-7 in intact mice also causes in a gradual loss of both naïve $CD4^+$ and $CD8^+$ T cells (Surh and Sprent, 2008). In the steady state, unmanipulated naïve T cells do not typically exhibit any self-renewing homeostatic proliferation in the periphery (Surh and Sprent, 2008).

In contrast to naïve T cells, the homeostasis of memory $CD4^+$ and $CD8^+$ T cells is characterized by a slow rate of self-renewing homeostatic proliferation in the steady state (Surh et al., 2006). Although memory $CD4^+$ T cells continue to rely on IL-7 for their survival and

homeostatic proliferation, memory $CD8^+$ T cells are more dependent on the related cytokine IL-15 (Surh et al., 2006). Rather than being secreted like most cytokines, other accessory BM-derived cells must "trans-present" IL-15 to memory $CD8^+$ T cells to sustain memory $CD8^+$ T cell homeostasis (Stonier et al., 2008). An additional difference is that memory $CD8^+$ T cells do not require MHCI interactions or TCR signaling in order to persist or proliferate. Although still controversial, TCR signaling and MHCII contacts are also dispensable long-term maintenance (Surh et al., 2006).

The same factors that govern the homeostasis of naïve and memory T cells also control the maintenance of Tregs. Thus, to understand Treg homeostasis, we will explore the roles of TCR/MHCII interactions, cytokines, and accessory cells.

Peripheral homeostasis of regulatory T cells

Initial *in vitro* studies showed that Tregs did not flux calcium (Ca^{2+}) or proliferate appreciably upon stimulation with IL-2 or TCR agonists, leading to the conclusion that Tregs were hyporesponsive to stimulation or anergic (Gavin et al., 2002; Thornton and Shevach, 1998). Therefore, it was surprising that immunization with cognate antigen promoted antigen-specific Treg proliferation *in vivo* (Klein et al., 2003). Adoptively transferred Tregs also proliferated extensively in lymphopenic hosts (Gavin et al., 2002). Moreover, examination of mice under steady state conditions revealed that peripheral Tregs are continuously dividing (Fisson et al., 2003). Despite this cell division, the total number of Tregs remained relatively constant over time, indicating that division of peripheral Tregs is balanced equally by cell death or exit from the Treg pool (Fisson et al., 2003). In this manner, Treg division resembles the homeostatic proliferation of memory T cells. Thymectomy experiments in adult mice suggest that this self-renewing Treg proliferation can sustain Treg homeostasis in the absence of thymic output (Rubtsov et al., 2010). Additionally, adoptive transfer of even a small number of WT Tregs into IL-2Rβ KO mice, which contain very few Tregs, restores the Treg numbers to WT levels and prevents autoimmunity for the life of the recipient (Cheng et al., 2011). Host-derived Tregs can also expand and maintain self-tolerance after lethal irradiation in BM chimeras re-constituted with only Scrufy BM (Komatsu

and Hori, 2007). Collectively, these studies demonstrate the importance of peripheral Treg proliferation for the maintenance of self-tolerance. Consequently, there is intense interest in determining the factors that control the self-renewing proliferation of peripheral Tregs.

Not unexpectedly, the same molecular pathways govern both the differentiation and the peripheral maintenance of Tregs. For instance, it was found that the addition of exogenous IL-2 could overcome the anergic phenotype of Tregs *in vitro* (Thornton et al., 2004). Likewise, endogenous IL-2 *in vivo* supports Treg homeostatic proliferation as neutralization of IL-2 by anti-IL-2 monoclonal antibodies (mAb) prevents Tregs from dividing (Setoguchi et al., 2005). Since Tregs cannot produce IL-2, they must rely on IL-2 produced by Tconvs to divide and survive (Almeida et al., 2002; Almeida et al., 2006; Cheng et al., 2011; Setoguchi et al., 2005). In support of this notion, the presence of WT T cells can rescue Treg numbers in IL-2 KO in a mixed BM chimera setting (Almeida et al., 2002; Almeida et al., 2006; Cheng et al., 2011). Moreover, anti-IL-2 mAb treatment dramatically reduces the number of peripheral Tregs in BALB/c mice and causes multi-organ autoimmunity in NOD mice (Setoguchi et al., 2005). Conversely, targeted stimulation of the IL-2R with IL-2 cytokine/anti-IL-2 mAb immune complexes causes massive peripheral Treg proliferation and expansion (Webster et al., 2009), indicating that the endogenous steady state of levels of IL-2 are not saturating.

In addition to IL-2, TCR signaling is a critical determinant of Treg homeostasis. In this respect, the behavior of Tregs differs from that of antigen-experienced memory T cells, which can be maintained in the absence of MHC-induced TCR signals. In support of this notion, experiments in lymphopenic hosts demonstrated that MHCII dependent signals are necessary for Treg survival and division *in vivo* (Bhandoola et al., 2002; Gavin et al., 2002). Additionally, conditional deletion of the TCR signaling molecules Lck or the linker of activation of T cells (LAT) in T cells impaired Treg survival and division (Kim et al., 2009; Shen et al., 2010). Thus, despite the *in vitro* hyporesponsiveness of Tregs to TCR stimulation, these data suggest that MHCII-dependent TCR signals are required for Treg division and maintenance *in vivo*.

Whereas MHCI molecules are ubiquitous, MHCII expression is restricted to APCs in the steady state, although MHCII expression can be induced on a handful of additional cell types by

inflammatory stimuli. Thus, it was not obvious which APCs provided MHCII-dependent signals to sustain Treg division. A logical candidate APC is the DC, which possesses the most potent ability to activate conventional T cell subsets. Indeed, early studies demonstrated that DCs presenting cognate antigen on MHCII stimulated antigen-specific Tregs to divide (Fehérvári and Sakaguchi, 2004; Yamazaki et al., 2003). Furthermore, targeting of cognate antigens to DCs *in vivo* also resulted in the division of antigen-specific Tregs (Yamazaki et al., 2008; Yamazaki et al., 2003). Collectively, these data implicate DCs as the accessory cell type that presents cognate antigen on MHCII to promote Treg proliferation and maintenance.

Besides TCR signaling induced by MHC molecules, B7 molecules provide costimulation to T cells through the CD28 receptor resulting in full T cell activation and effector function. Thus, it was unexpected that blockade or deletion of B7 molecules or deletion of the CD28 receptor resulted in accelerated development of spontaneous diabetes in the NOD mice (Salomon et al., 2000). Analysis of mice lacking B7 signaling to CD28 revealed a profound decrease in peripheral Treg numbers (Salomon et al., 2000). Additionally, adoptive transfer of WT Tregs into CD28 KO mice delayed diabetes development, identifying a relative deficiency of Tregs as the cause of the accelerated diabetes development (Salomon et al., 2000). Meanwhile, adoptive transfer experiments supported the notion that CD28 expression contributes to the maintenance of Treg homeostasis (Tang et al., 2003). Moreover, DC expression of the B7 molecules CD80 and CD86 was important for their ability to stimulate antigen-specific Treg division (Yamazaki et al., 2003). Finally, stimulation of CD28 through “superagonists” mAbs expands peripheral Tregs and helps to suppress disease in an EAE model (Beyersdorf et al., 2005). These studies show that CD28 costimulation is also supports the homeostasis of peripheral Tregs.

Altogether, similar to defects in Treg generation, the inability to sustain an adequate population of peripheral Tregs can also result in a breakdown of self-tolerance. Thus, the study of both Treg differentiation and peripheral maintenance are necessary to understand the homeostasis of this essential population. Despite the significant progress in our understanding of the requirements for peripheral Treg maintenance, some questions remain. For instance, a rigorous assessment of the importance of cell-autonomous TCR signaling in Tregs for the

maintenance of a polyclonal Treg population is lacking. In every experimental approach utilized thus far, TCR signaling is not eliminated specifically in Tregs, but in all T cells. Given the dependence of Treg maintenance on Tconv-derived IL-2, the Treg maintenance defects observed in previous studies may result from the inability of Tconvs to produce IL-2 in the absence of TCR signaling rather than a cell-autonomous requirement for TCR signaling in Tregs. Consistent with this notion, a study examining mutant mice expressing hypomorphic alleles of LAT suggests that TCR signaling may not be as critical for steady state Treg homeostasis as previous work indicates (Siggs et al., 2007). Moreover, previous studies showing that DCs promote Treg division examined only TCR transgenic antigen-specific Tregs that may not completely recapitulate the biology of polyclonal Tregs generated in a WT host. For these reasons, further experimentation is required to dissect the manner in which DCs promote Treg division and the necessity of TCR/MHCII interactions in Treg maintenance.

Structure of thesis

This thesis examines the signals that control Treg differentiation and peripheral maintenance, with a particular focus on the role of TCR signaling. While MHCII-bearing DCs and IL-2 producing Tconvs have been shown separately to be critical for Treg division and maintenance, an integrated model of Treg division describing the precise cellular interactions between DCs, Tconvs, and Tregs is lacking. Moreover, prior work utilized antigen-specific TCR transgenic Tregs to conclude that direct TCR stimulation is essential for Treg proliferation and maintenance (Fehérvári and Sakaguchi, 2004; Walker et al., 2003; Yamazaki et al., 2003). Although a valuable tool, antigen-specific TCR transgenic Tregs may not accurately reflect the behavior of polyclonal WT Tregs possessing diverse antigenic reactivity. Thus, we hypothesize that DCs promote polyclonal Treg division through distinct interactions with IL-2 producing Tconvs and Tregs. In particular, we hypothesize that direct TCR stimulation of polyclonal Tregs may be dispensable for their division. In Chapter II, we use *in vitro* studies to determine the specific cellular interactions between DCs, Tconvs, and Tregs that mediate Treg division. To clarify the requirement for direct TCR stimulation of Tregs, we designed experiments in which DC-derived

MHCII-dependent signals are accessible to only IL-2 producing Tconvs but not Tregs and vice versa. We find that DC-derived MHCII signals are required only to induce Tconvs to produce the IL-2 that is necessary for Treg division. In fact, in the presence of exogenous IL-2, Tregs require only MHCII-independent signals from DCs to divide. Collectively, these studies define the dynamic interactions between DCs, Tconvs, and Tregs that are necessary to support Treg division *in vitro*. Furthermore, the experiments presented in Chapter II suggest that MHCII-dependent TCR signals may be dispensable for Treg division.

The next Results chapter extends these *in vitro* observations to examine the TCR dependence of Treg division *in vivo*. Prior work has concluded that cell-autonomous TCR signaling is required for Treg division. However, the approaches utilized in these studies could not dissociate the requirement for TCR signaling in Tregs themselves from the TCR signals necessary to induce Tconvs to produce the IL-2 needed to sustain Treg division. As presented in Chapter III, we devise complementary approaches to test the requirement for cell-autonomous TCR signaling in Treg division *in vivo*. First, we utilize conditional deletion of the TCR signaling molecule SLP-76 to abrogate downstream TCR signaling specifically in Tregs, while preserving TCR signaling in Tconvs capable of producing endogenous IL-2. Alternatively, we put WT Tregs in an environment that is deficient in MHCII but contains ample quantities of exogenously administered IL-2. These studies reveal that while Tregs do require TCR signaling to divide at steady state IL-2 levels present in an unmanipulated host, cell-autonomous TCR signaling becomes dispensable for Treg division in the presence of IL-2 ICs that mimic high concentrations of this cytokine. These studies demonstrate for the first time that Tregs are capable of dividing in the absence of cell-autonomous TCR signaling *in vivo*.

During the course of investigating the TCR dependence of peripheral Treg division, we became interested in the central role of the TCR in Treg development. While the TCR instructive model of Treg differentiation holds widespread acceptance, the intracellular signaling pathways that link TCR stimulation to Treg differentiation remain incompletely defined. It is known, however, that TCR-induced signaling through Ras/ERK and NF-κB are involved in cell fate decisions during thymic T cell development. Seminal work has demonstrated an essential role for the Ras/ERK

pathway in positive and negative selection (Alberola-Ila et al., 1995; Daniels et al., 2006; Swan et al., 1995). Integrating the role of Ras/ERK signaling into the TCR affinity model of T cell development, it is possible that this pathway may operate as a sensor of TCR affinity that controls not only thymic selection checkpoints, but also Treg differentiation. Similarly, a number of studies have established a positive role for NF-κB signaling in Treg development. Notably, both Ras/ERK and NF-κB pathways are downstream of TCR-induced DAG signaling. Thus, we hypothesized that the DAG pathway links TCR signaling to Treg differentiation through activation of either Ras/ERK and/or NF-κB. The final chapter of the Results section investigates the role TCR-induced DAG signaling in promoting differentiation to the Treg lineage. Since direct modulation of DAG levels is difficult, we manipulate DAG signaling by deleting DAG kinase ζ (DGKζ), a negative regulator of this pathway. Although preliminary, we show in Chapter IV that enhanced TCR-induced DAG signaling promotes the preferential development of Tregs. Future studies will determine whether DAG signaling promotes Treg differentiation through activation of the Ras/ERK pathway or the NF-κB pathway.

In the final section of the thesis, we discuss the significance of our findings for our understanding of Treg development and homeostasis. Furthermore, we propose future avenues of experimentation based on our results.

Chapter II

Results

Dendritic cells induce regulatory T cell division through antigen-dependent and -independent interactions

Introduction

$CD4^+Foxp3^+$ Tregs are crucial for protecting the host from autoimmunity by suppressing self-reactive T cells and preventing immunopathology by restraining immune responses. The peripheral Treg pool is comprised of both nTregs that develop in the thymus and iTregs that are converted from Tconvs in the periphery (Curotto de Lafaille and Lafaille, 2009; Josefowicz and Rudensky, 2009; Piccirillo and Shevach, 2004; Sakaguchi et al., 2008). Tregs constitute 5-15% of the total $CD4^+$ T cell pool in the steady state (Sakaguchi et al., 1995; Sakaguchi et al., 2001). It is thought that emigration of newly matured nTregs from the thymus, conversion of iTregs, and the continuous self-renewing division of mature Tregs already present in the periphery sustains a stable population of functional Tregs (Curotto de Lafaille and Lafaille, 2009; Fisson et al., 2003; Jordan et al., 2001).

The importance of Tregs is illustrated by deficiencies in their number or function, which lead to widespread autoimmune disease (Liston and Rudensky, 2007; Sakaguchi et al., 1995). An inadequate number of Tregs can result either from the inability to produce nTregs in the thymus or from a defect in maintaining their survival and/or self-renewing cell division in the periphery (Liston and Rudensky, 2007). For example, mice or humans lacking a functional form of the Foxp3 are devoid of Tregs and develop a fatal autoimmune syndrome (Brunkow et al., 2001; Fontenot et al., 2003). Additionally, mice deficient in CD28, TGF-β, and IL-2, also display autoimmune manifestations due partially to an inability to maintain their peripheral Treg pool in the steady state (Liston and Rudensky, 2007).

Despite the importance of Tregs, the mechanisms that regulate peripheral Treg homeostasis remain incompletely defined. Using antigen-specific TCR transgenic Tregs, previous work has suggested a connection between DCs and the homeostasis of peripheral Tregs. These studies have shown that antigen-specific Tregs depend upon cognate antigen/MHCII and co-

stimulatory interactions with DCs to divide (Fehérvári and Sakaguchi, 2004; Yamazaki et al., 2008; Yamazaki et al., 2003). Furthermore, targeting antigen to CD8α^- DCs are able to induce antigen-specific Treg proliferation *in vivo* (Yamazaki et al., 2008). Of note, in addition to direct antigen stimulation of Tregs, MHCII expressing DCs can induce antigen-specific Treg division in the presence of only exogenous IL-2 (Yamazaki et al., 2003). Although the use of TCR transgenic mice has facilitated the study of Tregs, cells from these mice may not completely recapitulate the biology of Tregs that develop in the setting of a T cell compartment possessing diverse antigenic reactivity. This fact is of particular concern, as the antigenic specificity of polyclonal Tregs remains controversial (Hsieh et al., 2004; Hsieh et al., 2006; Pacholczyk et al., 2007). Studies examining polyclonal Tregs have demonstrated that Tregs display a form of self-renewing cell division in specific-pathogen free mice in the absence of overt antigenic stimulation (Fisson et al., 2003). This Treg division is dependent on IL-2, which likely derives from Tconvs activated by self-Ags or commensal organisms (Setoguchi et al., 2005). Recent work has identified the importance of DCs and their expression of MHCII in the maintenance of polyclonal Treg division and homeostasis (Darrasse-Jèze et al., 2009). From these studies, it is presumed that polyclonal Tregs are induced to divide by self-Ags presented on MHCII expressing DCs. However, the cellular and molecular interactions that underlie DC induction of polyclonal Treg division remain incompletely defined.

Several reports investigating the mechanisms that regulate the expansion and maintenance of Tregs have focused on the ability of the cytokine granulocyte-macrophage colony stimulating factor (GM-CSF) to expand this T cell population (Ganesh et al., 2009; Gaudreau et al., 2007; Kared et al., 2008; Sheng et al., 2008). Although GM-CSF can promote anti-tumor immunity and autoimmune disease (Hamilton, 2008), combined deficiency of GM-CSF and the related cytokine IL-3 results in the development of autoimmunity (Enzler et al., 2007). Moreover, *in vivo* administration of GM-CSF prevents or attenuates autoimmune disease in a variety of mouse models (Ganesh et al., 2009; Gaudreau et al., 2007; Sheng et al., 2008). The finding that GM-CSF treatment results in DC expansion and an accumulation of Tregs suggests a potential

mechanism for its function in immune tolerance (Ganesh et al., 2009; Gaudreau et al., 2007; Sheng et al., 2008).

Here, we show that CD8α^- DCs are uniquely able to induce polyclonal Tregs to undergo cell division in splenocyte cultures through both MHCII-dependent and -independent interactions. Molecules capable of activating or expanding DCs, including GM-CSF and toll-like receptor (TLR) ligands, enhanced this Treg division through direct effects on DCs. *In vivo* expansion of DCs also correlated with an increase in the frequency and proliferation of polyclonal Tregs. Treg division depended strictly on IL-2, which Tconvs produced upon receiving MHCII signals and B7 co-stimulation from DCs. Unlike Tconvs, Tregs need only IL-2, B7 co-stimulation, and unidentified interactions with DCs to divide. However, stimulation of Tregs through the TCR further augmented Treg division in the presence of IL-2 production by Tconvs. These data confirm a role for DCs in Treg homeostasis and provide a model in which the interactions between DCs, Tconvs, and Tregs are required to support polyclonal Treg division.

Results

GM-CSF selectively promotes the division of Treg *in vitro*

T cells proliferate when stimulated through their TCR upon encounter with their cognate antigen presented by MHC molecules on APCs. Since most T cells that react strongly against self-derived peptides are deleted in the thymus, peripheral T cells would not be expected to divide when cultured with syngeneic APCs in the absence of exogenous antigens. However, upon culture of both B6 and Balb/c splenocytes in cell culture media alone, we unexpectedly but consistently observed the selective division of a modest fraction of $CD4^+Foxp3^+$ Tregs (Figure 2.1A), suggesting that Tregs divide at a basal level in splenocyte cultures. This preferential Treg division increased significantly with the addition of GM-CSF (Figure 2.1B, C), in a dose-dependent manner (Figure 2.1D). Neither $CD4^+Foxp3^-$ Tconvs nor $CD8\alpha^+$ cytotoxic T cells divided to the same extent under these cell culture conditions (Figure 2.1A, B), even though all T cell subsets divided vigorously when stimulated with anti-CD3 mAb (Figure 2.1B). Furthermore, we observed no synergy between GM-CSF and anti-CD3 on Treg division **(**Figure 2.1B**)**. Although we consistently observed increased division of Tregs in B6 and Balb/c splenocytes, there was variability with regards to total Treg numbers. Total Tregs numbers were modestly increased in Balb/c but not B6 splenocytes cultured with GM-CSF compared to media alone (Figure 2.1E, F). Thus, the Treg division that is observed in GM-CSF-treated splenocyte cultures may model the self-renewing division that is necessary for the homeostatic maintenance of Tregs. These results show that Tregs display basal division in the absence of overt TCR stimulation in splenocyte cultures, which is enhanced by GM-CSF.

GM-CSF belongs to the common β chain (βc) cytokine family, which also includes IL-3 and IL-5 (Martinez-Moczygemba and Huston, 2003). Both IL-3 and IL-5 enhanced basal Treg division, albeit to a lesser extent than GM-CSF (data not shown), suggesting that the augmentation of basal Treg division extended to other βc cytokines. Since multiple splenic cell types can produce βc cytokines (Martinez-Moczygemba and Huston, 2003), we next tested whether endogenous production of βc cytokines was responsible for the basal level of Treg division. The addition of neutralizing anti-GM-CSF mAb did not affect this basal amount of Treg

cell division (Figure 2.1G). Furthermore, Tregs in splenocyte cultures from WT mice and mice doubly deficient in both the IL-3β and the βc signaling chains, IL-3β/βc double KO (DKO), exhibited a similar amount of basal cell division (data not shown), suggesting that endogenous production of βc cytokines was not responsible for basal Treg division. These results indicate that Tregs in cultured splenocytes display a basal level of division that does not depend on, but can be augmented by, βc cytokine signaling.

GM-CSF-expanded $CD4^+Foxp3^+$ cells do not arise from Tconvs

The dividing Tregs in the GM-CSF-treated cultures may have arisen from pre-existing Tregs or Tconvs that had converted to iTregs in our culture conditions. To identify the cell of origin of the dividing Tregs, we cultured either fluorescence-activated cell sorting (FACS)-purified Tregs or Tconvs with T-cell depleted splenocytes with or without GM-CSF. We found dividing $CD4^+$ T cells and $CD4^+Foxp3^+$ cells almost exclusively in cultures initially receiving $CD4^+CD25^+$ T cells (Figure 2.2A**)**, demonstrating that the dividing Tregs derived predominantly from a $CD4^+CD25^+$ population enriched in Tregs rather than the conversion of Tconvs to iTregs during culture.

GM-CSF-expanded $CD4^+Foxp3^+$ cells are bona fide Tregs with potent suppressive function

Although Foxp3 is considered a specific marker for Tregs, we wished to determine whether the dividing $CD4^+Foxp3^+$ T cells possessed phenotypic and functional properties of Tregs. Similar to freshly isolated Tregs, the dividing Tregs in our cultures expressed the IL-2 receptor α chain CD25, cytotoxic T lymphocyte antigen 4 (CTLA-4), folate receptor 4 (FR4), and GITR (Figure 2.2B**)**. To test their regulatory function, we co-cultured GM-CSF-expanded Tregs and freshly isolated Tregs with Tconv effectors at various Treg:Tconv ratios. Compared to freshly isolated Tregs, Tregs cultured in GM-CSF displayed potent suppressive function toward Tconvs stimulated with anti-CD3 (Figure 2.2C). These results suggest that the dividing $CD4^+Foxp3^+$ T cells in our splenocyte cultures were bona fide Tregs.

DCs induce polyclonal Treg division

Next, we investigated whether the effect of GM-CSF on enhancing Treg division was purely cell-intrinsic or if another splenic cell type was required for this process. To test this question, we cultured FACS-purified T cells either alone or together with T cell-depleted splenocytes in the presence of GM-CSF. Tregs failed to divide when T cells were cultured alone but divided robustly in the presence of T cell-depleted splenocytes (Figure 2.3A). These results suggest that Treg division required a non-T cell, accessory splenic cell type. To determine which cell population(s) in the spleen was responsible for inducing Treg division, we cultured FACS-purified T cells with equal numbers of FACS-purified $CD11c^+$ DCs, $CD19^+B220^+$ B cells, or $F4/80^+CD11b^+$ macrophages in the presence of GM-CSF. The cultures containing DCs stimulated Tregs to divide more robustly than those containing B cells or macrophages (Figure 2.3B). In fact, we observed that as few as 25,000 DCs were able to induce robust Treg division in the presence of GM-CSF (Figure 2.3C). Thus, these data suggest that DCs are sufficient to support Treg division in culture.

Although GM-CSF is known to act on DCs, it has also been shown to augment TCR-induced Treg division through direct effects on Tregs (Kared et al., 2008). To distinguish whether GM-CSF acted on DCs and/or T cells in enhancing Treg division, we utilized IL-3β/βc DKO mice, which are unable to signal through the GM-CSFR. To obtain sufficient numbers of DCs for our experiments, DCs were isolated from mice treated with fms-like tyrosine kinase 3 ligand (FLT3L) (Maraskovsky et al., 1996). $CD4^+$ T cells and DCs from WT and IL-3β/βc DKO were co-cultured in each possible T cell/DC combination with or without GM-CSF. Augmentation of Treg division by GM-CSF was abrogated when GM-CSF signaling was absent in DCs but remained intact when GM-CSF signaling was absent in T cells (Figure 2.3D), suggesting that GM-CSF acted directly through DCs, and not T cells, to enhance Treg division.

We next tested whether other inducers of DC activation such as the TLR ligands lipopolysaccharide (LPS) and CpG DNA could also promote Treg division. Co-culture of total $CD4^+$ T cells with DCs in the presence of LPS and CpG also significantly enhanced Treg division compared to untreated controls (Figure 2.3E). To test whether the effects of TLR signaling were

on DCs or on T cells, we co-cultured $CD4^+$ T cells and DCs from WT B6 and myeloid differentiation primary response gene 88 (MyD88) KO mice in all possible combinations in the presence or absence of CpG. We observed a trend suggesting that the effect of CpG on Treg division occurred in a MyD88-dependent manner and relied on signaling through DCs but not through T cells (data not shown). Thus**,** multiple factors that promote DC activation augmented DC-induced Treg division.

Having established that DCs promote polyclonal Treg division *in vitro*, we sought to investigate whether an increase in DCs may lead to enhanced polyclonal Treg division *in vivo*. We first attempted to increase DC numbers by treating with GM-CSF *in vivo*, but the administration of GM-CSF to WT B6 or Balb/c mice did not increase the frequency of DCs or Tregs and caused only a modest increase in the frequency of Tregs incorporating BrdU over PBS controls (data not shown). Continuous delivery of GM-CSF or polyethylene glycol modification of GM-CSF may be necessary to achieve increased DC numbers given the short $t_{1/2}$ of GM-CSF *in vivo* (Daro, 2000). Thus, as an alternative approach, we expanded DCs in WT B6 mice using B16 melanoma cells that produce FLT3L (B16-FLT3L) and observed an increase in the frequency and total number of DCs in the spleens of inoculated mice compared to untreated mice or those injected with B16 cells that do not express FLT3L (Figure 2.4A, data not shown). Concomitant with an increase in DC number, B16-FLT3L-treated mice contained an elevated frequency of splenic Tregs (Figure 2.4B). Moreover, BrdU incorporation studies revealed that Tregs in the B16-FLT3L-treated mice exhibited increased cell division compared to Tregs from control mice (Figure 2.4C). These data demonstrate that expansion of DCs *in vivo* correlates with Treg expansion and an increased rate of Treg division, suggesting that DC interactions with Tregs may regulate Treg homeostasis *in vivo*.

$CD8\alpha^-$ DCs stimulate Treg division through cell-to-cell contact

In the spleen, conventional DCs can be divided broadly into the $CD8\alpha^+$ and the $CD8\alpha^-B220^-$ DC subpopulations (Villadangos, 2007; Pulendra, 2008). To determine which subset could support Treg division, we co-cultured FACS-purified $CD4^+$ T cells with each population of

FACS-purified DCs in the presence of GM-CSF. CD8α^-B220$^-$ DCs promoted significantly more Treg division compared to CD8α^+ DCs (Figure 2.5A). Next, we asked whether DC-induced Treg division was contact-dependent. We cultured FACS-purified CD4$^+$ T cells with FACS-purified DCs either in the same chamber or in different chambers of a transwell separated by a cell-impermeable membrane that permitted diffusion of soluble factors. Tregs divided only when T cells were cultured together in the same chamber with DCs, suggesting that DC induction of Treg division required direct cell contact (Figure 2.5B). Together, these data indicate that CD8α^- splenic DCs support Treg division in a cell-contact dependent manner.

IL-2 production by Tconvs is necessary for Treg division

The co-culture experiments described thus far used populations of total T cells or CD4$^+$ T cells rather than purified Tregs. Thus, it remained unclear whether Tconvs were necessary for Treg division or if DCs stimulated Tregs directly to induce their division. We cultured FACS-purified Tregs with FACS-purified DCs alone or in the presence of Tconvs. Only a modest fraction of Tregs divided when cultured alone with DCs, whereas Treg division was robust when Tconvs were added back to the cultures (Figure 2.6A), suggesting that Tconvs were required for optimal Treg division. Because of the limitations of FACS, our sorted Treg populations usually contained ~5% contaminating Tconvs, which may explain the modest Treg division in co-cultures of Tregs and DCs.

Since cell contact between DCs and T cells appeared critical and cognate antigen has been implicated in promoting Treg division (Yamazaki, 2003; Josefowicz, 2008), we next tested whether MHCII expression by DCs was required to support Treg division by co-culturing FACS-purified CD4$^+$ T cells with either FACS-purified WT or MHCII KO DCs. We observed Treg division only in co-cultures of CD4$^+$ T cells and MHCII-expressing DCs (Figure 2.6B). However, it was unclear whether MHCII interactions were necessary between DCs and Tregs, between DCs and Tconvs, or both. To address this issue, FACS-purified Tregs and Tconvs were separated by a cell-impermeable membrane in a transwell plate and cultured alone or with either FACS-purified WT or MHCII KO DCs. We observed Treg division only when Tregs and DCs were in direct

contact and when Tconvs were co-cultured with MHCII-expressing DCs across the transwell (Figure 2.6C). These data suggest that DC-induced Treg division depended on a soluble factor produced from Tconv interactions with MHCII-expressing DCs. To our surprise, MHCII expression was not required by DCs that were in contact with Tregs (Figure 2.6C). Thus, Tregs required an MHCII-independent, cell contact-dependent interaction with DCs to divide.

We next sought to identify the soluble factor produced by Tconv-WT DC interactions that was required for Treg division. A logical candidate was IL-2, because of its central role in Treg survival and proliferation (Sakaguchi et al., 2008); (Burchill et al., 2007a; Setoguchi et al., 2005). Indeed, IL-2 was detected in supernatants of co-cultures of $CD4^+$ T cells with WT DCs, but was barely detectable in co-cultures of $CD4^+$ T cells and MHCII KO DCs (Figure 2.6D). Moreover, the addition of anti-IL-2 neutralizing mAbs to unfractionated splenocyte cultures decreased both the frequency of Tregs and their division significantly (Figure 2.6E). These data suggested that MHCII-independent signals from DCs and a source of IL-2 might be sufficient to stimulate Treg division. In support of this notion, although IL-2 had no effect on Treg division when Tregs were cultured alone, co-culture of Tregs with MHCII KO DCs in the presence of exogenous IL-2 was sufficient to induce Tregs to divide (Figure 2.6F). These data demonstrated that MHCII expression by DCs is required for stimulation of Tconvs to produce IL-2. This paracrine IL-2 cooperated with contact-dependent but MHCII-independent interactions between DCs and Tregs to induce Treg division.

TCR stimulation and B7 co-stimulation cooperate with IL-2 to stimulate Treg division

To determine which molecules on DCs might provide the MHCII-independent signals to Tregs, we examined the surface phenotype of FACS–purified DCs cultured with GM-CSF and TLR ligands. We first observed that only GM-CSF treatment, but not LPS or CpG treatment, increased the number of DCs in culture (Figure 2.7A), suggesting that increased DC numbers alone could not explain the effects of enhanced Treg division. Additionally, cultured DCs upregulated the expression of CD80, CD86, and MHCII in the presence of GM-CSF, LPS, and CpG compared to untreated media alone (Figure 2.7B). Blockade of B7 signals from DCs with

CTLA-4-Ig abrogated much of the Treg division observed in co-cultures of FACS-purified $CD4^+$ T cells and WT DCs (Figure 2.7C), likely resulting from the failure of Tconvs in these cultures to produce IL-2 (Figure 2.6D). In addition, CTLA-4-Ig partially but significantly attenuated the division of FACS-sorted Tregs co-cultured with MHCII KO DCs in the presence of IL-2 (Figure 2.7D). These data suggest that DC-derived B7 signals were important for both the production of IL-2 by Tconvs and also as a direct signal to Tregs that contributes to their MHCII-independent cell division.

Although MHCII expression on DCs was not necessary for Treg division, we consistently observed increased Treg division when Tregs were co-cultured with WT DCs compared to MHCII KO DCs in the presence of IL-2 (Figure 2.8A). These data implied that MHCII/TCR interactions might augment Treg division. To investigate the role of TCR stimulation by cognate antigen, we utilized the TS1×HA28 mouse, which possesses a transgenic TCR specific for the S1 peptide of HA and a second transgene expressing HA protein, resulting in the development of S1-specific Tregs. To dissociate paracrine IL-2 from TCR signals delivered to Tregs, TS1×HA28 Tregs were co-cultured with Tconvs from the DO11.10 TCR transgenic mouse, whose Tconvs respond not to S1 but instead to an ovalbumin (OVA)-derived peptide. Thus, stimulation with OVA peptide would result in TCR-mediated activation of DO11.10 Tconvs but not TS1×HA28 Tregs, providing a source of IL-2 but not direct TCR signals to the TS1×HA28 Tregs. We cultured FACS-purified TS1×HA28 Tregs and DO11.10 Tconvs in the presence of irradiated splenocyte feeder cells and stimulated the cultures with S1 peptide, OVA peptide, or both peptides. We observed TS1×HA28 Treg division in the presence of either S1 or OVA peptide, suggesting either TCR or IL-2 signals separately could induce Treg division (Figure 2.8B). However, the presence of both S1 and OVA peptide further enhanced TS1×HA28 Treg division (Figure 2.8B). These results demonstrate that although Tconv-derived IL-2 can support Treg division independent of MHCII signals, the combination of cognate antigen and IL-2 signals result in optimal stimulation of Treg division.

Discussion

Despite the large body of literature on Tregs, the process of Treg homeostasis remains incompletely understood. In this study, we demonstrated that CD8α^- DCs are critical for the initiation and coordination of polyclonal Treg division. DCs are involved in this process in multiple ways, requiring direct interactions with both Tconvs and Tregs. DCs stimulated Tconvs to produce IL-2 in an MHCII and B7-dependent manner. This IL-2, in conjunction with MHCII-independent co-stimulatory signals from DCs, was sufficient to induce Treg division. Although dispensable, TCR stimulation of antigen-specific Tregs by cognate antigen combined with paracrine IL-2 to promote optimal Treg division. Based on these data, we propose a model to describe the interactions among DCs, Tconvs, and Tregs that are required for optimal Treg division. This model may provide insight into the mechanisms underlying the self-renewing cell division polyclonal Tregs (Figure 2.9).

By culturing unfractionated splenocytes with GM-CSF, we observed the selective induction of polyclonal Treg division. To our knowledge, our report is the first to demonstrate the ability of Tregs to divide in splenocyte cultures without overt TCR stimulation or exogenous IL-2. This system allowed us to dissect the cellular and molecular interactions underlying the division of polyclonal Tregs. Several groups have reported that distinct subsets of DCs have roles in inducing antigen-specific Treg expansion (Tarbell et al., 2006; Yamazaki et al., 2008) or conversion of Tconvs into iTregs (Coombes et al., 2007; Sun et al., 2007). Our data add to these findings and demonstrate that DCs are crucial regulators of polyclonal Treg division. However, some have also argued that DCs may be dispensable for Treg generation and maintenance, since Treg frequency is normal in CD11c-DTa mice that constitutively lack DCs (Birnberg et al., 2008). In contrast, a recent report demonstrated that acute depletion of DCs *in vivo* results in decreased Treg numbers and cell division, which leads to an increase in T cells that are competent to produce inflammatory cytokines (Darrasse-Jèze et al., 2009). This apparent discrepancy may be explained by compensatory mechanisms that may be initiated in the constitutive absence of DCs, which allows a different cell type to substitute for DCs to maintain

Treg homeostasis. Moreover, the cell division of Tregs was not measured in the context of the constitutive absence of DCs (Birnberg et al., 2008).

The finding that GM-CSF can enhance a basal level of Treg division *in vitro* fits with its role in increasing the survival and division of monocyte lineage cells (Hamilton, 2008). Unlike GM-CSF and TLR ligands, FLT3L did not enhance DC-induced Treg division in unfractionated splenocyte cultures or co-cultures of $CD4^+$ T cells and DCs, despite promoting DC survival and/or proliferation *in vitro* (data not shown). Conversely, FLT3L has clear effects on DC expansion and Treg proliferation *in vivo* (Darrasse-Jèze et al., 2009; Swee et al., 2009). A potential explanation is that FLT3L treatment of DCs *in vitro* prevents their maturation, as these DCs do not upregulate B7 costimulation molecules (data not shown), and thus FLT3L-treated DCs may be inefficient stimulators of Treg division. Perhaps *in vivo* administration of FLT3L facilitates differentiation of DCs from hematopoietic progenitors and these DCs can mature in the appropriate microenvironment, away from FLT3L signals.

Two recent publications demonstrated a role for DCs in polyclonal Treg division, but they presented conflicting data on the requirement of MHCII expression by DCs in this process (Darrasse-Jèze et al., 2009; Swee et al., 2009). While one study indicated that DC-induced Treg division is MHCII-independent (Swee et al., 2009), the other argued that MHCII expression by DCs is required for Treg homeostasis (Darrasse-Jèze et al., 2009). Our data support the conclusions of both studies, since although DC expression of MHCII is not required for Treg division in our culture system, stimulation of the TCR through cognate antigen/MHCII can supplement IL-2 production by Tconvs to enhance Treg division further. Although the precise requirements for antigen/MHCII-independent division of Tregs is unclear at this time, we showed that DC-derived B7 costimulation plays a partial role in supporting Treg division both in the presence and absence of MHCII. The identification of additional DC-derived molecules involved in inducing Treg division and promoting Treg homeostasis warrants further study.

It has been suggested that Treg homeostasis is maintained, at least in part, by self-renewing division of peripheral Tregs to offset losses of this important T cell subset. We have identified the DC as a key cell type that initiates and coordinates this Treg division. Further

studies are required to address the relevance of these DC/Treg/Tconv interactions in the steady state *in vivo***.** In the next results chapter, we extend these *in vitro* findings to investigate whether Treg division requires MHCII *in vivo*. Using a number of complementary approaches, we test whether our model of Treg division may occur *in vivo*.

Figures

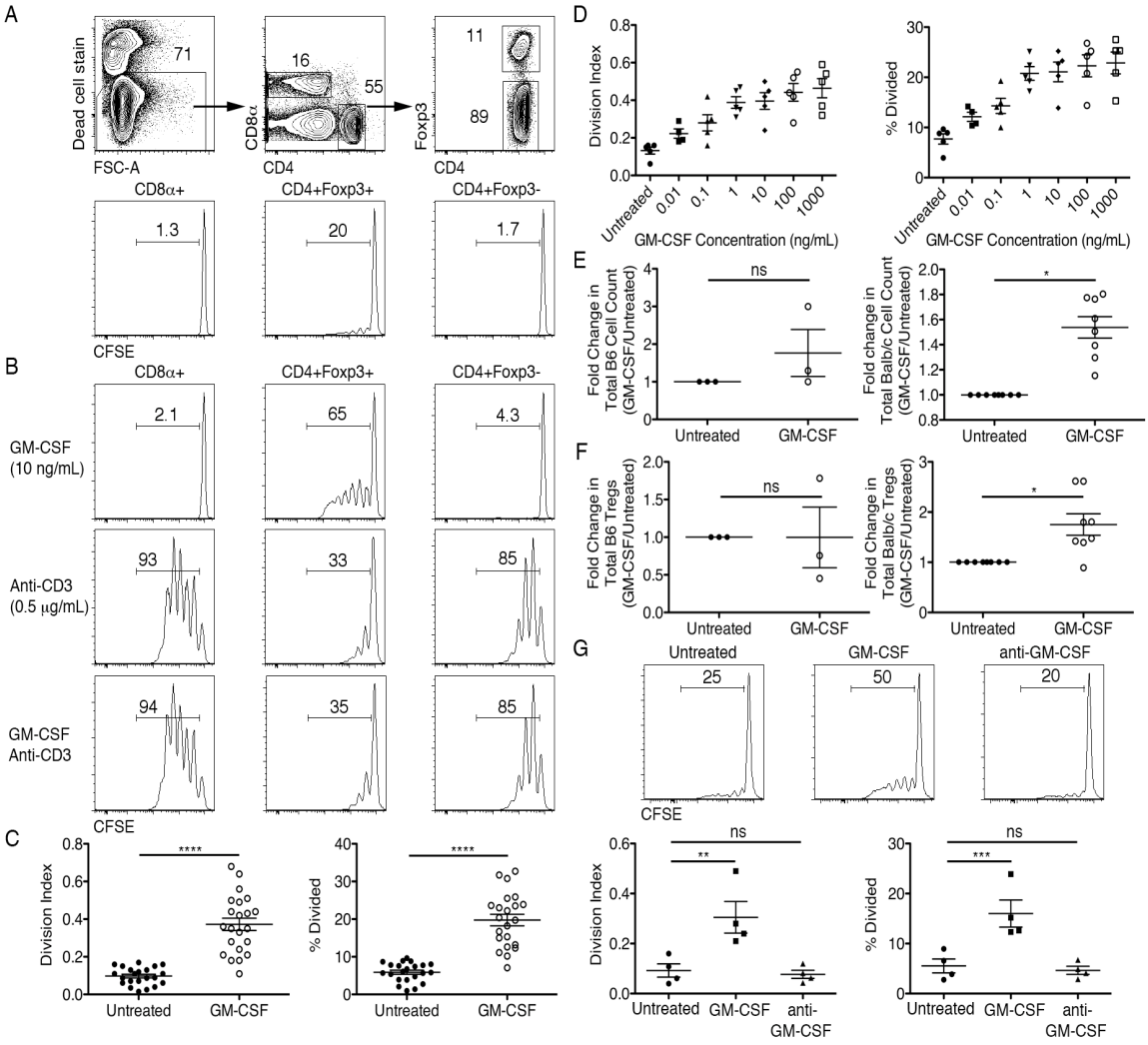

Figure 2.1. CD4$^+$Foxp3$^+$ cells in splenocyte cultures selectively undergo basal division that is enhanced by GM-CSF. (A) Splenocytes from Balb/c mice were labeled with CFSE and cultured in T cell media only, or (B) in the presence of anti-CD3 (0.5 mg/ml), GM-CSF (10 ng/ml), or both, or (G) anti-GM-CSF Ab (20 mg/ml). Four days later, cell division of various T cell subsets was analyzed by flow cytometry. (C) Treg Division Index and % Divided data were compiled from 22 independent experiments with B6 or Balb/c splenocytes cultured with or without GM-CSF. (D) A GM-CSF dose response was performed with B6 or Balb/c splenocytes and shown as Treg Division index and % Divided data compiled from five independent experiments. (E) Live cell count of B6 and Balb/c splenocytes cultured for four days with or without GM-CSF from three (B6) and seven (Balb/c) independent experiments. (F) Cultures were stained and analyzed by flow cytometry to determine the frequency of live, CD4$^+$Foxp3$^+$ cells after four days in culture. From these frequencies, the total number of Tregs after culture was determined for each condition. Data in (E, F) are shown as fold change in GM-CSF-treated compared to untreated control cultures. Histograms in (G) are gated on CD4$^+$Foxp3$^+$ cells and Treg Division Index and % Divided data (G) summarize three independent experiments. In all graphs, individual data points are shown with the mean ± SEM for each group. * p<0.05, ** p<0.01, and *** p<0.001, **** p<0.0001 by paired, two-tailed Student t test (C), Wilcoxon's signed-rank test (E, F) or one-way, repeated measures ANOVA and Tukey's post test (G)

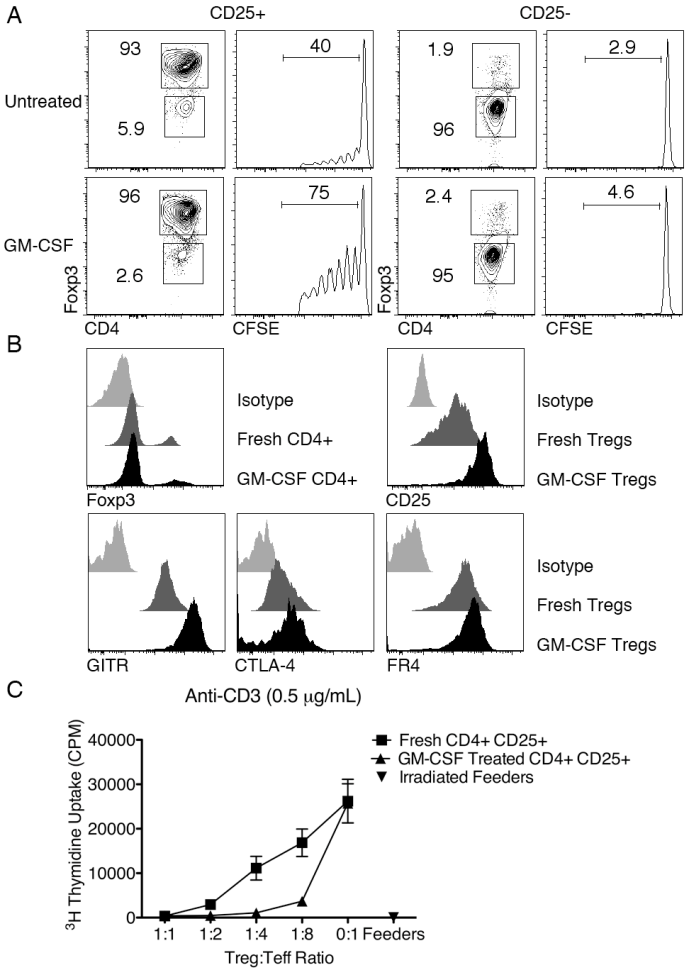

Figure 2.2. Cultured $CD4^+Foxp3^+$ cells derive predominantly from pre-existing Treg and exhibit potent suppressive function. (A) 1×10^4 CFSE-labeled, FACS-sorted Balb/c Tregs ($CD4^+CD25^+$) or Tconvs ($CD4^+CD25^-$) were co-cultured with 4×10^5 Balb/c T cell-depleted splenocytes with or without GM-CSF for 4 days followed by analysis of cell division by flow cytometry. The contour plots and histograms are gated on $CFSE^+CD4^+$ cells and are representative of two independent experiments. (B) CFSE-labeled Balb/c splenocytes were cultured in the presence of GM-CSF for 4 days and analyzed for Treg markers by flow cytometry. Representative histograms are gated on $CD4^+$ T cells for Foxp3 expression or on $CD4^+Foxp3^+$ Tregs for CD25, GITR, CTLA-4, and FR4 expression and show expression levels for the indicated proteins or isotype control (top), on freshly isolated (middle) and cultured GM-CSF treated cells (bottom). One representative of three independent experiments is shown. (C) Freshly isolated Balb/c Tregs (■) and Tregs from 4-day GM-CSF-treated Balb/c splenocyte cultures (▲) were FACS-sorted and co-cultured with FACS-sorted freshly isolated Balb/c Tconv effectors and Balb/c irradiated feeder cells at various Treg:Teff ratios for three days in the presence of anti-CD3 Ab (0.5 mg/ml). Cultures were pulsed with ^{3}H-thymidine and uptake was measured 16 h later to assess T cell proliferation. ^{3}H-thymidine uptake of irradiated feeders alone is shown (▼). The data are represented as mean ± SEM of triplicate determinations and is representative of four independent experiments.

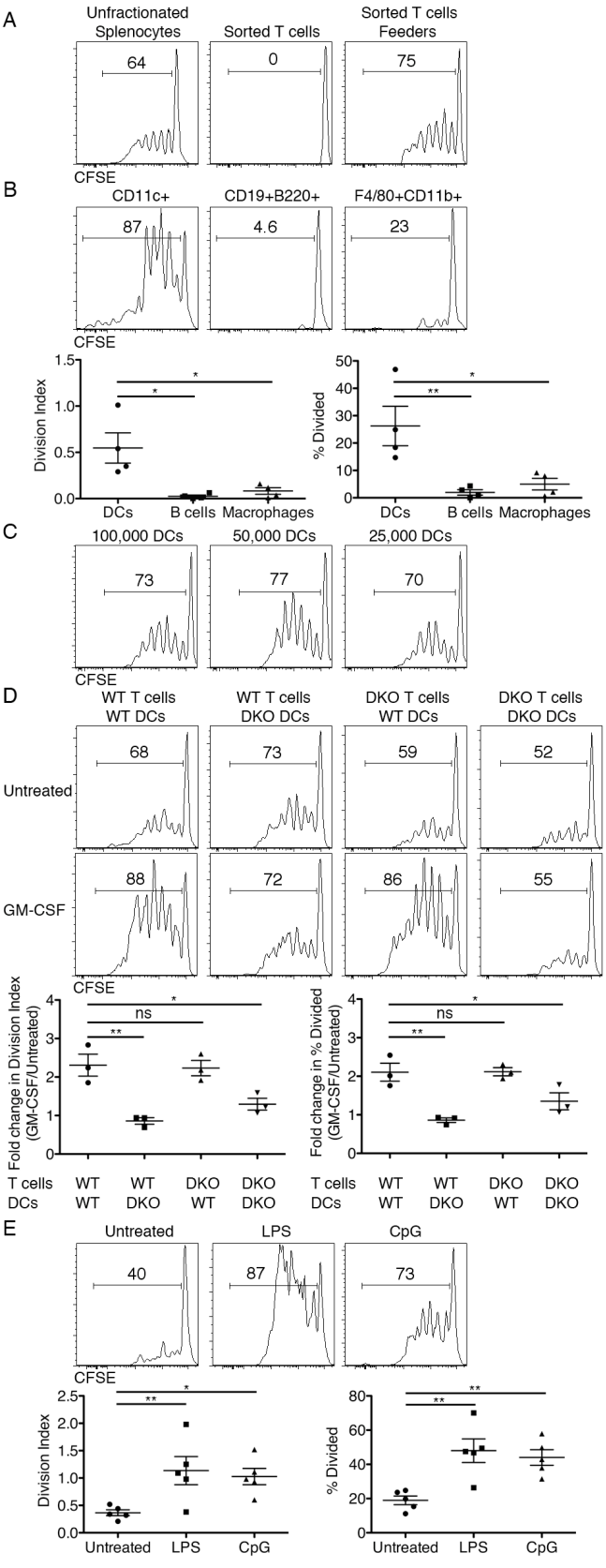

Figure 2.3. DCs preferentially induce Treg division. (A) CFSE-labeled unfractionated Balb/c splenocytes or 1 × 10^5 FACS-sorted CD90.2$^+$ Balb/c T cells cultured alone or with 4 × 10^5 Balb/c T cell-depleted splenocytes were treated with GM-CSF. (B) 1 × 10^5 FACS-sorted B6 CD11c$^+$ DCs, CD19$^+$B220$^+$ B cells, or F4/80$^+$CD11b$^+$ macrophages were co-cultured with 1 × 10^5 CFSE-labeled, FACS-sorted CD90.2$^+$ T cells in the presence of GM-CSF. (C) Decreasing numbers of FACS-sorted Balb/c DCs were co-cultured with 1 × 10^5 CFSE-labeled, FACS-sorted Balb/c CD90.2$^+$ T cells in the presence of GM-CSF. Data are representative of four independent experiments. (D) 7 × 10^4 CFSE-labeled, FACS-sorted CD4$^+$ T cells from WT B6 or IL-3β/βc DKO mice were co-cultured with 1 × 10^5 FACS-sorted WT B6 or IL-3β/βc DKO DCs with or without GM-CSF. To normalize Treg numbers from WT B6 and IL-3β/βc DKO mice, CD4$^+$CD25$^+$ Tregs and CD4$^+$CD25$^-$ Tconvs were FACS-sorted separately, CFSE-labeled, and co-cultured at a 1:6 ratio. Graphs show fold changes in division index comparing GM-CSF-treated to untreated controls. (E) 7 × 10^4 CFSE-labeled, FACS-sorted CD4$^+$ T cells from B6 mice were co-cultured with 1 × 10^5 B6 DCs with media alone, LPS (100 ng/ml), or CpG (100 ng/ml). All cell cultures were incubated for four days and analyzed for cell division by flow cytometry. Representative histograms are gated on CD4$^+$Foxp3$^+$ cells (B, D, E). Compiled cell division data are shown as Treg Division Index and % Divided data using B6 and Balb/c cells (B) or B6 cells only (D, E) from four (B), three (D), and five (E) independent experiments and represented as individual data points with the mean ± SEM. * p<0.05 and ** p<0.01 by one-way, repeated measures ANOVA with Dunnett's (B, E) or Tukey's (D) post tests.

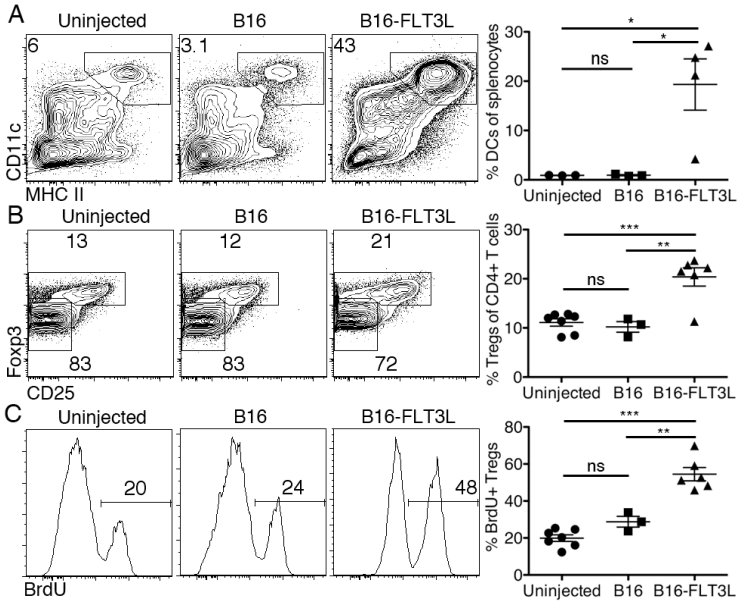

Figure 2.4. Expansion of DCs *in vivo* correlates with increased Treg frequency and proliferation. WT B6 Mice were injected s.c. with or without B16 or B16-FLT3L tumor cells and nine days later administered BrdU for three days. (A) Splenic DCs from these mice were analyzed by flow cytometry and the frequency of DCs is represented as individual data points with the mean ± SEM of three or four mice. Contour plots are gated on live, CD19/CD3-negative events. (B, C) The frequency (B) and BrdU incorporation (C) of splenic Tregs from these mice were measured by flow cytometry. Representative contour plots are gated on $CD4^+$ cells and representative histograms are gated on $CD4^+Foxp3^+$ cells. The graphs show compiled data of Treg frequency as a percentage of $CD4^+$ T cells and BrdU incorporation by Tregs (n = 3-7 mice/group combined from two independent experiments) and individual mice are represented along with the mean ± SEM. * $p<0.05$, ** $p<0.01$, and *** $p<0.001$ by one-way, repeated measures ANOVA with Tukey's post test.

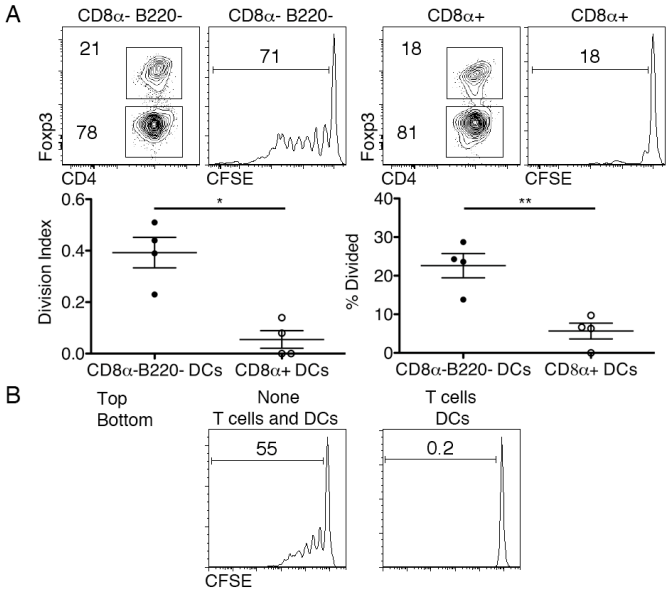

Figure 2.5. CD8α$^-$ DCs support Treg division in a cell-contact dependent manner. (A) (Top panel) 1 × 10^5 FACS-sorted B6 CD8α$^-$B220$^-$ or CD8α$^+$ DCs were co-cultured with 7 × 10^4 CFSE-labeled, FACS-sorted B6 CD4$^+$ T cells in the presence of GM-CSF for four days and CFSE dilution of CD4$^+$Foxp3$^+$ cells was analyzed by flow cytometry. The Treg Division Index and % Divided are represented as individual data points with the mean ± SEM of four independent experiments. * p<0.01, ** p<0.001 by paired, two-tailed Student's t test. (B) 7 × 10^5 CD11c$^+$ DCs were FACS-sorted and co-cultured with 5 × 10^5 CFSE-labeled, FACS-sorted B6 CD4$^+$ T cells either in the same chamber or across separate chambers of a 24-well transwell plate in the presence of GM-CSF for four days. The contents of each chamber of the transwell were analyzed separately by flow cytometry. All histograms are gated on CD4$^+$Foxp3$^+$ events and are representative of four independent experiments.

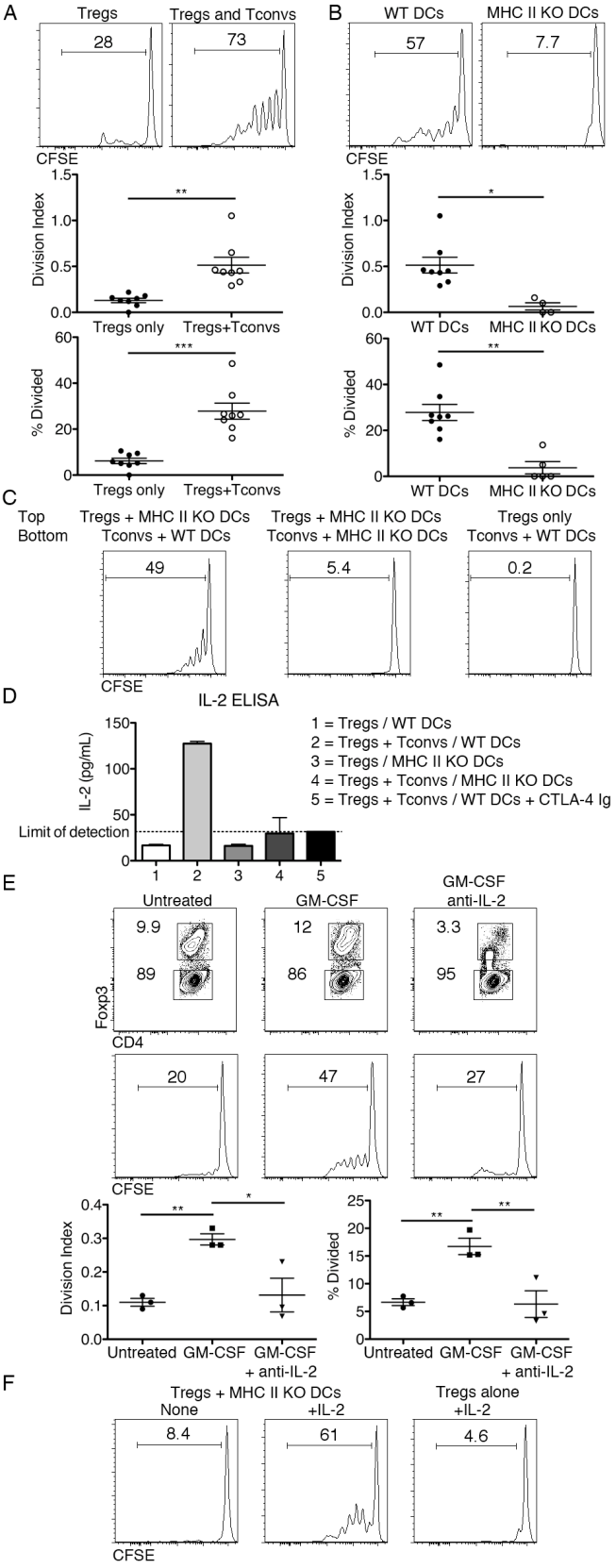

Figure 2.6. IL-2 production by Tconvs through interaction with MHC II expressing DCs is necessary for Treg division. (A) 1 × 10^4 CFSE-labeled, FACS-sorted B6 Tregs ($CD4^+CD25^+$) and 6 × 10^4 CFSE-labeled, FACS-sorted B6 Tconvs ($CD4^+CD25^-$) were co-cultured with 1 × 10^5 FACS-sorted B6 DCs in the indicated combinations in the presence of GM-CSF. (B) 7 × 10^4 CFSE-labeled, FACS-sorted B6 $CD4^+$ T cells were co-cultured with 1 × 10^5 FACS-sorted B6 WT or MHC II KO DCs in the presence of GM-CSF. (C) 7 × 10^4 Tregs and 5 × 10^5 Tconvs were FACS-sorted from B6 mice, CFSE-labeled, and co-cultured with 7 × 10^5 FACS-sorted B6 WT or MHC II KO DCs in the indicated combinations in separate chambers of transwell plates. (D) IL-2 content of supernatants from co-cultures of GM-CSF-treated B6 T cells and WT or MHC II KO DCs were detected by ELISA. Results are expressed as mean IL-2 concentration ± SEM of triplicate determinations. (E) CFSE-labeled Balb/c splenocytes were cultured in GM-CSF in the presence or absence of anti-IL-2 neutralizing Ab (20 mg/ml). (F) 1 × 10^4 CFSE-labeled FACS-sorted B6 Tregs were cultured alone with IL-2 (50 U/ml) or co-cultured with 1 × 10^5 FACS-sorted B6 MHC II KO DCs in the presence or absence of IL-2. All cell cultures contained GM-CSF (10 ng/ml) and were incubated for four days. Representative histograms are gated on $CD4^+Foxp3^+$ cells are shown in the top panels of (A, B, E), while compiled cell division data are shown as Treg Division Index and % Divided data using B6 and Balb/c cells (A) or B6 cells only (B, E) from eight (A), five or eight (B), and three (E) independent experiments. In all graphs, individual data points are shown with the mean + SEM. * p<0.05, ** p<0.01, *** p<0.001 by paired, two-tailed Student's t test (A, B) or by one-way, repeated measures ANOVA with Dunnett's (E) post test.

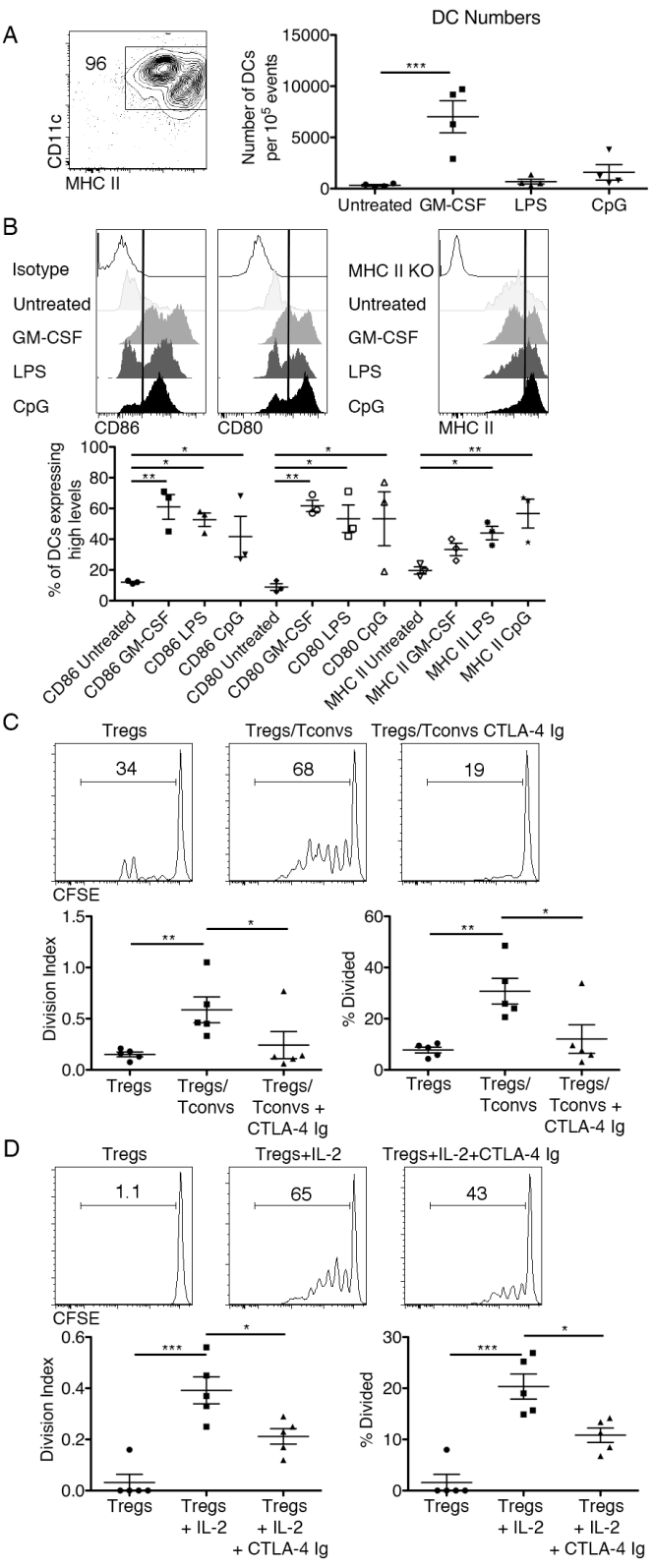

Figure 2.7. DCs deliver B7 co-stimulatory signals to promote Treg division in conjunction with IL-2. (A) 1 × 10^5 FACS-sorted B6 DCs were cultured with media alone, GM-CSF (10 ng/ml), LPS (100ng/ml), or CpG (100 ng/ml) for four days. $CD11c^+MHC\ II^+$ DCs were analyzed by flow cytometry. DC numbers were calculated based on the number of $CD11c^+MHC\ II^+$ cells per 1 × 10^5 acquired events and are represented as individual data points with the mean ± SEM from three independent experiments. (B) Representative histograms of cell surface marker expression for each treatment condition are shown along with appropriate isotype controls or staining of knockout cells. The vertical lines in each histogram delineate the threshold used to calculate the percentage of DCs that express high levels of each cell marker analyzed. Cumulative data from three independent experiments are represented as individual data points with the mean ± SEM in the bottom panel. (C, D) 1 × 10^4 CFSE-labeled FACS-sorted B6 Tregs ($CD4^+CD25^+$) were co-cultured with 1 × 10^5 FACS-sorted B6 WT DCs with or without 6 × 10^4 FACS-sorted Tconvs ($CD4^+CD25^-$) (C) or MHC II KO DCs with or without IL-2 (50 U/mL) (D) in the presence of GM-CSF with or without CTLA-4-Ig (20 μg/mL) for four days. Representative histograms in top panels of (C, D) are gated on $CD4^+Foxp3^+$ cells and compiled cell division data are shown as Treg Division Index and % Divided data using B6 cells from five independent experiments (C, D). Individual data points are shown with the mean ± SEM. * p<0.05, ** p<0.01, *** p<0.001 by one-way, repeated measures ANOVA with Dunnett's post test.

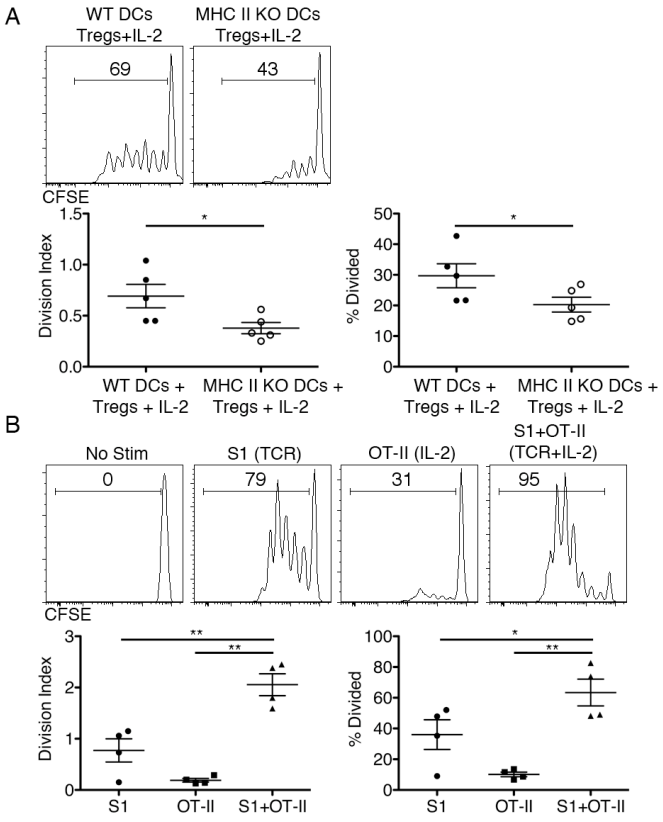

Figure 2.8. TCR and IL-2 signals synergize to induce optimal Treg division. (A) 1 × 10^4 CFSE-labeled, FACS-sorted B6 Tregs ($CD4^+CD25^+$) and 1 × 10^5 FACS-sorted B6 WT or MHC II KO DCs were co-cultured in the presence of GM-CSF with or without IL-2 (50 U/mL) for four days. (B) 2 × 10^4 CFSE-labeled, FACS-sorted TS1×HA28 nTregs ($CD4^+CD25^+$) and DO11.10 Tconvs ($CD4^+CD25^-$) were co-cultured with 1 × 10^5 irradiated Balb/c or Balb/c Rag2 KO feeder splenocytes in media alone, with S1 peptide (1 mM), $Ova_{323-339}$ peptide (1 mM), or both peptides together for four days. Cell division was analyzed by flow cytometry. Representative histograms in top panels are gated on (A) $CD4^+Foxp3^+$ cells or (B) $CD4^+Foxp3^+6.5^+$ cells. 6.5 is an anti-clonotypic TS1 TCR Ab. Compiled data from five (A) or four (B) independent experiments are shown as individual data points and with the mean ± SEM of Treg Division Index or % Divided in the bottom panels. * $p<0.05$ and ** $p<0.001$ by paired, two-tailed Student's *t* test (A) or one-way, repeated measures ANOVA with Dunnett's post test (B).

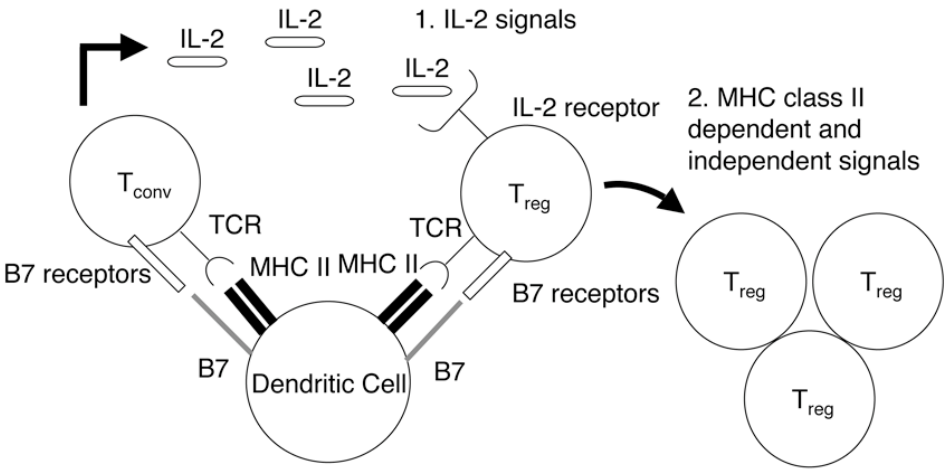

Figure 2.9. A model for the complex interplay between Tregs, Tconvs, and DCs required for Treg division. DCs induce Tconvs to produce IL-2 through MHC II and B7 co-stimulation-dependent signals. This paracrine IL-2 (A) stimulates Tregs to divide in the presence of B7 co-stimulatory and other unidentified signals from DCs (B). When present, cognate Ag/MHC II complexes can enhance Treg division further, leading to optimal Treg division.

Chapter III

Results

IL-2 signals determine the degree of TCR signaling necessary to support regulatory T cell division *in vivo*

Introduction

Suppression of immune responses against both self and foreign antigens by $CD4^+Foxp3^+$ Tregs is a critical mechanism of self-tolerance (Sakaguchi et al., 2008). To maintain immune tolerance, a sufficient number of Tregs must be present in the periphery of the host. A combination of newly generated thymic Tregs, the peripheral conversion of naïve Tconvs into iTregs, and the division/survival of existing Tregs contribute to the steady state maintenance of this essential population (Curotto de Lafaille and Lafaille, 2009; Liston and Rudensky, 2007; Sakaguchi et al., 2008). A defect in any facet of Treg homeostasis can disrupt immune tolerance and lead to autoimmunity. For example, mutations in the *foxp3* gene, which is the crucial transcription factor that enforces Treg cell fate, prevent the generation of Tregs and results in the fatal autoimmune diseases observed in Scurfy mice and human patients with IPEX (Bennett et al., 2001; Brunkow et al., 2001; Wildin et al., 2001). Furthermore, mice deficient in proteins such as CD28 and TGFβ have defects in both the thymic production and the peripheral maintenance of Tregs, rendering them prone to autoimmunity (Liston and Rudensky, 2007).

A striking feature of peripheral Tregs is their continuous cell division in the steady state (Fisson et al., 2003). This basal rate of homeostatic proliferation appears to be balanced by an equal rate of cell death or cell exit from the Treg pool, as the total number of Tregs remains fairly constant throughout the adult life of a mouse, accumulating only in old age (Fisson et al., 2003). This steady state Treg division is reminiscent of the process of homeostatic proliferation observed in memory $CD4^+$ and $CD8^+$ T cells, which is believed to be an essential feature that promotes the long-term persistence of these populations (Surh et al., 2006). Therefore, several groups have interpreted steady state Treg division to be a process that is essential to the maintenance of the peripheral Treg pool (Fisson et al., 2003; Rubtsov et al., 2010; Setoguchi et al., 2005).

IL-2 is critical for the proliferation and maintenance of Tregs. Although Tregs do not make their own IL-2, they constitutively express CD25, the high affinity α subunit of the IL-2R, which allows them to respond to low levels of IL-2 produced by other cells (Setoguchi et al., 2005). Mice deficient in IL-2 or components of the IL-2R have decreased Tregs and develop autoimmunity (Cheng et al., 2011). This outcome is due, in part, to the defective peripheral maintenance of Tregs. Indeed, the acute neutralization of IL-2 in adult mice recapitulates IL-2 or IL-2R deficiency by disrupting Treg homeostasis and causing autoimmunity (Cheng et al., 2011; Liston and Rudensky, 2007). Complementation studies suggest that Tconvs are the source of IL-2, since the combination of WT, but not TCRα KO, BM with IL-2 KO BM rescues Treg numbers in mixed BM chimeras (Almeida et al., 2002; Almeida et al., 2006). Furthermore, recent work has shown that IL-2:anti-IL-2 mAb immune complexes (IC) can augment Treg division and cause expansion of this population (Boyman et al., 2006; Webster et al., 2009).

In addition to IL-2, current dogma asserts that Treg division and homeostasis are absolutely dependent on TCR signaling in Tregs (Bhandoola et al., 2002; Darrasse-Jèze et al., 2009; Gavin et al., 2002). It is believed that since Treg differentiation is initiated through high affinity TCR signaling with its cognate self-antigen presented on MHCII (Jordan et al., 2001), the presence of these cognate self-antigen:MHCII complexes in the periphery also sustains Treg survival, division, and homeostasis (Fisson et al., 2003). Consistent with this notion, adoptively transferred Tregs fail to divide in MHCII KO (Bhandoola et al., 2002; Gavin et al., 2002) and conditional deletion of the TCR signaling proteins Lck or LAT in T cells results in decreased Treg division and survival *in vivo* (Kim et al., 2009; Shen et al., 2010). More recent work has identified expression of MHCII specifically on DCs to be crucial for Treg homeostasis *in vivo* (Darrasse-Jèze et al., 2009). However, in each of the experimental approaches utilized to date, the loss of TCR signaling is not confined to the Treg population but also occurs in Tconvs, which are the major source of IL-2 that supports Treg division. Thus, the failure of Tregs to divide in previous studies may be attributable to the lack of IL-2 production by Tconvs rather than a cell-autonomous requirement for TCR stimulation in Tregs. We propose this notion based on *in vitro*

studies presented in the previous chapter demonstrating that Tregs do not need MHCII to divide if exogenous IL-2 and contact with MHCII KO DCs are provided.

Here, we examined the necessity of cell-autonomous TCR signaling in Tregs for their division *in vivo*. Through the complementary approaches of restricting the peptide repertoire presented on MHCII and disrupting TCR signal transduction in Tregs, we find that Treg division is partially dependent on a diverse peptide repertoire and is completely dependent on TCR signaling at steady state IL-2 levels. However, strong IL-2R stimulation induced by IL-2 ICs obviated the need for TCR signaling in Treg division, indicating that TCR signals are not absolutely necessary in every setting. In the absence of exogenous IL-2R agonists and MHCII, the activation of the IL-2R-induced STAT5 pathway alone is capable of supporting Treg division. These data demonstrate for the first time that while steady state Treg division requires TCR signaling, targeted IL-2R stimulation can overcome this requirement to promote Treg division *in vivo*. We propose that the combination of TCR and IL-2R/STAT5 signaling together determine the extent of cell division individual Tregs undergo and ultimately control the homeostasis of this population.

Results

TCR signaling is required for Treg division in the steady state

Previous studies have shown that TCR transgenic Tregs divide in the presence of cognate antigen *in vivo* (Walker et al., 2003; Yamazaki et al., 2003), lending experimental support to the idea that cell-autonomous TCR interactions with cognate self-antigens presented on MHCII sustain Treg division and homeostasis. To test this notion further, we utilized H-2DMα KO mice, which are defective in the ability to exchange peptides from maturing MHCII molecules (Martin et al., 1996). Consequently, these mice display a severely restricted MHCII peptide repertoire where almost all surface MHCII molecules are loaded with a single peptide, the class II-associated invariant chain peptide (CLIP). We reasoned that Tregs that have developed in H-2DMα KO mice would have TCR specificities that react most suitably with CLIP. On the other hand, Tregs from WT mice, which were selected on a diverse array of peptides, would lose nearly all cognate antigen-induced TCR reactivity when adoptively transferred into H-2DMα KO hosts. To test whether the loss of these TCR signals in Tregs hinders their division, we adoptively transferred magnetic bead purified, CFSE-labeled WT and H-2DMα KO T cells bearing different congenic markers into the same H-2DMα KO recipients expressing a third set of congenic markers. One month later, donor WT Tregs divided significantly less than donor H-2DMα KO Tregs (Figure 3.1A). Thus, although cell-autonomous TCR interactions with their selecting antigen/MHCII complexes appear important for optimal Treg division, some Tregs can still divide without this stimulation.

To investigate further the requirement of TCR signaling in Treg division, we devised a strategy to abrogate TCR signaling specifically in Tregs. This approach was needed to dissociate the TCR signaling capacity of Tregs from that of Tconvs, since Tconvs produce IL-2 in a TCR/MHCII-dependent manner to support Treg division. We utilized mice in which the TCR signaling molecule SLP-76 could be inducibly deleted by a tamoxifen-inducible cre recombinase (Maltzman et al., 2005). Conditional deletion of SLP-76 abrogates downstream TCR signaling and prevents T cell activation (Wu et al., 2011). A yellow fluorescent protein (YFP) reporter was used to mark cells with a history of cre-mediated recombination and thus deletion of the floxed

SLP-76 allele. To create a mixture of SLP-76 deficient Tregs and WT Tconvs, we employed a mixed BM chimera approach in which congenically marked WT competitor BM was mixed with BM expressing different congenic markers from either a heterozygous SLP-$76^{+/-}$ (Het) or conditional heterozygous SLP-$76^{flox/+}$ (cHet), or a conditional KO SLP-$76^{flox/-}$ (cKO) donor and transplanted into irradiated WT recipients expressing a third set of congenic markers. Tamoxifen was then administered to all BM chimeras to generate either YFP^{+}SLP-$76^{+/-}$ or YFP^{+}SLP-$76^{-/-}$ Tregs. Five days after the last tamoxifen dose, the mice were administered BrdU to assess the extent of Treg division (Figure 3.1B, data not shown). In each of the BM chimeras, 10-25% of the WT competitor Tregs incorporated BrdU, suggesting that sufficient IL-2 was being produced by WT Tconvs to sustain Treg division. While a large fraction of the YFP^{+}SLP-76 Het/cHet Tregs also incorporated BrdU, the YFP^{+}SLP-76 cKO Tregs were nearly completely defective in BrdU incorporation (Figure 3.1B, data not shown). Normalization of BrdU incorporation by YFP^{+} Tregs to WT competitor Tregs demonstrated a significant reduction in BrdU incorporation by YFP^{+}SLP-76 cKO Tregs compared to YFP^{+}SLP-76 Het/cHet Tregs. Thus, these data suggest that cell-autonomous TCR signaling is required to sustain Treg division at steady state levels of endogenous IL-2.

MHCII-dependent TCR signals are not required for Treg division in the presence of IL-2R agonists *in vivo*

A recent study demonstrated although naïve $CD8^{+}$ T cells normally do not divide at steady state levels of IL-2, very high concentrations of IL-2 can induce these cells to proliferate intensely (Cho et al., 2007). Based on these data, we hypothesized that while Tregs required TCR signaling to divide at endogenous levels of IL-2, perhaps exogenous provision of very high concentrations of this cytokine could bypass the TCR dependence of Treg division. To test this notion, CFSE-labeled T cells B6.SJL mice were FACS-sorted to greater than 99% purity and adoptively transferred into either congenically marked WT or MHCII KO recipients. The recipient mice were then given IL-2 ICs, which have been reported to enhance the biological activity of exogenously administered IL-2 *in vivo* (Boyman et al., 2006). As previously reported (Gavin et al.,

2002), without IL-2 IC administration, adoptively transferred Tregs divided in WT but not in MHCII KO recipients (Figure 3.2A). However, with IL-2 IC administration, donor Tregs divided equally well in WT and MHCII KO hosts, indicating that IL-2 IC-induced Treg division can occur independently of MHCII-mediated TCR signaling (Figure 3.2A). We also observed an increase in the absolute number of donor Tregs in IL-2 IC-treated compared to untreated WT and MHCII KO recipients (Figure 3.2B). Unlike the effect on cell division, IL-2 IC-induced Treg expansion appeared to be partially dependent on MHCII, as the frequency and total numbers of donor Tregs were significantly higher in WT versus MHCII KO recipients (Figure 3.2B). We obtained similar results in two strains of MHCII KO mice, both the I-$A^b\beta$ KO strain and mice that are completely devoid of all MHCII genes (Figure 3.2B). These data suggest that in the presence of an IL-2R agonist, Tregs proliferate *in vivo* in the absence of MHCII and hence antigen-induced TCR signals.

IL-2R agonists can restore Treg division in the absence of SLP-76-mediated TCR signaling

Because IL-2 IC treatment allowed Tregs to divide in the absence of MHCII-mediated TCR signaling, we next examined whether IL-2 ICs could overcome the loss of SLP-76-mediated TCR signaling. We administered tamoxifen to WT+SLP-76 Het/cHet and WT+SLP-76 cKO mixed BM chimeras, then treated these mice with PBS or IL-2 ICs at the start of a BrdU pulse. While PBS-treated YFP^+SLP-76 cKO Tregs did not take up BrdU, IL-2 IC-treated YFP^+SLP-76 cKO Tregs incorporated BrdU at a rate similar to PBS-treated YFP^+SLP-76 Het/cHet Tregs (Figure 3.3A), suggesting that strong IL-2 signals can overcome the need for TCR signaling in Treg division. However, BrdU incorporation by IL-2 IC-treated YFP^+SLP-76 cKO Tregs was considerably less than that observed in both WT competitor Tregs within the same mice and YFP^+SLP-76 Het/cHet Tregs from IL-2 IC-treated mice (Figure 3.3A). Thus, in the absence of SLP-76-mediated TCR signaling, IL-2 IC treatment restores the ability of Tregs to divide. Moreover, although IL-2 IC treatment increased the frequency and total number of Tregs in both WT competitor and YFP^+SLP-76 Het/cHet $CD4^+$ T cell compartments, we did not observe a concomitant increase in the frequency or total number of Tregs in the YFP^+SLP-76 cKO $CD4^+$ T

cell pool (Figure 3.3B, data not shown). These data suggest that strong IL-2R signaling can substitute for a loss of SLP-76-mediated TCR signaling to induce Treg division.

STAT5 activation is sufficient to sustain Treg division in the absence of MHCII *in vivo*

To provide a mechanism for the ability of IL-2 ICs to promote TCR-independent Treg division, we tested the involvement of the STAT5 pathway, since IL-2R signaling in Tregs preferentially activates STAT5 (Bensinger et al., 2004). Moreover, transgenic expression of a constitutively active form of STAT5b (STAT5b-CA) is sufficient to induce thymic Treg differentiation and maintain Treg homeostasis in the absence of IL-2Rβ (Burchill et al., 2007b). Thus, we examined whether activation of the STAT5 pathway alone supports Treg division in a TCR-independent manner. First, we tested whether STAT5 activation downstream of IL-2R stimulation was still intact in the absence of TCR signaling. *In vitro* stimulation of whole splenocytes and LN cells with IL-2 resulted in equivalent levels of phospho-STAT5 in both YFP^+SLP-76 cHet and YFP^+SLP-76 cKO Tregs (Figure 3.4A). Next, to investigate whether selective activation of STAT5 supports Treg division in the absence of TCR signaling, we adoptively transferred a 1:1 mixture of FACS-purified, CFSE-labeled WT T cells and STAT5b-CA T cells expressing different congenic markers into WT or MHCII KO hosts expressing a third set of congenic markers. After two weeks, we observed that while both donor Treg populations divided in WT hosts, only STAT5b-CA expressing Tregs divided in MHCII KO hosts (Figure 3.4B). However, the rescue of Treg division by STAT5b-CA in MHCII KO mice was partial, given that STAT5b-CA expressing Tregs divided significantly more in WT compared to MHCII KO hosts (Figure 3.4B). Moreover, the recovery of total STAT5b-CA donor Tregs was significantly lower in MHCII KO compared to WT recipients (Figure 3.4C). These data suggest that MHCII-dependent TCR signals are not required for the division of Tregs. Thus, the selective activation of IL-2-induced STAT5 signaling is minimally sufficient to sustain Treg division.

Discussion

Although it is believed that peripheral Treg division and maintenance are dependent on TCR stimulation by antigen/MHCII (Bhandoola et al., 2002; Darrasse-Jèze et al., 2009; Fisson et al., 2003; Gavin et al., 2002), our studies in Chapter II have shown that Tregs can divide *in vitro* in the absence of MHCII. In this chapter, we demonstrated that Tregs can also divide *in vivo* in the absence of MHCII. At steady state levels of IL-2, Treg division required TCR signaling. However, with exogenous administration of IL-2R agonists, MHCII expression and cell-autonomous TCR signaling were both dispensable for Treg division. Moreover, expression of STAT5b-CA in Tregs partially restored Treg division in the absence of MHCII. These data suggest that the selective activation of the STAT5 pathway downstream of the IL-2R is the minimal requirement for Treg division *in vivo*. Based on these data, we propose a model in which Treg division and homeostasis can be at least partially maintained by any degree of TCR signaling, depending on the degree of IL-2R stimulation that is also present.

The experiments shown here demonstrate for the first time that in settings where IL-2 signals are abundant, TCR/MHCII contacts are dispensable for Treg division. We propose a model where progressively stronger IL-2R stimulation is required to offset the loss of cell-autonomous TCR signals in Tregs in order to sustain the division and homeostasis of this population. In limiting amounts of IL-2, as is the case in the steady state, Tregs encountering their cognate antigens may divide preferentially over Tregs of other TCR specificities. However, in situations where ample IL-2 is produced, such as during an inflammatory process, Tregs of various TCR specificities may be induced to proliferate and expand. The recruitment of Tregs with multiple TCR specificities may be important for optimal control of immune responses (Föhse et al., 2011).

Although Tregs receiving diminished TCR signaling continue to divide in the presence of IL-2R signaling, the extent to which this residual cell division is able to maintain these Treg may correlate directly with the degree of TCR signaling that they experience. For example, with IL-2 IC administration, the recovery of total Tregs was consistently lower in MHCII KO compared to WT recipients. Similar results were obtained upon adoptive transfer of STAT5b-CA expressing Tregs

into WT versus MHCII KO hosts. Moreover, even though IL-2 IC administration partially restored the division of SLP-76 cKO Tregs, this treatment failed to expand SLP-76 cKO Tregs compared to PBS-treated controls. The decreased recovery of Tregs receiving suboptimal TCR stimulation may represent a new homeostatic "set point" for the pool size of those Treg subsets. Meanwhile, Tregs that receive the strongest TCR signals may have a proliferative advantage that allows them to be preferentially maintained in the Treg pool.

The mechanisms by which TCR signals and IL-2 cooperate to mediate Treg homeostatic proliferation are unknown. There may be a role for the TCR in modulating the Treg responsiveness to IL-2 signaling. In naïve $CD8^+$ T cells, TCR signals augment the amount and organization of lipid rafts, which in turn determine the degree of naïve $CD8^+$ T cell proliferation in response to IL-2 (Cho et al., 2010). However, our data show that proximal IL-2 signaling in Tregs, as measured by phospho-STAT5 levels, is intact even in the absence of SLP-76, suggesting that TCR signaling may not affect IL-2 responsiveness. Still, we cannot entirely exclude the possibility that SLP-76 mediates signaling downstream of the IL-2R through other intermediates.

A related caveat is that while a major function of SLP-76 is to integrate TCR signals for T cell activation (Zou et al., 2010), it is also responsible for signaling through integrins (Smith-Garvin et al., 2009). Integrins mediate T cell adhesion to APCs and transmit important activation cues to T cells (Smith-Garvin et al., 2009). Thus, defective integrin signaling in SLP-76 cKO Tregs may contribute to their impaired cell division. In fact, a recent study suggests that integrin-mediated signaling may be crucial for Treg suppressive function (Au-Yeung et al., 2010). Thus, it is possible that Treg division and homeostasis may also depend on integrin signaling. Although these unknown factors represent a limitation of our studies of SLP-76 deficient Tregs, if SLP-76 deficiency impairs Treg division through a receptor other than the TCR, it would only further diminish the importance of the TCR in this process.

Stimulation of the IL-2R activates three major signaling pathways. One pathway activated by IL-2R stimulation is JAK-STAT signaling, occurring specifically through STAT5. Additionally, two Shc dependent pathways that activate phosphoinositide 3-kinase (PI3K)/Akt and Ras/mitogen-activated protein kinases (MAPKs) are triggered (Cheng et al., 2011). We focused

our studies on STAT5, since IL-2R signaling in Tregs preferentially activates STAT5 due to inhibition of the PI3K/Akt axis by phosphatase and tensin homolog (PTEN) in these cells (Bensinger et al., 2004). Moreover, transgenic expression of STAT5b-CA is sufficient to induce thymic Treg differentiation, maintain Treg homeostasis, and suppress autoimmunity in the absence of IL-2Rβ (Yu et al., 2009). WT transgenic STAT5b-CA mice also have elevated percentages of Tregs (Burchill et al., 2007b). Importantly, naïve $CD4^+$ T cells from STAT5-CA mice can divide *in vivo* in the absence of MHCII (Taylor et al., 2006). Similarly, we found that expression of STAT5b-CA allowed Tregs to divide in the absence of MHCII. An outstanding question is whether MHCII-independent TCR signaling is necessary for Treg division in the setting of STAT5b-CA expression. To test this notion, we will examine whether expression of STAT5b-CA can sustain Treg division in the absence of SLP-76. It is possible that these experiments will uncover an additional role for MHCII-independent TCR signaling in Treg division. MHC-independent or ligand-independent TCR signaling has been implicated in β selection of DN3 thymocytes, in which the pre-TCR complex is thought to signal in a cell-autonomous fashion to complete this development checkpoint (Haks et al., 2003). However, no role for MHC-independent TCR signaling has been demonstrated for mature T cells expressing a complete TCR. Indeed, if MHCII-independent TCR signaling can sustain Treg division, it is possible that Treg homeostasis may be maintained through a different mechanism than both naïve and memory T cells, which are maintained independent of TCR signaling and through MHC-dependent tonic TCR signaling, respectively (Surh and Sprent, 2008).

Collectively, our data demonstrate that peripheral Tregs can divide in the absence of TCR signaling *in vivo*. These findings challenge the long-held notion that peripheral Tregs are maintained by continuous TCR signals induced by self-antigen/MHCII complexes. Tregs are pivotal to the maintenance of self-tolerance. While these cells have remarkable therapeutic potential to modulate human disease, the appropriate manner in which they should be manipulated remains undefined. An understanding of the mechanisms that govern their division and homeostasis will be crucial for properly harnessing the immunosuppressive functions of Tregs for therapeutic interventions.

The first two chapters of the Results section have investigated the signals that govern the behavior of mature peripheral Tregs both *in vitro* and *in vivo*. In particular, we focused on the dependence of Treg division on TCR signaling. During the course of our studies on TCR signaling in mature Tregs, we became interested in the TCR signals that control their generation in the thymus. Although the prevailing model of Treg development proposes an instructive role for high affinity TCR signaling, the specific intracellular pathways that translate TCR signaling to Treg differentiation is unknown. In Chapter IV, we examine the role of a particular TCR-induced signaling pathway in promoting Treg differentiation.

Figures

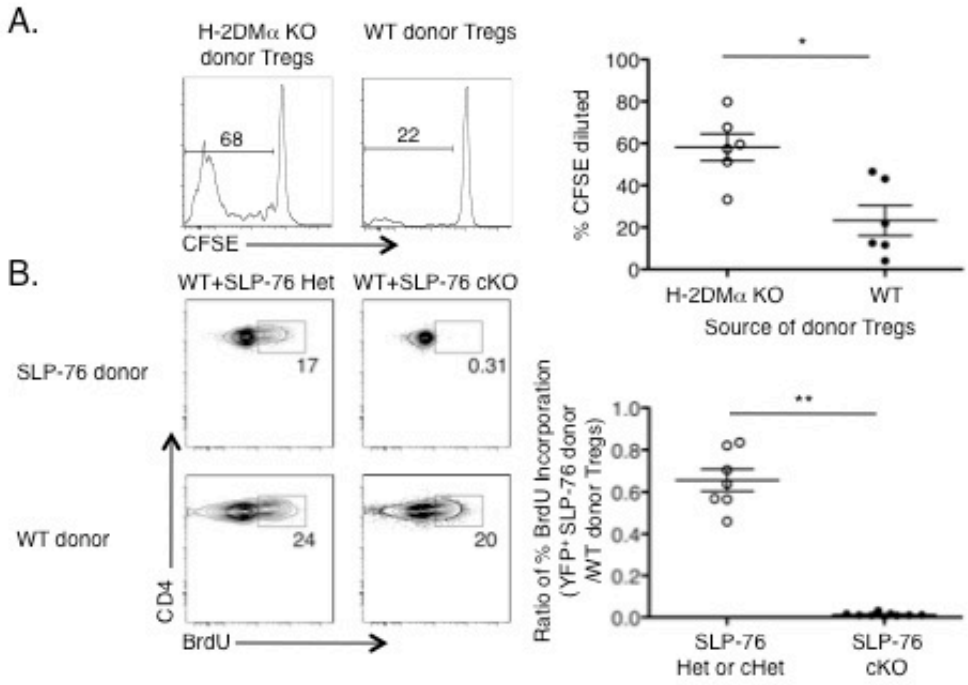

Figure 3.1. TCR signaling is required for Treg division in the steady state (A) CFSE-labeled Magnetic-activated cell sorting (MACS)-purified $CD4^+$ T cells from WT ($CD90.1^+CD45.2^+$) and H-2DMa KO ($CD90.2^+CD45.1^+$) mice were mixed and injected i.v. into H-2DMa KO mice ($CD90.2^+CD45.2^+$). One month later, CFSE dilution of splenic donor Tregs was analyzed by flow cytometry. One representative CFSE dilution plot of donor WT (right plot) or H-2DMa KO (left plot) $CD4^+Foxp3^+$ Tregs is shown. Data from two independent experiments are represented as the mean ± SEM with a total of 6 mice per group (right). (B) WT+SLP-76 Het/cHet and WT+SLP-76 cKO mixed BM chimeras were administered tamoxifen and treated with BrdU for 13-14 days. BrdU incorporation by splenic Tregs was analyzed by flow cyometry. One representative contour plot gated on $CD90.1^-CD45.2^+YFP^+CD4^+Foxp3^+$ cells for SLP-76 Het (top left plot) or cKO (top right plot) and $CD90.1^+CD45.2^+CD4^+Foxp3^+$ for WT donor Tregs (bottom plots). Data from two independent experiments are represented as the mean ± SEM with a total of 7-8 mice per group (right graph). BrdU incorporation by SLP-76 Het/cHet or cKO YFP^+Tregs was normalized to that of WT donor Tregs in the right graph. * $p<0.05$ and ** $p<0.001$ by two-tailed, unpaired Student's *t* test.

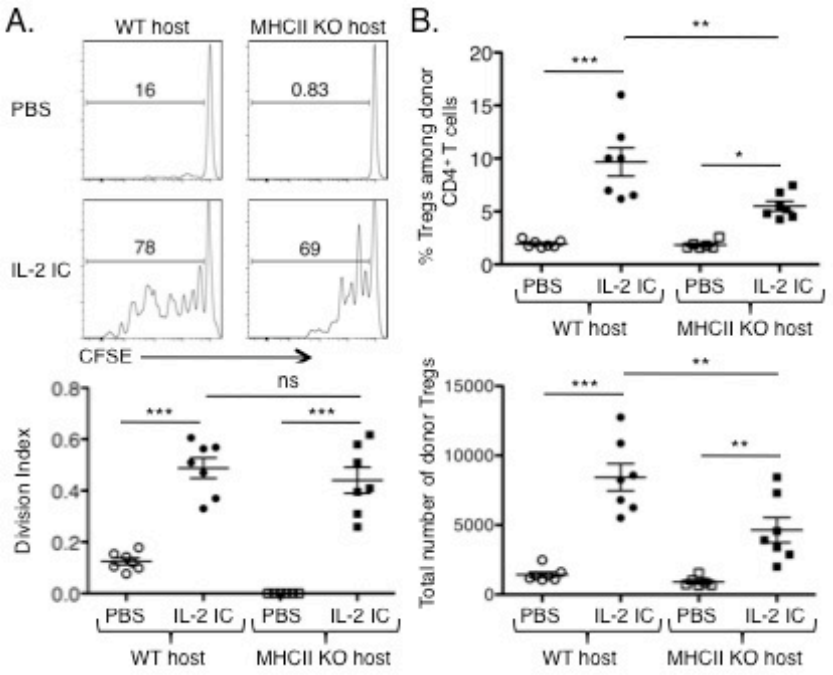

Figure 3.2. IL-2 ICs induce Treg proliferation in the absence of MHCII *in vivo*. (A) FACS-sorted CFSE-labeled CD45.1$^+$ total WT T cells were adoptively transferred into CD45.2$^+$ WT or MHCII KO mice and treated with IL-2 ICs for three days. Three days after the last IL-2 IC treatment, CFSE dilution by donor splenic Tregs was analyzed by flow cytometry. (A) Histograms show representative CFSE profiles of WT donor Tregs (CD45.1$^+$CD4$^+$Foxp3$^+$) in the indicated recipients treated with PBS (top plots) or IL-2 ICs (bottom plots). The division index of Tregs was determined and represented as the mean ± SEM from two independent experiments with a total of 7-8 mice per group (bottom graph). (B) The frequency of donor Tregs among total donor CD4$^+$ T cells (top) and the total number (bottom) of donor Tregs from WT and MHCII KO recipient mice described in (A) are plotted as the mean ± SEM from two independent experiments with a total of 7-8 mice per group. We obtained similar results in two strains of MHCII KO mice, both the I-A^{b}b KO strain and mice that are completely devoid of all MHCII genes. * p<0.05, ** p<0.01, and *** p<0.001, by one-way analysis of variance (ANOVA) with Tukey's post test.

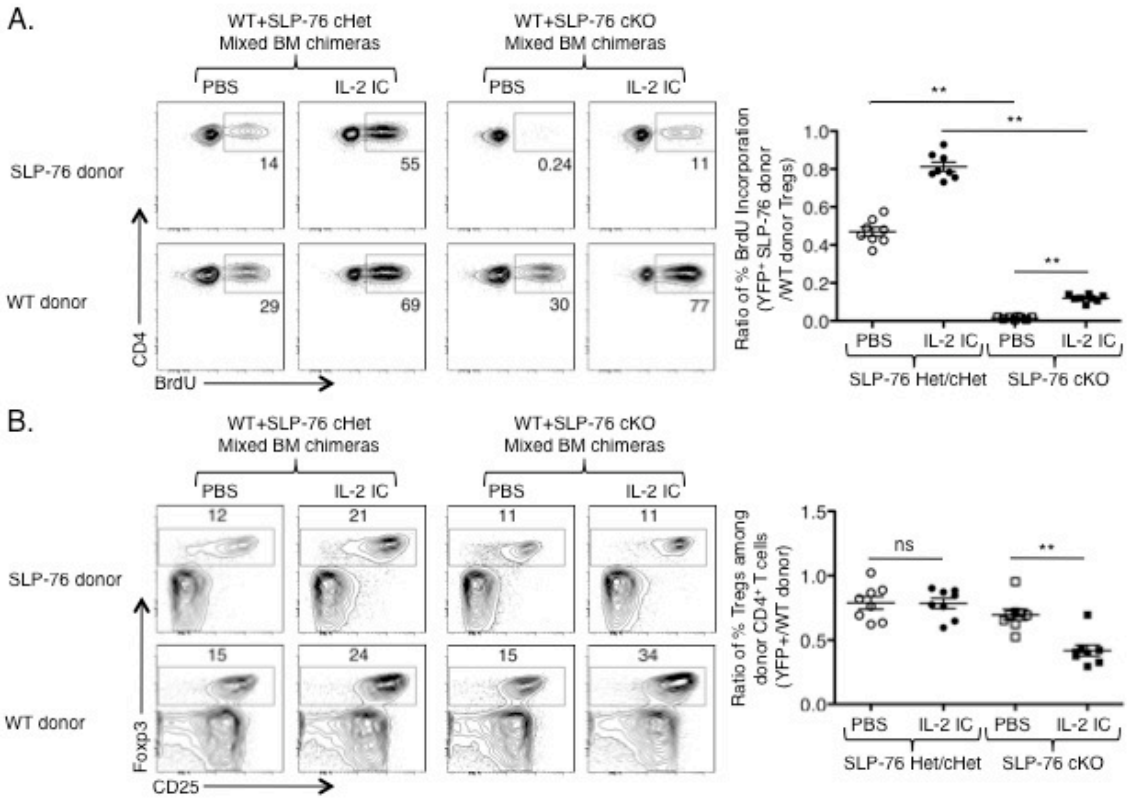

Figure 3.3. IL-2 ICs restore Treg division in the absence of SLP-76-mediated TCR signaling. WT+SLP-76 Het/cHet and WT+SLP-76 cKO mixed BM chimeras were administered tamoxifen and treated with PBS or IL-2 IC for three days and BrdU for five days. (A) BrdU incorporation by $CD90.1^{-}CD45.2^{+}YFP^{+}CD4^{+}Foxp3^{+}$ SLP-76 Het/cHet or cKO Tregs (top row) and $CD90.1^{+}CD45.2^{+}CD4^{+}Foxp3^{+}$ WT donor Tregs (bottom row) was analyzed by flow cytometry and is shown as representative contour plots (left). BrdU incorporation by either SLP-76 Het/cHet or cKO Tregs was normalized to that of WT donor Tregs within the same mouse and data from two independent experiments is represented as the mean ± SEM with a total of 8 mice per group (right graph). (B) The frequency of $Foxp3^{+}$ Tregs among total $CD4^{+}$ splenic T cells derived from SLP-76 Het/cHet or cKO (top row) and WT donor BM (bottom row) is shown as representative contour plots (left). The Treg frequency in SLP-76 Het/cHet or cKO $CD4^{+}$ T cells was normalized to that of the WT donor $CD4^{+}$ T cells within the same mouse and data from two independent experiments is represented as the mean ± SEM with a total of 8 mice per group (right). * p<0.01 and ** p<0.001 by one-way ANOVA with Tukey's post test.

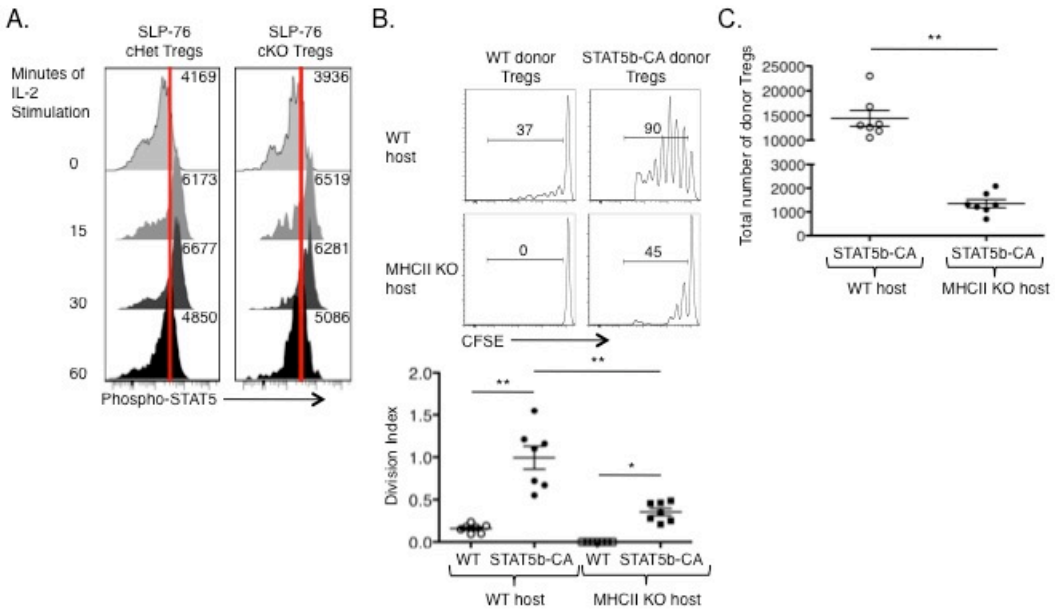

Figure 3.4. Selective activation of STAT5 signaling is sufficient to sustain Treg division in the absence of MHCII. (A) Splenocytes and LNs from tamoxifen-treated SLP-76 cHet and SLP-76 KO mice were stimulated with IL-2 (100 U/mL) for 0, 15, 30, and 60 minutes and stained for phospho-STAT5. Histograms are gated on $YFP^+CD4^+Foxp3^+$ Tregs and the MFI for phospho-STAT5 staining is shown at the upper right hand corner of each histogram. The red line helps to emphasize differences in phospho-STAT5 staining. One representative of a total of three SLP-76 cHet and two SLP-76 cKO mice analyzed in two independent experiments is shown. (B) A 1:1 mixture of FACS-purified CFSE-labeled $CD90.1^+CD45.2^+$ WT and $CD90.2^+CD45.2^+$ STAT5b-CA total T cells were adoptively transferred into $CD90.2^+CD45.2^+$ WT or MHCII KO recipient mice. Two weeks later, CFSE dilution of $CFSE^+$ donor $CD4^+Foxp3^+$ Tregs in spleen and LNs was analyzed by flow cytometry and shown as representative histograms. The division index of Tregs was determined and represented as the mean ± SEM from two independent experiments with a total of 7 mice per group. (D) The total number of STAT5b-CA $CD4^+Foxp3^+$ Tregs recovered from the spleens and LNs of WT or MHCII KO recipients is represented as mean ± SEM of two independent experiments with a total of 7 mice per group. * $p<0.01$ and ** $p<0.001$ by unpaired, two-tailed Student's *t* test or one-way ANOVA with Tukey's post test.

Chapter IV

Results

The role of the diacylglycerol pathway in regulatory T cell differentiation

Introduction

Self-tolerance is mediated by $CD4^{+}Foxp3^{+}$ Tregs that patrol the tissues of the host, suppressing both appropriate and harmful immune responses. The thymus is the major producer of Tregs, which are exported along with other T cell subsets to the periphery. Seminal studies identified a critical role for TCR interactions with antigen/MHCII in thymic nTreg development (Jordan et al., 2001). Strongly stimulatory agonist peptide antigens that induce high affinity TCR interactions promoted nTreg formation preferentially *in vivo*, whereas partial agonist peptides did not (Jordan et al., 2001). Based on these studies, Treg differentation has been incorporated into the TCR affinity model of thymic T cell development (Liston and Rudensky, 2007). In this model, low affinity TCR interactions with antigen/MHC are required for positive selection, while high affinity TCR interactions with antigen/MHC result in the negative selection of developing thymocytes (Liston and Rudensky, 2007). Initially, it was proposed that the TCR affinity needed to instruct thymocytes to the Treg lineage was above the affinity threshold for positive selection but below the threshold for negative selection (Liston and Rudensky, 2007). However, recent work indicates that the TCR affinity that instructs Treg differentiation is even higher than the threshold needed for negative selection (Cozzo Picca et al., 2011). Despite the importance of TCR affinity in Treg differentiation, the precise signaling pathways that sense and translate high affinity TCR stimulation into Treg lineage commitment are incompletely defined.

Studies in mutant or KO mice have implicated a role for a number of TCR signaling proteins in Treg development. Upon TCR stimulation, recruitment of multiple PTKs leads to the activation of PLC-γ1, which initiates downstream signal transduction. A critical event in the activation of PLC-γ1 is binding to the signaling protein LAT at tyrosine 136 (Koonpaew et al., 2006). Genomic knock-in mice harboring a tyrosine to phenylalanine substitution at position 136 of LAT (LAT^{Y136F}) possess diminished PLC-γ1 function (Koonpaew et al., 2006). The LAT^{Y136F}

mutation also results in a cell-intrinsic defect in Treg generation, indicating both LAT and PLC-γ1 function are necessary for Treg development (Koonpaew et al., 2006). The function of PLC-γ1 is to cleave the membrane lipid PIP_2 into the second messengers IP_3 and DAG, which together propagate downstream signaling. IP_3 signaling results in intracellular Ca^{2+} flux and the nuclear translocation of the transcription factor NFAT. Importantly, NFAT can bind to the *foxp3* enhancer region to promote transcription after TCR stimulation (Tone et al., 2008), suggesting that the critical function of PLC-γ1 in Treg differentiation is to promote NFAT nuclear translocation and trans-activation of the *foxp3* gene.

Meanwhile, one important pathway that DAG signaling activates is Ras/ERK signaling. The Ras/ERK pathway is critical for mediating both positive and negative selection during thymic T cell development (Alberola-Ila et al., 1995; Daniels et al., 2006; Swan et al., 1995). Thymocytes from ERK1 and ERK2 double KO mice fail positive selection and generate few mature T cells (Fischer et al., 2005). A pattern of Ras/ERK activation distinct from that observed in positive selection also appears to participate in the negative selection of developing thymocytes (Daniels et al., 2006). Thus, the Ras/ERK pathway may operate as a gauge of the strength of TCR stimulation that converts TCR affinity to intracellular signals that alter cell fate decisions during thymic T cell development. Given the proven role of TCR affinity in Treg differentiation, it is possible that TCR-induced Ras/ERK signaling may direct Treg differentiation. Despite these studies, direct examination of the contribution of either Ca^{2+} or DAG activated pathways to Treg differentiation is lacking.

Downstream of PLC-γ1 activation, the NF-κB pathway has been implicated in controlling Treg differentiation. Mice expressing a dominant negative form of IKK2 or those with a combined deficiency of the NF-κB family members p50 and c-Rel contain very few Tregs (Schmidt-Supprian et al., 2003; Zheng et al., 2003). Additionally, mice lacking signaling proteins important for TCR-induced NF-κB activation, such as transforming growth factor-b-activated-kinase-1 (TAK1), PKCθ, CARMA1, and TRAF6, all possess cell-intrinsic defects in Treg generation (Barnes et al., 2009; Gupta et al., 2008; Shimo et al., 2011; Wan et al., 2006). Furthermore, deletion of CD28, the co-stimulatory receptor that augments TCR-induced NF-κB activation, results in impaired

Treg development (Schulze-Luehrmann and Ghosh, 2006; Tai et al., 2005). Recent studies have identified the transcription factor c-Rel as the NF-κB family member that is responsible for inducing *foxp3* gene expression and Treg lineage differentiation (Long et al., 2009; Ruan et al., 2009).

Despite the well-established role of NF-κB activity in Treg development, the signaling pathway that links TCR-induced PLC-γ1 activation to NF-κB-dependent Treg differentiation remains undefined. Notably, biochemical studies have identified DAG signaling as an important pathway that links PLC-γ1 function to the activation of PKCθ, a positive regulator of NF-κB activity, and Ras/ERK signaling. Previously, we generated mice deficient in DGKζ, a negative regulator of DAG-mediated signal transduction that is expressed in T cells. T cells from DGKζ KO mice display enhanced activation of DAG-mediated signaling pathways downstream of TCR stimulation, such as the phosphorylation of the ERK (Zhong et al., 2003). Importantly, this alteration in the DAG pathway appears specific, as Ca^{2+} signaling remains grossly normal in DGKζ KO mice. Based on the role of Ras/ERK in translating TCR affinity to cell fate decisions and the known role of NF-κB signaling on Treg development, we hypothesized that the upstream signaling through DAG may link high affinity TCR stimulation to Treg differentiation.

This chapter provides a preliminary analysis of the role of DAG signaling in Treg generation. DGKζ KO mice contain an increased frequency of Tregs in both the thymus and the spleen. This phenotype is cell-intrinsic as demonstrated through BM chimeras reconstituted with a mixture of WT and DGKζ KO BM. Additionally, CD4 SP thymocytes from DGKζ KO mice differentiate preferentially into Tregs *in vitro* as compared to those from WT mice, suggesting that enhanced TCR-induced DAG signaling augments nTreg differentiation. Moreover, deletion of DGKζ partially restores Treg development in CD28 KO mice, implying that augmented DAG signaling through the TCR may compensate partially for the absence of co-stimulation to induce Treg differentiation. Future studies will address our hypothesis that the preferential Treg differentiation observed in DGKζ KO mice results from enhanced activation of the NF-κB family member c-Rel by TCR-induced DAG signaling.

Results

Cell-intrinsic loss of DGKζ results in enhanced Treg differentiation

To examine the impact of enhanced DAG signaling on Treg development, we compared Treg numbers in WT and mice with a germline deletion of DGKζ. We found an elevated frequency of Foxp3$^+$ Tregs in both the thymus and spleen of DGKζ KO mice as compared to WT controls (Figure 4.1A). Preliminary studies indicate this Treg phenotype does not seem to develop as the mice age, as three-week-old DGKζ KO mice also possess higher frequencies of Tregs than WT mice (Figure 4.1A). While DGKζ KO mice contain decreased frequencies of Foxp3$^-$ Tconvs (Figure 4.1A), the total numbers of Foxp3$^-$ Tconvs remained the similar in DGKζ KO mice compared to WT controls (data not shown). Conversely, the total number of splenic Tregs was higher in DGKζ KO mice compared to WT controls (Figure 4.1B), suggesting that the increased frequency of Tregs in DGKζ KO mice is not merely a result of decreased frequencies of Tconvs, Although there is only a trend toward an elevated total number of thymic Tregs in DGKζ KO compared to WT mice, analysis of more mice will likely reveal a statistically significant difference (Figure 4.1B). These data suggest that augmented DAG signaling may enhance Treg development *in vivo*.

Since DGKζ KO mice harbor a germline deletion, the observed increase in Treg numbers may result from the loss of DGKζ in non-hematopoietic cells, in non-T cell lineage hematopoietic cells, or in the thymocytes themselves. To distinguish between these possibilities, we generated BM chimeras that received a mixture of congenically labeled WT and DGKζ KO BM cells after lethal irradiation. Loss of DGKζ did not grossly affect early T cell development in the thymus at eight to ten weeks after BM transplant, as cells derived from DGKζ KO BM were represented in similar proportions in BM hematopoietic stem cells and DN and DP thymocyte populations (Figure 4.2A). Meanwhile, CD4$^+$ SP thymocytes and splenocytes derived from DGKζ KO donor BM contained increased frequencies of Foxp3$^+$ Tregs as compared to WT-derived CD4$^+$ SP controls (Figure 4.2B), revealing a cell-intrinsic role for DGKζ in controlling Treg differentiation. These data suggest that DGKζ may normally function to restrain Treg differentiation in a cell-intrinsic fashion.

Over-expression of DGKζ inhibits Treg differentiation in DGKζ KO, but not WT, cells

To confirm that absence of DGKζ is responsible for enhanced Treg differentiation, we tested whether re-expression of DGKζ protein is able to reverse this phenotype. To accomplish this, BM cells from DGKζ KO mice were transduced with a modified mouse stem cell retrovirus (MIGR) expressing either a green fluorescent protein (GFP) reporter alone or together with DGKζ. Transduced BM cells were transplanted to congenically labeled, lethally irradiated mice and allowed to reconstitute the host for nine to ten weeks. Although DGKζ over-expression did not appear to alter early T cell development (data not shown), the frequency of thymic and splenic $Foxp3^+$ Tregs was substantially decreased in GFP^+CD4^+ T cells over-expressing DGKζ as compared to either non-transduced GFP^-CD4^+ T cells from the same mice or with GFP^+CD4^+ T cells from MIGR-GFP transduced control mice (Figure 4.3A). Although these differences did not reach statistically significance for thymic Tregs, analysis of more mice is ongoing. Concurrently, in splenic T cells, over-expression of DGKζ limited the levels of TCR-induced phospho-ERK1/2 (data not shown), which is a surrogate measure of DAG-mediated signaling. Consistent with our hypothesis, TCR-induced phospho-ERK levels correlated directly with the frequency of Tregs within each mouse (data not shown). Of note, the frequencies of Tregs observed in this experiment are likely artificially low due to technical limitations. Regardless, these data confirm that loss of DGKζ alters both DAG-mediated signaling and Treg development concomitantly.

Since DGKζ deficiency enhances Treg development, we reasoned that inhibition of DAG-mediated signaling through over-expression of DGKζ might restrain Treg generation in WT T cells. To test this notion, BM cells from Foxp3 red fluorescent protein (RFP) reporter mice were transduced with MIGR-GFP or with MIGR-DGKζ-GFP and transplanted to congenically labeled, lethally irradiated mice. DGKζ over-expression did not alter early T cell development (data not shown). To our surprise, despite a threefold increase in DGKζ protein levels and a concomitant decrease in TCR-induced phopsho-ERK1/2 levels between GFP^+ and GFP^- splenic T cells (data not shown), over-expression of DGKζ did not change the frequency of thymic or splenic $Foxp3^+$ Tregs within GFP^+CD4^+ T cells as compared to either GFP^-CD4^+ T cells from the same mice or to GFP^+CD4^+ from control mice transduced with MIGR-GFP (Figure 4.3B). One explanation for this

result is that below a certain threshold of DAG signaling, changes in DAG levels do not impact Treg differentiation. Alternatively, perhaps a threefold increase in DGKζ protein levels is insufficient to lower DAG signaling enough to alter Treg development. Collectively, these data demonstrate that although loss of DGKζ function augments Treg differentiation, enhanced function DGKζ cannot inhibit Treg development in WT cells.

DGKζ deficient CD4 SP thymocytes differentiate preferentially into Tregs *in vitro*

From the experiments presented thus far, the mechanism through which augmented DAG-mediated signaling produces elevated Treg frequencies remains unclear. One possibility is that thymic nTreg differentiation is directly augmented in the absence of DGKζ. However, naïve Tconvs can also become activated in peripheral tissues and differentiate into iTregs. On the other hand, deletion of DGKζ may enhance iTreg generation in the periphery to increase Treg numbers in the spleen. In turn, peripheral iTregs may re-circulate back to the thymus resulting in elevated frequencies of thymic Tregs. To test whether nTreg differentiation is affected in the absence of DGKζ, we employed an *in vitro* assay in which immature $Foxp3^{-}CD69^{+}CD44^{low}CD4^{+}$ SP thymocytes can be induced to differentiate into Tregs (Wirnsberger et al., 2009). To simulate nTreg differentiation, immature $Foxp3^{-}CD69^{+}CD44^{low}$CD4 SP thymocytes from WT and DGKζ KO Foxp3 GFP reporter mice were FACS-purified and cultured in the presence of anti-CD3 with or without IL-2 for three days. While CD3 stimulation alone did not induce Treg differentiation, the combination of CD3 and IL-2 induced a higher frequency of $CD4^{+}Foxp3^{+}$ Tregs in cultures of DGKζ $CD4^{+}$ SPs compared to WT controls (Figure 4.4). Although more replicates of this experiment are necessary to assess whether the observed difference is statistically significant, these data suggest that augmentation of TCR-induced DAG signaling may preferentially enhance nTreg differentiation in the thymus.

Concurrent deletion of DGKζ rescues Treg numbers in CD28 KO mice

TCR-induced DAG signaling promotes activation of the NF-κB pathway, which is critical for Treg differentiation. Thus, we hypothesized that the loss of DGKζ results in increased DAG-

mediated NF-κB activation downstream of TCR stimulation, leading to augmented Treg development. To test this notion, albeit indirectly, we intercrossed DGKζ KO mice to CD28 KO mice, which display impaired activation of the NF-κB pathway and a cell-intrinsic defect in Treg differentiation (Schulze-Luehrmann and Ghosh, 2006; Tai et al., 2005). We predicted that loss of DGKζ would restore NF-κB signaling to CD28 deficient thymocytes, thereby normalizing Treg numbers in CD28/DGKζ DKO mice. Preliminary data indicate that while CD28 KO mice contained very low frequencies of Foxp3$^+$ Tregs in the thymus and spleen compared to WT controls, concurrent deletion of both CD28 and DGKζ partially restored the frequency of Tregs toward WT levels (Figure 4.5A). Moreover, while both DGKζ KO and CD28/DGKζ DKO mice possessed diminished numbers of naïve thymic Tconvs compared to CD28 KO and WT controls, the total numbers of thymic Tregs were also increased in CD28/DGKζ DKO compared to CD28 KO mice (Figure 4.5B). While analysis of more mice is needed to determine the statistical significance of these findings, these data demonstrate that DGKζ deficiency can compensate for a loss of CD28 costimulatory signals to promote Treg differentiation *in vivo*.

Discussion

In the previous chapter, we examined the TCR signaling requirements of mature peripheral Tregs. Those studies piqued our interest in the role of TCR signaling during Treg lineage commitment. Given the fundamental importance of TCR stimulation in Treg generation, in this chapter, we investigated the intracellular signaling pathways downstream of the TCR that govern Treg differentiation. We have demonstrated that deletion of DGKζ, a negative regulator of DAG signaling, results in elevated Treg frequencies *in vivo*. DGKζ controls Treg development in a cell-intrinsic manner, as Treg frequencies remain elevated in $CD4^+$ T cells derived from DGKζ-deficient, but not WT, BM in the setting of a mixed BM chimera. Loss of DGKζ in immature $CD4^+$ thymocytes results in preferential Treg differentiation upon stimulation with TCR stimulation and IL-2 *in vitro*, suggesting that enhanced TCR-induced DAG signaling promotes nTreg generation. Deletion of DGKζ in CD28 KO mice partially rescued Treg development *in vivo*, suggesting that enhanced DAG signaling may promote NF-kB activity and Treg development independently of CD28 co-stimulation. Collectively, these data suggest that DAG signaling may link TCR stimulation to downstream activation of the Ras/ERK or NF-kB pathways in order to induce Treg differentiation.

Deletion of DGKζ not only enhances Treg development, but also appears to impair the generation of Tconvs in the thymus. Combined loss of DGKα and ζ, which are the only isoforms expressed in appreciable quantities in T cells, leads to a severe, but incomplete, developmental block at the DP stage, likely resulting from defects in thymic selection (Guo et al., 2008). Thus, we cannot exclude the possibility that DGKζ deletion enhances negative selection of Tconvs thereby elevating thymic Treg frequencies artificially. However, the total number of splenic Tregs is significantly increased in DGKζ KO mice and the total number of thymic Tregs is also elevated, albeit not significantly, suggesting that impaired negative selection may not fully account for this phenotype. An important caveat to this latter hypothesis is that developing thymocytes in DGKζ KO mice may compensate for impaired thymic selection by adjusting their TCR affinities through further receptor re-arrangements, thereby maintaining normal or near-normal thymic output. One approach to examine the precise role of DGKζ deletion in positive or negative

selection is to restrict the thymocyte TCR repertoire in DGKζ KO mice through concurrent transgenic expression of a single TCR and deletion of the RAG genes to disable re-arrangement of endogenous TCR loci. Unfortunately, the *dgkζ* gene is located within 1 centimorgan of the *rag1/2* genes, rendering the assessment of the impact of DGKζ deletion on positive or negative selection difficult. The genetic linkage of *dgkζ* and *rag1/2* all but precludes the generation of TCR transgenic DGKζ/rag1/2 DKO mice through simple intercrossing. One approach to overcome this confounding factor is to generate DGKζ/rag1/2 DKO mice through targeted mutation of *rag1/2* in the germline of DGKζ KO mice. These DGKζ/rag1/2 DKO mice must be intercrossed onto a transgenic background expressing a single TCR. However, this strategy is time-intensive and costly. An alternative approach is to intercross DGKζ KO mice to transgenic mice over-expressing Bcl family members. In these mice, it may be possible to evaluate whether DGKζ deficient Tregs are generated preferentially since the elimination of negatively selected Tconvs will be prevented. However, transgenic over-expression of Bcl family members may also bypass the requirement of positive selection, thus precluding a definitive interpretation of the results. Therefore, it remains difficult to dissect completely the differential effects of DGKζ deletion on negative selection and Treg differentiation.

Experiments examining the effect of DGKζ over-expression yielded potentially paradoxical results. While DGKζ over-expression in DGKζ deficient BM reduced the frequency of thymic and splenic Tregs to levels well below those observed in control $CD4^+$ T cells, DGKζ over-expression in WT Foxp3-RFP reporter BM did not alter Treg frequencies when compared to controls. Although these results appear contradictory, it is likely that the technical limitations of co-staining for GFP and Foxp3 are partly responsible for findings in DGKζ deficient BM. The Foxp3 staining procedure diminishes the fluorescence of GFP, thereby potentially skewing the true frequency of Tregs in the transduced GFP^+ cell fraction. We have bred DGKζ KO mice onto a Foxp3-RFP reporter background to facilitate identification of Tregs that have been transduced with the MIGR-GFP. We will repeat the DGKζ over-expression experiments using these mice and assess the resulting degree of Treg development. However, it is unclear why DGKζ over-

expression does not diminish Treg numbers in WT T cells. It is possible that the extent of DGKζ over-expression that we achieved with MIGR is insufficient to reduce DAG signaling levels below the minimal threshold necessary to promote Treg generation. Future experimentation will explore this issue more rigorously.

Previous studies of DGKζ deficient T cells have showed that they divide more than WT controls upon TCR stimulation (Zhong et al., 2003). Thus, one caveat complicating the interpretation of the *in vitro* Treg differentiation assay is that the elevated frequency of Tregs found in cultures of DGKζ deficient T cells may result from the increased proliferation of these cells rather than an enhancement in differentiation. To test this question, we labeled immature $Foxp3^{-}CD69^{+}CD44^{low}CD4^{+}$ SP thymocytes from both WT and DGKζ KO mice with a cell proliferation dye and examined the extent of cell division after anti-CD3 and IL-2 stimulation. Preliminary data indicate that immature $Foxp3^{-}CD69^{+}CD44^{low}CD4^{+}$ SP thymocytes from WT and DGKζ KO mice divide to a similar extent (data not shown), suggesting that DGKζ deficient immature $CD4^{+}$ SP thymocytes may differentiate preferentially into Tregs compared to WT controls.

Although immature thymocytes lacking DGKζ preferentially generated Tregs *in vitro*, enhanced nTreg differentiation may not be the sole cause for the elevated Treg numbers in DGKζ KO mice. Deletion of DGKζ may also cause an increased rate of iTreg conversion and a preferential expansion of peripheral Tregs, which may both contribute to an overall increase in Treg numbers. It will be necessary to compare the rate of iTreg conversion in WT and DGKζ deficient Tconvs with *in vitro* Treg skewing assays, using a dose titration of exogenously added TCR agonists, CD28 agonists, IL-2, and TGFβ. Furthermore, preliminary studies show that DGKζ deficient Tregs divide at a higher rate compared to WT Tregs in mixed BM chimeras, as measured by incorporation of the thymidine analog 5-bromo-2'-deoxyuridine (BrdU) (data not shown). Although these differences are not statistically significant, analysis of more mice is ongoing. To determine whether DGKζ deficient Tregs expand preferentially in the periphery, adoptive transfer studies will be performed in which WT and DGKζ deficient Tregs will be

transferred into the same recipient mice and tracked over time. These approaches may implicate other mechanisms that contribute to the elevated number of Treg in DGKζ KO mice.

Although we hypothesize that DAG signaling downstream of TCR stimulation promotes Treg differentiation, there is some evidence that IL-2R stimulation can also modulate the activity of DGKs and therefore DAG signaling (Flores et al., 1996). In fact, the *in vitro* Treg differentiation assay we utilized requires both stimulation of the TCR and the IL-2R. To examine directly whether DAG signaling is enhanced downstream of IL-2R stimulation, we will assess the levels of ERK phosphorylation in WT and DGKζ deficient Tregs. Furthermore, to examine whether the loss of DGKζ alters Treg differentiation through the IL-2R, we will test the degree of IL-2-induced Treg differentiation from direct Treg precursors *in vitro*. It remains possible that deletion of DGKζ augments Treg generation through enhanced DAG signaling downstream of both the TCR and the IL-2R.

While engagement of the TCR alone normally results in only weak NF-κB signaling, the presence of CD28 signals leads to full activation of the NF-κB pathway (Schulze-Luehrmann and Ghosh, 2006). Since NF-κB signaling is critical for Treg differentiation, previous studies have suggested that the Treg developmental defects observed in CD28 KO mice are a consequence of inefficient activation of the NF-κB pathway. Since TCR-induced DAG signaling can also activate NF-κB signaling through PKCθ and the CBM complex, it is likely that this pathway also contributes to Treg generation. In support of this notion, we demonstrated that concurrent deletion of DGKζ partially restores Treg numbers in CD28 KO mice. We hypothesize that developing thymocytes in CD28/DGKζ DKO mice experience enhanced DAG-mediated activation of the NF-κB pathway downstream of the TCR. In this manner, deletion of DGKζ compensates for the loss of CD28 co-stimulation and can partially restore Treg development.

Both biochemical and genetic approaches will be necessary to examine this hypothesis. To test the notion that DGKζ deficiency causes enhanced NF-κB signaling, we will measure the degree of TCR-induced NF-κB activity in WT and DGKζ deficient $CD4^+$ thymocytes by western blot and electrophoretic mobility shift assay. We expect to find enhanced NF-κB signaling in DGKζ deficient $CD4^+$ thymocytes as compared to WT controls. Recent work has demonstrated

the essential role of c-Rel in directly activating *foxp3* transcription and promoting Treg differentiation (Long et al., 2009; Ruan et al., 2009). Thus, as a direct evaluation of NF-κB-induced c-Rel activity, we will perform chromatin immunoprecipitation for c-Rel at the *foxp3* locus in WT and DGKζ deficient $CD4^+$ T cells. We expect that c-Rel binding at the *foxp3* locus will be augmented in the absence of DGKζ. Using a genetic approach, we are breeding DGKζ KO mice onto a c-Rel KO background to test whether enhanced DAG signaling acts through c-Rel to induce Treg development. If our hypothesis is correct, we do not expect to observe an elevated frequency of Tregs in c-Rel/DGKζ DKO mice. With these approaches, we hope to demonstrate that DAG signaling links TCR stimulation to NF-κB activation and subsequent Treg differentiation.

However, our data are too preliminary at this time to make a firm prediction on the signaling pathway downstream of DAG signaling that is responsible for mediating Treg differentiation. Indeed, as mentioned in the Introduction, much evidence indicates that Ras/ERK signaling acts as a sensor for TCR signal strength and converts TCR signals into cell fate decisions in developing thymocytes. Notably, DAG signaling also enhances both the degree and duration of Ras/ERK signaling in T cells (Zhong et al., 2003). Thus, it will be necessary to examine whether augmented Ras/ERK signaling mediates enhanced Treg differentiation in the absence of DGKζ. To test this possibility, we will assess the ability of immature $CD4^+$ SP thymocytes from DGKζ KO mice to differentiation in Tregs *in vitro* in the presence of chemical inhibitors of ERK or Ras signaling. Genetic approaches involving breeding of DGKζ KO mice to mice with defects in TCR-induced Ras/ERK signaling, such as ERK1/2 DKO or RasGRP1 KO mice, may provide additional insight in the dependence of Treg differentiation on this pathway. In fact, it is conceivable that both NF-κB and Ras/ERK signaling are involved in Treg differentation downstream of TCR-induced DAG signaling.

Although preliminary, our data suggest that DAG rather than Ca^{2+} signaling links TCR stimulation to Treg lineage commitment. If validated, our hypothesis may provide a connection between PLC-γ1 activation and NF-κB-induced Treg differentiation. Given the importance of Tregs in self-tolerance, a more complete understanding of the pathways that mediate their development will be critical for manipulation of these cells for therapeutic benefit.

Figures

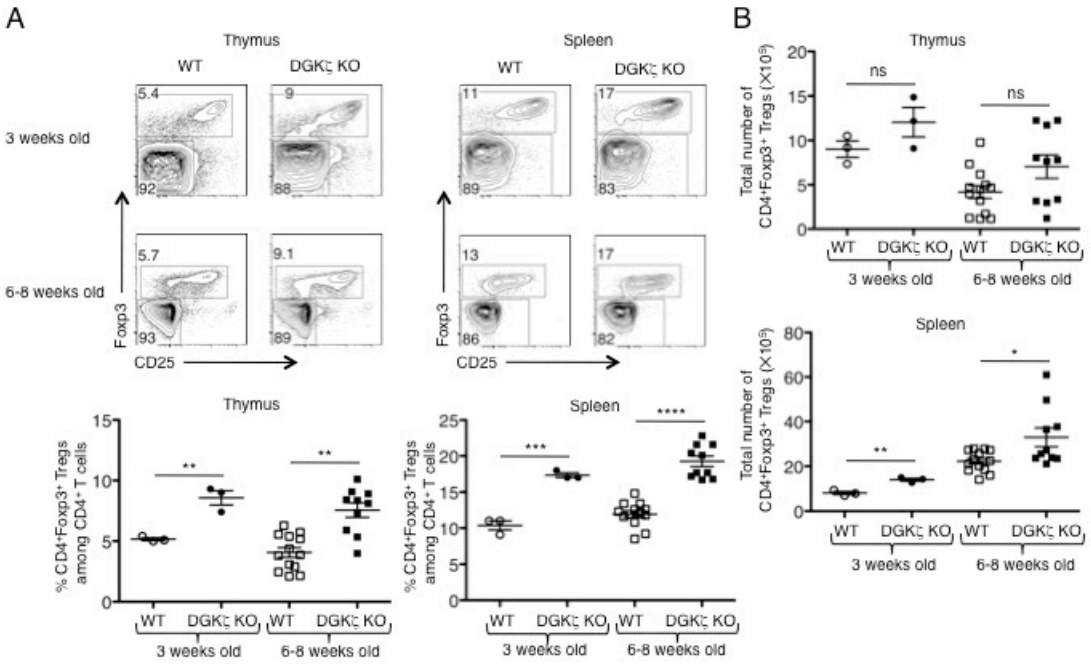

Figure 4.1. DGKζ KO mice contain elevated numbers of Tregs. (A) Contour plots (top panels) show the frequency of Foxp3$^+$ Tregs among total CD4+ T cells in the thymuses (left plots) and spleens (top plots) of WT and DGKζ KO mice at three weeks (top row) and 6-8 weeks (bottom row) of age. Cumulative data are shown in scatter plots (bottom panels). Contour plots are gated on CD4$^+$CD8$^-$ cells. (B) Total numbers of CD4$^+$Foxp3$^+$ Tregs in the thymuses (top) and spleens (bottom) of WT and DGKζ KO mice at 3 weeks and 6-8 weeks of age as in (A). Three (three weeks old) and 9-13 (6-8 weeks old) mice were analyzed and are shown as individual data points with the mean ± SEM for each group and summarize one (three weeks old) or four (6-8 weeks old) independent experiments. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 by unpaired, two-tailed Student's t test.

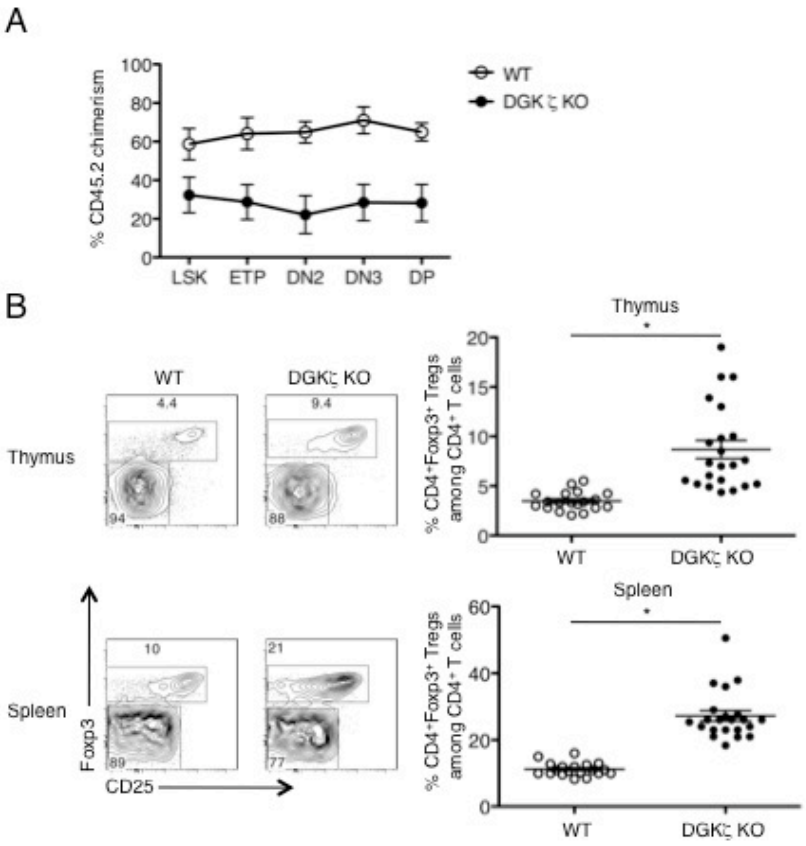

Figure 4.2. Cell-intrinsic loss of DGKζ in thymocytes results in elevated Treg frequencies. A mixture of CD45.1$^+$ WT donor BM and CD45.2$^+$ WT or DGKζ KO BM was transplanted into lethally irradiated CD45.1$^+$ recipient mice to create mixed BM chimeras. Recipient mice were analyzed eight to nine weeks after BM transplant. (A) The graph shows the frequency of CD45.2$^+$ donor WT or DGKζ KO BM-derived cells found within the multipotent Lineage$^-$ Sca-1$^+$c-kit$^+$ (LSK) stage in the BM and the DN1 or early thymic progenitor (ETP), DN2, DN3, and DP stages in the thymus. No statistically significant difference between CD45.2$^+$ donor chimerism was observed between developmental stages. (B) Contour plots show the frequency of Foxp3$^+$ Tregs among donor CD45.2$^+$ CD4$^+$ T cells in the thymuses (left, top row) and spleens (left, bottom row) of the mixed BM chimeras receiving either WT (left column) or DGKζ KO (right column) BM. Cumulative results are shown as individual data points with the mean ± SEM for each group for Treg frequencies among total CD4$^+$ T cells in the thymus (top right graph) and spleen (bottom right graph). A total of 19 and 22 mixed BM chimeras receiving WT or DGKζ KO donor BM were analyzed in three independent experiments. * p<0.0001 by unpaired, two-tailed Student's t test.

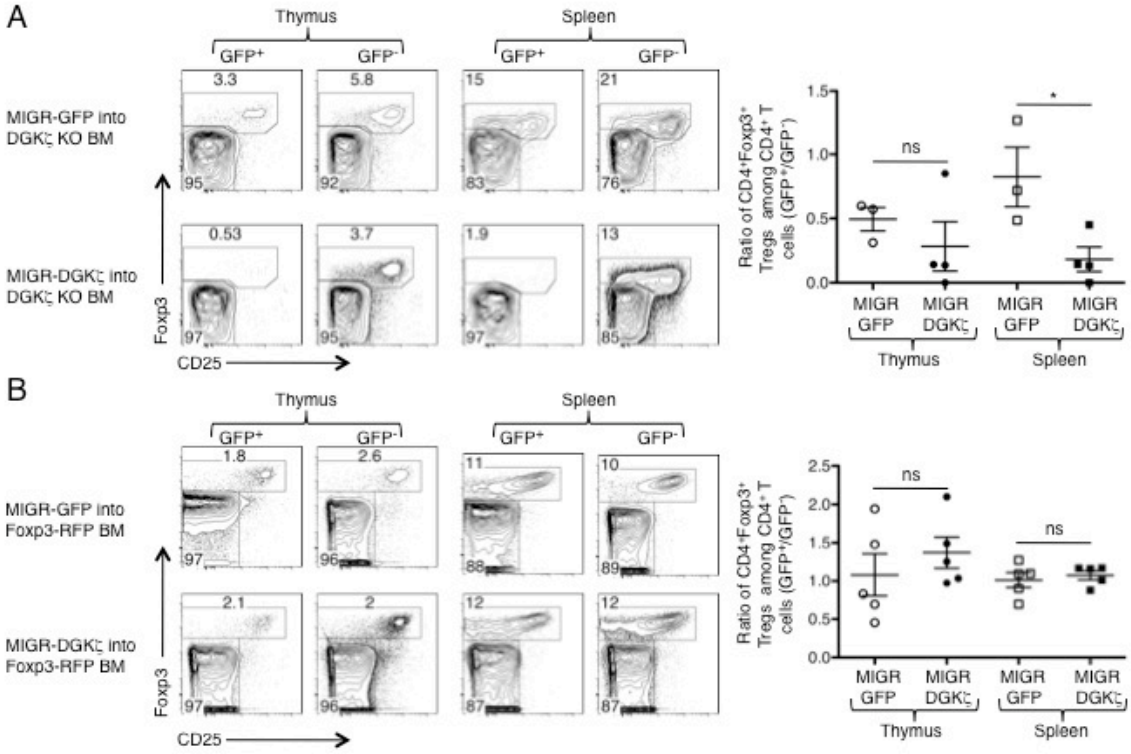

Figure 4.3. Over-expression of DGKζ inhibits Treg differentiation in DGKζ KO, but not WT, cells. (A) CD45.2$^+$ DGKζ KO BM was harvested and transduced *in vitro* with either MIGR-GFP or MIGR-DGKζ-GFP and transplanted into lethally irradiated CD45.1$^+$ recipient mice, which were analyzed eight to ten weeks later. Contour plots show the frequency of Foxp3$^+$ Tregs among total CD4$^+$ within transduced GFP$^+$ and untransduced GFP$^-$ thymocytes (left columns) or splenocytes (right columns) derived from DGKζ KO BM transduced with MIGR-GFP (top row) or MIGR-DGKζ-GFP (bottom row). The frequency of Tregs among transduced donor CD45.2$^+$GFP$^+$CD4$^+$ T cells were normalized to the frequency of Tregs among untransduced donor CD45.2$^+$GFP$^+$CD4$^+$ T cells from the same mice and the resulting ratios are shown as individual data points with the mean ± SEM for each group in the right graph. Three to four mice were analyzed per group in one experiment. (B) CD45.2$^+$ WT Foxp3-RFP reporter BM was harvested and transduced *in vitro* with either MIGR-GFP or MIGR-DGKζ-GFP and transplanted into lethally irradiated CD45.1$^+$ recipient mice, which were analyzed eight to ten weeks later. Contour plots show the frequency of Foxp3$^+$ Tregs among total CD4$^+$ within transduced GFP$^+$ and untransduced GFP$^-$ thymocytes (left columns) or splenocytes (right columns) derived from WT Foxp3 RFP reporter BM transduced with MIGR-GFP (top row) or MIGR-DGKζ-GFP (bottom row). Five mice were analyzed per group and the ratios of Treg frequencies between GFP$^+$ and GFP$^-$ fractions are shown as individual data points as in (A) with the mean ± SEM for each group in the right graph, which summarizes one experiment. Statistics were performed using unpaired, two-tailed Student's *t* test.

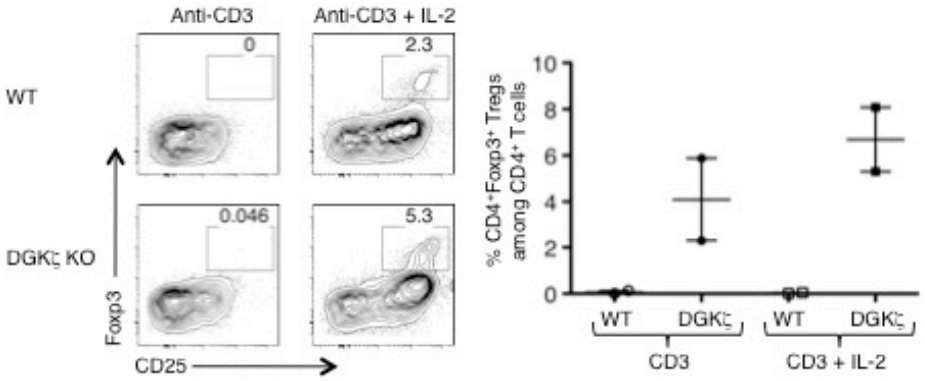

Figure 4.4. DGKζ deficient CD4 SP thymocytes differentiate preferentially into Tregs *in vitro*. Foxp3^{-}CD69^{+}CD44^{low}CD4^{+}CD8^{-} SP thymocytes were FACS-purified from the thymuses of WT and DGKζ KO Foxp3-GFP reporter mice and cultured in the presence of 10μg/mL of plate-bound anti-CD3 with or without 100 U/mL of recombinant human IL-2 for three days at 37 degrees C. Contour plots show the frequency of Foxp3^{+}CD25^{+} Tregs among total CD4^{+} T cells after culture with anti-CD3 alone (left columns) together with IL-2 (right columns) for Foxp3^{-} CD69^{+}CD44^{low}CD4^{+}CD8^{-} SP thymocytes from WT (top row) and DGKζ KO (bottom row) mice. Treg frequencies among total CD4^{+} T cells are shown as individual data points with the mean ± SEM for each group in the right graph, which summarizes two independent experiments.

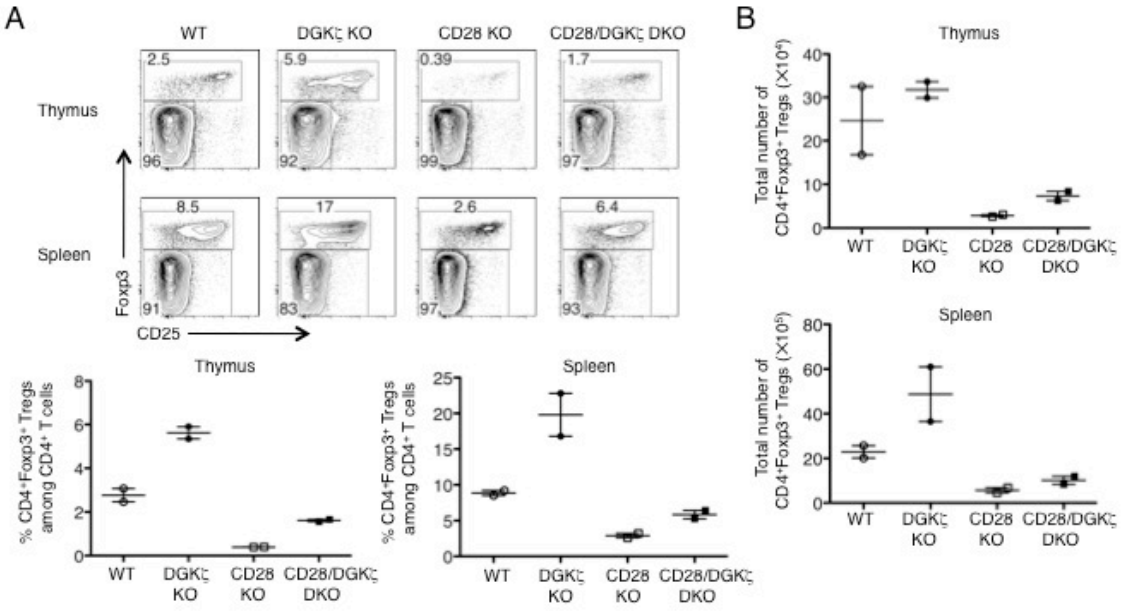

Figure 4.5. Concurrent deletion of DGKζ rescues Treg numbers in CD28 KO mice. (A) Contour plots (top) show the frequency of Foxp3$^+$ Tregs among total CD4$^+$ T cells in the thymuses and spleens of (starting from the left most column) WT, DGKζ KO, CD28 KO, and CD28/DGKζ DKO mice. Treg frequencies among total CD4+ T cells in the thymus (bottom left graph) and spleen (bottom right graph) are shown as individual data points with the mean ± SEM for each group in the graphs, which summarize a total of two mice per group in one experiment. (B) The total number of Tregs in the thymus (top graph) and spleen (bottom group) of each group of mice from (A) are shown as individual data points with the mean ± SEM for each group in the graphs, which summarize a total of two mice per group in one experiment.

Chapter V

Discussion and future directions

In this thesis, we first examined the accessory cells and signals that promote Treg differentiation *in vitro*. The results of these studies led to a model in which DCs coordinate Treg division through interactions with both Tconvs and Tregs. While DCs stimulated Tconvs to produce IL-2 in an MHCII-dependent manner, this Tconv-derived IL-2 cooperated with MHCII-independent signals from DCs to promote Treg division. Although dispensable, stimulation of the Treg TCR with cognate antigen presented on MHCII enhanced Treg division beyond that observed with IL-2 and MHCII KO DCs alone. As an extension of these findings, we next tested the hypothesis that Tregs can divide in the absence of TCR signaling *in vivo*. While Tregs do require TCR signaling for their division at steady state levels of endogenous IL-2, we demonstrated that Tregs are capable of dividing *in vivo* in the absence of both MHCII-induced and SLP-76-mediated TCR signaling when an exogenous IL-2R agonist is provided. Notably, the selective activation of the IL-2-induced STAT5 pathway was sufficient to support Treg division in the absence of MHCII *in vivo*. Shifting focus from the TCR signals that govern peripheral Treg division, we investigated the intracellular signaling pathways downstream of the TCR that promote Treg development. We concentrated on the DAG pathway, which is upstream of both Ras/ERK and NF-κB signaling, which are known to mediate thymic selection and Treg generation, respectively. We find that deletion of a DGKζ, negative regulator of DAG signaling, results in a cell-autonomous enhancement in Treg differentiation. Preliminary results support the notion that augmented TCR-induced DAG signaling may mediate Treg differentiation through NF-κB signaling. However, further experimentation will be required to validate this hypothesis. The findings presented herein generate a number of questions and potential avenues for further investigation that are outlined below.

Conventional $CD4^+$ T cells contribute to regulatory T cell homeostasis

Tconvs are the major source of IL-2 *in vivo* and thus support both Treg differentiation and homeostatic maintenance (Almeida et al., 2002; Almeida et al., 2006). In the steady state, endogenous IL-2 levels appear to be limiting, as administration of IL-2 ICs expand endogenous Treg populations (Boyman et al., 2006; Webster et al., 2009). Thus, Tconv derived IL-2 controls Treg homeostasis. The data presented in chapter III demonstrate that DCs induce Tconvs to produce IL-2 in an MHCII and B7 costimulation dependent manner. These studies are reminiscent of work performed in the 1970's and 1980's on the syngeneic MLR (Von Boehmer and Adams, 1973; Von Boehmer et al., 1972). The syngeneic MLR describes a phenomenon in which co-culture of T cells and non-T cells or APCs of the same genetic background results in IL-2 production and T cell proliferation. Our data potentially provide a mechanistic explanation for the syngeneic MLR, as Tconvs are induced by self-antigen/MHCII on DCs to produce IL-2, which promotes the division of Tregs.

An unexplored aspect of this process is the origin and identity of the Tconvs that produce IL-2 in response to antigen/MHCII and B7 costimulation provided by DCs. To characterize the Tconvs responsible for producing IL-2 in our *in vitro* cultures, we used the division of co-cultured Tregs as a surrogate measure of IL-2 production. Preliminary experiments show that both naïve $CD44^{low}$ and memory phenotype $CD44^{high}$ polyclonal Tconvs are capable of mediating DC-induced Treg division in culture (data not shown). Moreover, no difference in Treg division was observed upon co-culture with $CD5^{high}$ or $CD5^{low}$ Tconvs (data not shown), suggesting that the strength of TCR signaling received by Tconvs do not affect their ability to mediate Treg division. In contrast, compared to polyclonal WT Tconvs, OT-II $CD4^{+}$ Tconvs specific for a non-self peptide are less efficient in promoting DC-induced Treg division (data not shown), indicating that antigen specificity affects the ability of Tconvs to mediate this process. Future studies will examine the phenotype of these IL-2 producing Tconvs and the TCR and costimulatory signals that are required to promote their production of IL-2.

While Tconv production of IL-2 is dependent on MHCII and B7 costimulation, it remains unclear if these IL-2 producing Tconvs are truly autoreactive against specific self-antigens presented on MHCII or whether non-specific MHCII signals are sufficient to induce their IL-2

production. To test this question, we will compare IL-2 production by Tconvs co-cultured with DCs presenting a limited peptide repertoire, such as DCs from H2-DMα KO mice or WT DCs presenting a diverse peptide repertoire. Moreover, it will be necessary to clone the TCRs of IL-2 producing Tconvs and test whether Tconvs over-expressing these TCRs are pathogenic *in vivo*. It is unclear why these potentially auto-reactive, IL-2 producing Tconvs even exist in the host. A conventional explanation to explain their presence is that not all auto-reactive pathogenic T cells are deleted through negative selection. However, rather than a result of failed negative selection, perhaps one role for positive selection is to generate these potentially auto-reactive Tconvs to ensure that enough IL-2 is produced to maintain peripheral Tregs. Further study of these IL-2 producing Tconvs will be critical for a complete understanding of peripheral Treg maintenance.

Temporal and spatial organization of dendritic cell, conventional $CD4^+$ T cell and regulatory T cell interactions *in vivo*

Our studies demonstrate that DCs must contact both Tconvs and Tregs to promote Treg division, suggesting that these interactions must be properly coordinated in time and space within the secondary lymphoid organs and/or the peripheral tissues of the host. While the exact anatomic location of DC/Treg interactions has not been formally identified, it is notable that DCs, Tconvs, and Tregs all utilize the chemokine receptor CCR7 for migration into lymph nodes and trafficking to the T cells zones within secondary lymphoid tissues (Forster et al., 2008; Worbs and Forster, 2007). Given the highly organized structure of secondary lymphoid tissues, it is likely that the interaction of DCs, Tconvs, and Tregs is an ordered process. In support of this notion, CCR7 deficient Tregs do not migrate efficiently to lymph nodes and cannot appropriately suppress immune responses upon adoptive transfer into WT mice (Schneider et al., 2007). A few studies have also examined Treg interactions with DCs *in vivo*. Upon adoptive transfer, Tregs specific for a pancreatic islet self-antigen localize at the T cell/B cell boundary in pancreatic draining lymph nodes, but are randomly distributed within the T cell zone in lymph nodes draining other tissues (Tang et al., 2006). Of interest, many tissue-migratory DCs reside at the T cell/B cell boundary (Tang et al., 2006). Additionally, two-photon microscopy of explanted pancreatic lymph nodes

from NOD mice showed stable interactions between Tregs specific for pancreatic antigens and pancreatic-antigen expressing DCs (Tang et al., 2006).

While these studies examined the behavior of antigen-specific Tregs and DCs presenting cognate antigens, it will be important to define the exact location and timing of DC interactions with both IL-2 producing Tconvs and Tregs. *Ex vivo* microscopy of co-cultures of differentially marked DCs, Tconvs, and Tregs will help define the kinetics of DC/Tconv and DC/Treg interactions that are necessary to induce Treg division. For instance, it is not known whether DCs contact Tconvs and Tregs concurrently or whether DCs interact first with Tconvs and subsequently with Tregs to induce Treg division. Alternatively, the same DC may not need to contact both Tconvs and Tregs. Perhaps Tregs can interact with one DC and receive an IL-2 signal from a nearby Tconv that is interacting with a separate DC. For *in vivo* studies, two-photon microscopy can be used to examine the encounters of fluorescently labeled DCs with differentially labeled Tconvs and Tregs within secondary lymphoid organs. These studies will facilitate the identification of chemokine receptors or trafficking molecules required for DCs and T cells to home to their sites of interaction within lymphoid tissues. Taken together, the experiments proposed here will provide a better understanding of the spatial and temporal interactions between DCs, Tconvs, and Tregs that are necessary to sustain Treg homeostasis.

Antigen-independent regulatory T cell division

Previous studies have determined that cell-autonomous TCR signaling in Tregs is required for their division and maintenance. Utilizing TCR transgenic Tregs, these studies showed that DCs pulsed with cognate antigens stimulated Treg proliferation *in vitro* whereas control DCs did not (Fehérvári and Sakaguchi, 2004; Yamazaki et al., 2008; Yamazaki et al., 2003). In addition, exogenous administration of cognate antigen, targeting of cognate antigen to DCs *in vivo*, or tissue-specific expression of cognate antigen all promoted TCR transgenic Treg proliferation, suggesting that TCR stimulation can induce Treg proliferation and expansion directly (Fehérvári and Sakaguchi, 2004; Yamazaki et al., 2008; Yamazaki et al., 2003). Importantly, TCR transgenic Tregs only proliferated and expanded *in vivo* in those tissues containing their cognate

antigen, but not in tissues devoid of cognate antigen (Fehérvári and Sakaguchi, 2004; Yamazaki et al., 2008; Yamazaki et al., 2003). One flaw of these previous studies is that they utilized adoptively transferred Tregs identified through CD25, a marker expressed by only a subset of Tregs as well as by activated Tconvs. Thus, the exact composition of the $CD4^+CD25^+$ Treg populations analyzed was likely a heterogeneous group of T cells. Moreover, TCR transgenic Tregs may not completely recapitulate the behavior and biology of Tregs selected on a diverse range of self-antigens and possessing an array of antigen specificities. Lastly, in prior studies, the amount of cognate antigen present *in vivo* to stimulate TCR transgenic Treg proliferation was likely in excess of the concentrations of those self-antigens that are believed to maintain the homeostasis of WT Treg *in vivo*. Collectively, it remains unclear if Tregs require cell-autonomous TCR signaling to divide and be maintained in the homeostatic state.

In contrast, the experiments in chapter III show that WT Tregs do not require MHCII to divide *in vitro*. Instead, the presence of MHCII KO DCs and a source of IL-2 are sufficient to induce WT Treg division in culture. In chapter IV, we extended these studies to demonstrate that Treg division is not strictly dependent on cell-autonomous TCR signaling *in vivo*. While TCR signaling is required for Treg division at steady levels of IL-2, stimulation of the IL-2R with exogenously administered IL-2 agonists can overcome the TCR dependence of Treg division. Thus, although our studies confirm the notion that Tregs likely need TCR signaling to be sustained in most *in vivo* contexts, it raises the question of whether there are any physiologic situations where TCR signaling is dispensable for Treg maintenance.

A direct approach to test this question is to transfer antigen-specific Tregs into an environment free of their cognate antigen and track Treg division and persistence over time. However, a rigorous test of this notion requires the generation of RAG deficient antigen-specific Tregs in sufficient numbers for adoptive transfer. Even if adequate numbers of antigen-specific Tregs are obtained, it is impossible to rule out the presence of host self-antigens or antigens derived from commensal organisms capable of providing TCR signaling to the donor antigen-specific Tregs. Despite these confounding factors, this experiment will be informative.

It remains possible that small populations of peripheral Tregs specific for rare or sequestered self-antigens may be maintained without TCR signaling. However, these Tregs may exist in such small numbers that direct assessment of their division and persistence is difficult. One physiologic situation where Tregs may be maintained in the absence of TCR signaling is during settings of inflammation, where Tconvs are producing high concentrations of IL-2. The presence of high concentrations of IL-2 may overcome the TCR dependence of Treg division to sustain Tregs that do not receive antigen/MHCII induced TCR signaling. This situation most closely approaches our experiments using IL-2 ICs, which renders TCR signaling dispensable for Treg division. To test this notion, one can adoptively transfer RAG deficient TCR transgenic Tregs into a host free of their cognate antigens. Subsequently, an active inflammatory process, such as an infection or autoimmune reaction, must be induced to generate a high concentration of IL-2. Donor Treg division and persistence can then be tracked over time.

Although we examined the requirements for Treg division extensively in chapters III and IV, none of our experiments directly address whether Tregs can be maintained without antigen-dependent TCR stimulation in the long-term. Despite studies suggesting that the self-renewing division of peripheral Tregs is critical for their maintenance, this question is difficult to test directly. It is conceivable that Treg division contributes to, but is not absolutely necessary for, their peripheral maintenance. Conversely, it is possible that the Tregs in our experiments that divide without TCR signaling may not survive over time. To test whether Tregs can be maintained in the steady state without TCR signaling, we can use the SLP-76 cKO mouse to track the persistence of peripheral Tregs incapable of TCR signal transduction. Using a mixed BM chimera approach, we can generate mice containing both WT donor and SLP cHet or cKO T cells. After administering tamoxifen to delete the floxed SLP-76 allele, we can monitor the frequency of YFP^+ SLP-76 cKO Tregs over time in the peripheral blood. However, it will be necessary to thymectomize these BM chimeras to eliminate continuous thymic output of WT Tregs. This experiment will directly address whether peripheral Tregs require TCR signaling to be maintained over time.

Manipulation of regulatory T cell homeostasis as therapy for disease

Since defects in peripheral Treg numbers and function have been correlated with a number of autoimmune conditions and the expansion of Treg populations are therapeutic in many mouse models of autoimmunity and transplantation (Cools et al., 2007), the ability to modulate Treg numbers and function may prove valuable for the treatment of related clinical diseases. The studies presented in chapters III and IV demonstrate that exogenous provision of the cytokines GM-CSF, FLT3L, and IL-2, in the form of ICs, enhanced the division and expansion of Tregs. Based on these data, we plan to use GM-CSF and IL-2 ICs to expand Treg populations for therapeutic purposes.

Numerous mouse models of autoimmunity such as autoimmune diabetes and myasthenia gravis can be ameliorated through administration of GM-CSF (Gaudreau et al., 2007; Sheng et al., 2008). Although unmodified recombinant GM-CSF was ineffective at expanding DCs or Tregs *in vivo* in our hands, formulations of polyethylene glycol-modified GM-CSF with enhanced $t_{1/2}$ may hold promise for this purpose. To test this notion, we have engineered B16 melanoma cells to produce GM-CSF so that inoculation of mice with these B16-GM-CSF expressing cells provides a continuous supply of GM-CSF *in vivo*. Preliminary studies show that this B16-GM-CSF cell line is just as effective as the B16-FLT3L cell line in expanding DCs and Tregs *in vivo* (data not shown). An advantage to cytokine therapy is that it exploits endogenous mechanisms controlling Treg homeostasis to expand the recipient's own Tregs. Alternatively, GM-CSF can be used to expand autologous Tregs *in vitro* for subsequent adoptive transfer therapy. In fact, our data showed that GM-CSF induced a modest degree of Treg expansion in culture. The benefit of *ex vivo* Treg expansion is that it requires only autologous DCs and T cells, without the addition of supraphysiologic doses of TCR stimuli or IL-2. Tregs generated in this manner may more closely resemble host derived Tregs and may be more appropriate for therapy. This approach is potentially more cumbersome and prone to complications, as the *in vitro* expanded Tregs must be infused back into the recipients. Future studies will explore the ability of GM-CSF to modulate autoimmunity through *in vitro* and *in vivo* approaches.

In autoimmunity and transplant rejection, an imbalance exists between the activity of effector Tconvs and that of Tregs. One broad approach to fix this imbalance is to inhibit TCR signaling in all T cells with calcineurin inhibitors such as cyclosporine and FK506. These drugs are used widely in transplantation to prevent graft rejection through inhibition of T cell activation and thus IL-2 production. However, the loss of IL-2 may disrupt Treg homeostasis leading to a breakdown of tolerance to the transplanted graft. Indeed, despite the effectiveness of cyclosporine and FK506, some transplantation patients on these medications still reject their grafted organs. Moreover, these drugs are strong immunosuppressive agents that render the transplant patients susceptible to infections. Based on our findings that Tregs can divide in the absence of TCR signaling, we propose that IL-2 ICs may be useful as adjuvant therapy for drugs that inhibit TCR signaling to prevent T cell activation. The co-administration of IL-2R agonists may permit the specific maintenance of Tregs even in the presence of calcineurin inhibitors that abrogate TCR signaling. This combination therapy might allow for a reduction in the cyclosporine dose, limiting its significant toxicity. Although IL-2 therapy has side effects in humans, even very low levels of IL-2R signaling, characterized by transient STAT5 activation, are sufficient to ensure Treg homeostasis and suppressive function (Yu et al., 2009). Thus, the doses of IL-2 ICs needed for adjuvant therapy might be correspondingly low. Although IL-2R stimulation may enhance the function of effector Tconvs, several mouse models of autoimmunity and human studies suggest that IL-2 therapy may preferentially expand Treg populations and alter this imbalance in favor of immune tolerance (Tang et al., 2008; Zhang et al., 2005). To explore the effectiveness of IL-2 therapy to treat clinical disease, experiments investigating the efficacy of IL-2 ICs in conjunction with cyclosporine in models of autoimmunity are currently underway.

Signal integration in regulatory T cell differentiation

Treg lineage commitment has been shown to depend on both high affinity TCR interactions with agonist peptides and signaling through the NF-kB pathway (Jordan et al., 2001; Schmidt-Supprian et al., 2003; Zheng et al., 2003). However, the intracellular signals that connect high affinity TCR engagement to Treg differentiation is poorly understood. The data presented in

chapter II provide evidence that TCR-induced DAG signaling promotes Treg differentiation. Since DAG signaling activates the NF-κB pathway, which promotes Treg development, we hypothesize that DAG signaling may activate the NF-kB family transcription factor c-Rel to bind to the *foxp3* locus thereby inducing transcription and subsequent Treg differentiation. If this hypothesis is confirmed, it will establish DAG signaling as the pathway that links TCR stimulation to Treg differentiation. In addition to the experiments we proposed in chapter II, we hope to examine whether enhanced DAG signaling underlies the ability of agonist peptides to preferentially induce Treg differentiation *in vivo*. To test this notion, we will examine whether Treg-inducing agonist peptides generate enhanced DAG signaling in antigen-specific $CD4^+$ thymocytes *in vitro* as compared to partial agonists. Although the appropriate reagents to measure DAG signaling directly do not yet exist, establishing a correlation between high affinity TCR interactions induced by agonist peptides and enhanced DAG signaling would validate our conclusions further.

Despite evidence to suggest that the NF-κB pathway mediates Treg differentiation downstream of TCR-induced DAG signaling, it is possible that Ras/ERK signaling may be solely responsible or may cooperate with NF-κB signaling to induce Treg generation. Indeed, it is known that the pattern of Ras/ERK activation is different upon stimulation with low and high affinity agonist peptides that induce positive versus negative selection (Daniels et al., 2006), suggesting that the Ras/ERK pathway is a sensor for the strength of TCR signaling and converts TCR affinity to intracellular signals that determine cell fate. Given that Treg differentiation is incorporated in the TCR affinity model of thymic development, it is conceivable that Ras/ERK signaling is also involved in converting high affinity TCR signaling to Treg differentiation. Future experiments will address whether NF-κB and/or Ras/ERK signaling mediates Treg development downstream of DAG signaling as outlined in the Discussion section of Chapter IV.

While our data suggest that enhanced TCR-induced DAG signaling potentiates Treg differentiation, it is likely that the absolute magnitude of the DAG signal does not determine the efficiency of Treg generation. Concurrent with our studies of DGKζ KO mice, we analyzed Treg development in a knock-in mouse that harbors a tyrosine to phenylalanine mutation at position 145 of SLP-76 (SLP^{Y145F}) (Jordan et al., 2008). This mutation results in severely impaired function

of PLC-γ1 and T cells from SLP^{Y145F} mice display significant defects in both Ca^{2+} and DAG signaling (Jordan et al., 2008). We predicted that because thymocytes from SLP^{Y145F} mice possess impaired DAG signaling, they would generate fewer Tregs. In preliminary experiments, SLP^{Y145F} expression caused a cell-intrinsic increase in Treg frequencies (data not shown), suggesting that the DAG signal needed for Treg generation is more complex than a simple increase in magnitude and/or duration. Since DGKζ terminates DAG signaling, deletion of DGKζ causes an increase in both the magnitude and duration of TCR-induced DAG signals (Zhong et al., 2003). Thus, a combination of qualitative and quantitative changes in DAG signaling may cause enhanced Treg differentiation in DGKζ deficient T cells.

Alternatively, perhaps DAG signaling must be considered in the context of other signaling pathways. For instance, it is possible that strong and/or prolonged DAG signaling must synergize with relatively weaker Ca^{2+} signaling to induce Treg differentiation. Thus, the relative, rather than the absolute, degree of DAG signaling may be critical for Treg development. To understand how DAG signaling leads to enhanced Treg generation, the integration of multiple signaling pathways must be examined. TCR engagement activates a number of signaling cascades that have been implicated in Treg lineage choice. TCR-induced intracellular Ca^{2+} flux causes nuclear translocation of NFAT, which is known to promote *foxp3* transcription (Tone et al., 2008). Another important pathway is Akt/mammalian target of rapamycin (mTOR) signaling, which has been shown to inhibit Treg generation (Haxhinasto et al., 2008; Liu et al., 2009). Akt/mTOR inhibition of Treg differentiation may occur through the inactivation of forkhead-box O (Foxo) family transcription factors, which also trans-activates the *foxp3* gene (Ouyang et al., 2010). To complicate matters further, signals emanating the IL-2R and the TGF-βR also promote *foxp3* transcription through STAT5 and Smad3, respectively (Burchill et al., 2007b; Tone et al., 2008). At the same time, it will be necessary to examine the behavior of the signaling pathways and transcription factors that prevent Treg differentiation. One pathway to consider is the induction of retinoic acid related receptor (ROR)-γt by IL-6, which has been shown to oppose Treg formation (Mucida et al., 2007). Simultaneous analysis of multiple signaling pathways that contribute to or

inhibit Treg differentiation may be necessary to uncover a balanced pattern of signals that control Treg development in both DGKζ KO and SLPY^{145F} mice.

Presently, it is not known how the TCR signals described above and the transcription factors they activate are integrated to direct Treg differentiation at the transcriptional level. For example, in DGKζ deficient T cells, while both TCR-induced DAG and Akt signaling are enhanced ((Gorentla et al., 2011), data not shown), the net outcome of concurrent signaling through these opposing pathways is preferential commitment to the Treg lineage. Despite this complexity, new developments in intracellular phosphoprotein detection will allow for the analysis of multiple signaling pathways on the single cell level (Bendall et al., 2011). To understand the integration of all these signaling pathways, one can analyze the kinetics of transcription factor binding at the *foxp3* locus. Moreover, protein-protein interactions between these transcription factors may also contribute to the stabilization or disruption of the transcriptional machinery at the *foxp3* locus. Examination of the interactions between the network of signaling pathways and transcription factors that determine CD4$^+$ T cell lineage choice will be necessary for a complete understanding of Treg differentiation.

The role of the T cell receptor in determining regulatory T cell niche size

An intriguing, yet puzzling, aspect of the TCR instructive model of Treg lineage commitment is the finding that out of a large pool of developing thymocytes bearing the same Treg-derived TCR, only a few differentiate into Tregs (Bautista et al., 2009; Leung et al., 2009). Lowering the number of clonal precursor thymocytes results in formation of a higher frequency of Tregs, implicating the existence of small TCR-specific Treg niches that become saturated at low precursor frequencies (Bautista et al., 2009; Leung et al., 2009). These data suggest that intraclonal competition between thymocytes of the same TCR specificity limits Treg differentiation (Bautista et al., 2009; Leung et al., 2009). While this process may ensure a diverse Treg TCR repertoire, the parameters that determine which thymocytes become Tregs are not known. One possibility is that thymocytes bearing the same TCRs compete for specific antigen/MHCII complexes on thymic APCs (Bautista et al., 2009; Leung et al., 2009). Merging this hypothesis

with the notion that high affinity TCR interactions are required for Treg differentiation, we propose that only those developing thymocytes that receive enough cumulative TCR signals from antigen/MHCII within a developmental window can differentiate into Tregs, whether these TCR signals are from many low affinity interactions or fewer high affinity encounters with MHCII. Thus, if specific antigen/MHCII complexes were limiting in the thymus, then this model would fit with the data showing an inverse correlation between clonal precursor frequency and the efficiency of Treg differentiation.

To test the hypothesis that cumulative TCR signals above a certain threshold enable differentiation to the Treg lineage, Treg TCR transgenic mice must first be intercrossed with DGKζ KO mice. If our conclusions regarding the role of DAG signaling in Treg differentiation are correct, then upon each encounter with the appropriate antigen/MHCII molecule, DGKζ deficient precursor thymocytes should experience increased TCR-induced NF-κB signaling and/or Ras/ERK signaling as compared to WT controls. This enhanced NF-kB and/or Ras/ERK signaling may allow DGKζ deficient precursor thymocytes to achieve the threshold of TCR signals necessary for Treg differentiation with fewer antigen/MHCII encounters. Thus, in the absence of DGKζ, we would expect a higher efficiency of Treg generation for each Treg TCR clone at all precursor frequencies. However, to complicate this analysis, intraclonal competition may not occur just between clonal precursor thymocytes, but may include clonal mature Tregs as well. In this case, a population of mature Tregs must remain in the thymus to obstruct the generation of new Tregs of the same specificities. To compete with clonal precursor thymocytes, mature Tregs with the same specificity may limit access to cognate antigen/MHCII complexes by direct binding of their TCRs. In support of this notion, a portion of thymic Tregs may reside within the organ, as about 40% of thymic Tregs are RAG2-GFP negative in RAG2 reporter mice, suggesting that they are developmentally distant from the DP stage (McCaughtry et al., 2007).

Regardless of the mechanism, if each population of antigen-specific Tregs is constrained by a small niche size, it is unclear how the Treg population is expanded in DGKζ KO mice. One possibility is that within each TCR clone, more $CD4^+$ become Tregs in the absence of DGKζ. An alternative explanation is that TCRs that would not normally cause Treg differentiation are able to

instruct developing thymocytes to the Treg lineage in the absence of DGKζ. While a combination of both mechanisms may be operational in DGKζ KO mice, a detailed analysis of Treg TCR sequences in these mice may be informative. Collectively, these proposed studies with DGKζ KO mice may extend and refine the TCR instructive model of Treg differentiation.

Conclusions

The work presented here describes how signaling from the TCR and the IL-2R control Treg differentiation and peripheral maintenance. Despite inauspicious beginnings, Tregs are now recognized as the most critical cellular factor in the maintenance of self-tolerance. Although much progress has been made in understanding Treg formation and homeostasis, many more questions must be answered before therapies aimed at manipulating their number or function can be utilized in a clinical setting.

Appendix I

Abbreviations

A

Ab, antibody

APC, antigen-presenting cell

B

βc, common β chain

Bcl10, B-cell lymphoma/leukemia 10

BM, bone marrow

BrdU, 5-bromo-2'-deoxyuridine

C

Ca^{2+}, calcium

CARMA1, caspase recruitment domain and membrane-associated guanlyate kinase-containing scaffold protein

CFSE, carboxyfluorescein succinimidyl ester

cHet, conditional heterozygous

cKO, conditional knockout

CLIP, class II-associated invariant chain peptide

cTEC, cortical thymic epithelial cells

CTLA-4, cytotoxic T lymphocyte antigen 4

D

DAG, diacylglycerol

DC, dendritic cell

DGKζ, diacylglycerol kinase z

DKO, double knockout

DN, double negative

DP, double positive

E

EAE, experimental autoimmune encelphalomyelitis

ETP, early thymic progenitor

F

FACS, fluorescence-activated cell sorting

FLT3L, fms-like tyrosine kinase 3 ligand

Foxp3, forkhead box P3

FR4, folate receptor 4

G

γ_c, common γ chain

GFP, green fluorescent protein

GITR, glucocorticoid-induced tumor necrosis factor receptor-related protein

GM-CSF, granulocyte-macrophage colony stimulating factor

H

HA, hemagglutinin

Het, heterozygous

I

i.p., intraperitoneal

i.v., intravenous

IC, immune complex

IKK, IκB kinase

IL-, interleukin

IP_3, inositol 1,4,5-trisphosphate

IPEX, immune dysregulation, polyendocrinopathy, enteropathy, X-linked

iTreg, inducible regulatory T cell

J

JNK, Jun amino-terminal kinase

K

KO, knockout

L

LAT, linker of activation of T cells

LPS, lipopolysaccharide

LSK, $Lineage^-$ $Sca\text{-}1^+$ $c\text{-}kit^+$ multipotent BM progenitor

M

mAb, monoclonal antibody

MACS, magnetic-activated cell sorting

MALT1, mucosa-associated lymphoid tissue lymphoma translocation gene 1

MAPK, mitogen-activated protein kinase

MHCI or II, major histocompatibility class I or class II

MIGR, modified mouse stem cell retrovirus

MLR, mixed lymphocyte reaction

mTEC, medullary thymic epithelial cells

MyD88, myeloid differentiation primary response gene 88

N

NFAT, nuclear factor of activated T cells

NF-κB, nuclear factor of κ-light-chain-enhancer of activated B cells

NOD, non-obese diabetic

nTreg, natural Tregs

O

Ova, ovalbumin

P

PIP_2, phosphatidylinositol 4,5-bisphosphate

PI-3K, phosphoinositide 3-kinase

PKCθ, protein kinase C θ

PLC-γ1, phospholipase C-γ1

PTEN, phosphatase and tensin homolog

PTK, protein tyrosine kinase

R

R, receptor

RFP, red fluorescent protein

S

SLP-76, Src homology domain 2-containing leukocyte protein of 76 kilodaltons

SP, single positive

STAT, signal transducer and activation of transcription

STAT5b-CA, constitutively active form of STAT5b

T

TAK1, transforming growth factor-b-activated-kinase-1

Tconv, conventional $CD4^+$ T cell

TCR, T cell receptor

TGF-β, transforming growth factor b

TLR, toll-like receptor

TRAF6, tumor necrosis factor receptor-associated factor 6

Treg, Regulatory T cell

W

WT, wild-type

Y

YFP, yellow fluorescent protein

Z

ZAP-70, ζ-chain-associated protein kinase 70

Appendix II

Materials and Methods

Chapter II

Mice

WT C57BL/6, WT Balb/c, and IL-3β/βc DKO mice were purchased from The Jackson Laboratory. I-A^b (MHC II) KO and and Balb/c RAG2 KO mice were purchased from Taconic Farms. DO11.10, MyD88 KO, and Foxp3 GFP-reporter mice were gifts from Drs. David Artis, David LaRosa, and Vijay Kuchroo through Dr. Laurence Turka, respectively. TS1×HA28 mice have been previously described (Jordan et al., 2001) and were also bred onto a Balb/c Foxp3 GFP-reporter mouse. Mice were 6 weeks to 6 months of age at time of sacrifice. Mice were housed in specific pathogen-free conditions and treated in strict compliance with Institutional Animal Care and Use Committee regulations of the University of Pennsylvania.

Reagents and Abs

Anti-IL-2 neutralizing Ab (R&D Systems), CTLA-4 immunoglobulin fusion protein (CTLA-4-Ig) (R&D Systems), and anti-GM-CSF (Pierce-Endogen) neutralizing Abs were used at 20 μg/mL. LPS (Sigma), human rFLT3 ligand (FLT3L) (R&D Systems), and CpG DNA (Integrated DNA Technologies) were used at 100 ng/mL. Mouse rGM-CSF (Peprotech) was used at 10 ng/mL. Human rIL-2 (Peprotech) was used at 50 U/mL. Mouse and rat IgG were purchased from Sigma. Abs for flow cytometry were purchased from BD Pharmingen (San Diego, CA): Fc block (2.4G2), anti-I-A^b-biotin (KH74), anti-CD86 (GL1) FITC, Rat IgG2a κ isotype control FITC, anti-CD25 PE (PC61), anti-CTLA-4 PE (UC10-4F10-11), anti-CD90.2 PE (53-2.1), Armenian hamster IgG isotype control PE, rat IgG1 isotype control PE, rat IgG2b λ isotype control PE, anti-CD4 PerCP-Cy5.5 (RM4-5), anti-B220 PerCP-Cy5.5 (RA3-6B2), anti-BrdU allophycocyanin, anti-CD25 allophycocyanin-Cy7 (PC61), and streptavidin-PE-Cy7; Biolegend (San Diego, CA): anti-CD80 PE (16-10A1), anti-folate receptor 4 (FR4) PE (TH6), anti-glucocorticoid-induced tumor necrosis factor (GITR) PE (DTA-1), anti-CD8α AlexaFluor700 (53-6.7), anti-CD11c allophycocyanin

(N418), anti-CD3 Pacific Blue (17A2), and anti-CD19 Pacific Blue (6D5); eBiosciences (San Diego, CA): anti-BrdU FITC (PRB-1), anti-Foxp3 allophycocyanin (FJK-16s), and rat IgG2a κ isotype control allophycocyanin; or Molecular Probes, Invitrogen (Carlsbad, CA): CFSE, LIVE/DEAD Fixable Red Dead Cell Stain Kit. The 6.5 clonotype Ab was previously described (Jordan et al., 2001).

Flow cytometry, cell sorting, and data analysis

Cells were stained in PBS with Dead Cell stain and blocked with either 2.4G2 or mouse and rat IgG, followed by staining with Abs against surface Ags in staining buffer [2% FBS (HyClone), 0.05% sodium azide in PBS] on ice for 25-30 min, ending with two washes. Isotype control staining was performed where appropriate. For cell sorting, T cells and DCs were purified with CD90.2 and CD11c magnetic beads using MACS columns (Miltenyi Biotec) prior to cell surface staining. Intracellular Foxp3 (eBioscience) and BrdU (BD Pharmingen) staining were performed according to manufacturer's protocol. Flow cytometry of cells was performed with an LSR II or a FACSCalibur (BD Biosciences, San Jose, CA). Fluorescence activated cell sorting (FACS) was performed with a FACSAria cell sorter (BD Biosciences, San Jose, CA) at the UPenn Flow Cytometry and Cell Sorting Core. FACS-sorted populations were typically of >90-95% purity. Data were analyzed with FlowJo software (TreeStar). Dead cells were excluded from analysis with LIVE/DEAD Fixable Red Dead Cell staining. Doublets were excluded using FSC-H by FSC-W and SSC-H and SSC-W parameters.

Isolation of unfractionated splenocytes and splenic DCs

Spleens were harvested from mice and prepared in single cell suspension. RBCs were lysed with 0.83% ammonium chloride. For unfractionated splenocyte cultures, spleens were not treated with collagenase. B16 melanoma cells expressing FLT3L were a gift from Dr. Terri M. Laufer. 5×10^6 B16 melanoma FLT3L cells (B16-FLT3L) (Mach et al., 2000) were injected s.c. into mice. After two weeks, spleens were harvested and injected with cell culture media (MEM-a with 10% FBS) containing 1 mg/mL collagenase D (Sigma) and 50 μg/mL DNase I (Sigma), cut into small pieces,

and incubated for 30 min at 37°C prior to manual disruption. In some experiments, spleens from untreated WT mice were used for isolation of DCs.

***In vitro* Treg proliferation assay**

Unfractionated splenocytes, FACS-sorted $CD90.2^+CD8\alpha^-CD25^+$ or Foxp3-GFP^+ Tregs, or $CD90.2^+CD8\alpha^-CD25^-$ Tconvs cells were labeled with CFSE by washing with room temperature PBS, resuspending at 10^7 cells/mL of PBS, and mixing with an equal volume of PBS containing CFSE (750 nM final concentration). Cells were continuously shaken for 9 min with intermittent vortexing. The reaction was quenched with 100% FBS and washed with T cell media [MEM-a (Invitrogen) with 10% FBS, 1% penicillin/streptomycin, 10 mM HEPES, and 1×10^{-5} M 2-ME] before use. CFSE-labeled unfractionated splenocytes were plated at 5×10^5 cells/well in 200 μL of T cell media in 96 well flat bottom plates. When using FACS-sorted cells, varying numbers of T cells and DCs or T cell-depleted splenocytes were co-cultured depending on the experimental setup, although the ratio of each T cell subset to DCs was kept constant, except where indicated. T cell-depleted splenocytes were obtained from the $CD90.2^-$ flow-through fraction of CD90.2 MACS purification. In transwell experiments, cell populations were co-cultured in 24 well transwell plates (Corning) containing a 0.4 μm porous membrane insert. Cells were placed either in the insert in 100 μL or in the well beneath in 600 μL of media. Cell numbers were scaled up accordingly. Cells were cultured untreated in T cell media or treated with the indicated factors in a 37°C tissue culture incubator. On the fourth day, cells were harvested, stained, and analyzed by flow cytometry. Multiple cultured wells were combined for staining. Live cell counts were performed using a hemocytometer and Trypan Blue dead cell exclusion. IL-2 content of cell-free culture supernatants derived from splenocyte cultures was measured by ELISA, according to manufacturer's protocol (R&D Systems).

Treg suppression assay

$CD90.2^+CD8\alpha^-CD25^+$ Tregs and $CD90.2^+CD8\alpha^-CD25^-$ Tconvs cells were FACS-sorted from WT Balb/c mice or GM-CSF-treated 4 day Balb/c splenocyte cultures and were co-cultured at various

Treg:Tconv ratios with T cell-depleted, irradiated splenocytes in the presence of 0.5 mg/mL anti-CD3 stimulating Ab (2C11, BD Pharmingen) in 200 μL T cell media in a 96 well flat bottom plate at 37°C. Starting cell numbers for the suppression assay were 20,000 Tregs, 20,000 Tconvs, and 100,000 feeder cells. Three days later, each well was pulsed with 1 μCi ^{3}H-thymidine (Amersham). Sixteen hours later, nuclei were harvested onto filter paper and ^{3}H-thymidine incorporation was measured with a b-plate scintillation counter (PerkinElmer).

In vivo BrdU incorporation

WT B6 mice were untreated or injected s.c. with B16 or B16-FLT3L melanoma. Nine days later, a three-day pulse of BrdU was initiated with a 2 mg i.p. injection of BrdU, followed by feeding of BrdU in the drinking water at 1 mg/mL until time of sacrifice on day 12. Intracellular Foxp3 and BrdU staining were performed sequentially according to each manufacturer's protocol.

Statistical analyses

Data from independent repeats of experiments were graphed as individual data points with a mean $\pm$ SEM for each group. Proliferation data from Treg CFSE dilution profiles, gated on live CD4^{+}Foxp3^{+} cells, were transformed into "Division Index" and "% Divided" data using Flowjo. The "Division Index" measures the average number of divisions a cell that was present in the input population has undergone, while the "% Division" measures the percentage of cells in the input population that has undergone at least one cell division. These measurements do not take into account death of the input cell population. These values were graphed and analyzed for statistical significance using Prism (GraphPad). The statistical test used to calculate each *p* value is indicated in the figure legends. *p* values < 0.05 were considered significant.

Chapter III

Mice

WT C57BL/6, WT B6.SJL, and complete MHCII KO mice were purchased from The Jackson Laboratory. I-$A^b\beta$ KO mice were purchased from Taconic Farms. SLP-76 cKO mice were generated as described (16). All SLP-76 mice backcrossed onto a C57BL/6 background for at least 6 generations. STAT5b-CA transgenic mice were generated as described (20) and were intercrossed onto a SLP-76 cKO background. H-2DMα KO mice were a generous gift from Dr. Terri Laufer. Mice were 6-16 weeks of age at time of use and were housed in pathogen-free conditions and treated in strict compliance with Institutional Animal Care and Use Committee regulations of the University of Pennsylvania.

Reagents and antibodies

Recombinant mouse and human IL-2 were purchased from eBioscience (San Diego, CA) and Peprotech (Rocky Hill, NJ), respectively. Anti-mouse monoclonal IL-2 antibody (JES6-1) was purchased from BioXCell (West Lebanon, NH) and anti-CD3 (2C11) was purchased from BD Pharmingen (San Diego, CA). Antibodies for flow cytometry were purchased from either BD Pharmingen: anti-I-A^b-biotin (KH74), anti-phospho-STAT5 Y694 (47), anti-CD4 (RM4-5), anti-CD25 (PC61), anti-B220 (RA3-6B2), anti-CD69 (H1.2F3), anti-CD90.1 (OX-7), and streptavidin-PE-Cy7; Biolegend (San Diego, CA): anti-CD8α (53-6.7), anti-CD11c (N418), anti-CD11b (M1/70), anti-CD44 (IM7), anti-CD45.2 (104); eBioscience: anti-BrdU (PRB-1), anti-Foxp3 (FJK-16s), anti-CD45.1 (A20); or Molecular Probes, Invitrogen (Carlsbad, CA): CFSE, DAPI, LIVE/DEAD Fixable Dead Cell stain, anti-GFP AlexaFluor488. BrdU, mouse and rat IgG, and tamoxifen were purchased from Sigma-Aldrich.

Flow cytometry, cell sorting, and data analysis

Cells were stained in FACS buffer (2% FBS (HyClone), 0.05% sodium azide in PBS) on ice for 30 minutes with antibodies against surface antigens, followed by two washes with FACS buffer. For cell sorting, T cells were purified with CD90.2 magnetic beads using MACS columns (Miltenyi

Biotec) according to manufacturer's protocol prior to cell surface staining. Intracellular Foxp3 (eBioscience) and BrdU (BD Pharmingen) staining were performed with kits according to manufacturer's protocol. Flow cytometry was performed with an LSR II or FACSCanto (BD Biosciences). FACS was performed with a FACSAria cell sorter (BD Biosciences) at the University of Pennsylvania Flow Cytometry and Cell Sorting Core. FACS-sorted populations were typically of 96-99% purity. Data were analyzed with FlowJo software (TreeStar). Cell division data from Treg CFSE dilution profiles, gated on live $CD4^+Foxp3^+$ cells, were transformed into "Division Index" data using Flowjo's "Proliferation" function whenever possible. In cases where the Flowjo algorithms could not calculate "Division Indices," values measuring the percentages of CFSE diluted Tregs were used. The "Division Index" measures the average number of divisions a cell that was present in the input population has undergone.

CFSE labeling

Cells were washed twice with room temperature PBS and resuspended at 10^7 cells/mL in room temperature PBS. CFSE was added at a final concentration of 5 mM and incubated in the dark. Cells were shaken for 9 minutes, vortexing intermittently. The reaction was quenched with 100% FBS and washed with T cell media (RPMI 1640 (Invitrogen) with 10% FBS, 1% penicillin/streptomycin, HEPES (10 mM), and 1×10^{-5} M 2-ME).

Phospho-STAT5 staining

Mouse spleen and LNs were harvested and dissociated into single cell suspension. Cells were washed with T cell media, resuspended in T cell media at 5×10^6 cells/mL, and rested in a 37 C incubator for 2 hours. After the rest period, 5×10^6 cells were aliquoted into 5 mL FACS tubes and stimulated with 100 U/mL human recombinant IL-2 for the desired intervals in a 37 C incubator. Cells were fixed at the indicated timepoints with fresh paraformaldehyde to a final concentration 1.5%, and incubated for 10 minutes at room temperature. After washing, cells were resuspended and fixed with 1 mL of ice cold methanol for 20 minutes at 4 C. Cells were washed with FACS buffer and stained for surface antigens followed by staining for intracellular Foxp3.

IL-2 IC treatment

Mouse IL-2/IL-2 mAb IC were prepared as follows: for each injection, 5 mg of JES6-1 anti-mouse IL-2 antibody was incubated with 1 mg of recombinant mouse IL-2 for 30 minutes on ice prior to resuspension in PBS. PBS or IL-2 IC injections were performed for three consecutive days and mice were sacrificed for analysis on the sixth day after the first injection. Mice were administered BrdU (described below) on the first day of IL-2 IC treatment.

***In vivo* BrdU incorporation and staining**

Mice were administered BrdU with an initial bolus of 2 mg BrdU i.p. at the start of the labeling period. Concurrently, mice were given drinking water containing 1 mg/mL BrdU until the time of sacrifice. To detect BrdU incorporation in SLP-76 cHet or cKO Tregs, MACS-purified T cells were FACS-sorted into YFP^+ and YFP^- fractions prior to surface staining and subsequent intracellular staining for Foxp3. Thereafter, BrdU staining was performed. To preserve Foxp3 staining, the cells were resuspended in freezing media, 90% FBS and 10% DMSO, placed at -80 C, and stained for BrdU at a later time.

Mixed BM chimeras and tamoxifen administration

Donor BM was depleted of T cells by incubation with anti-CD4 and anti-CD8 antibodies, followed by labeling and depletion with goat anti-rat magnetic beads (Qiagen). T cell-depleted BM from $CD90.1^+CD45.2^+$ WT donor mice were mixed at a 1:1 ratio with $CD90.2^+CD45.2^+$ SLP-76 Het, SLP-76 cHet, or SLP-76 cKO BM and a total of 3-4 $\times$ 10^6 BM cells were injected i.v. into $CD45.1^+$ recipient mice that were lethally irradiated (9.5 Gy) at least four hours prior to injection. Since similar results were obtained with Het and cHet donor mice, they were used interchangeably as controls. Recipient mice were kept on antibiotic (trimethoprim/sulfamethoxazole) supplemented drinking water for three weeks after BM transplantation. After eight to ten weeks, recipient mice were bled to detect donor BM engraftment. For deletion of the floxed SLP-76 allele in SLP-76 cHet or SLP-76 cKO mice or mixed BM chimeras, mice were given 200 mg/g body weight of tamoxifen in corn oil every day for 5 days by oral gavage. Mice were then “rested” for at least 5

days after the last dose of tamoxifen. The efficiency of cre-mediated deletion of SLP-76 was at least 97% in YFP^{+}SLP-76 cKO T cells as demonstrated by failed CD69 upregulation after overnight anti-CD3 stimulation (data not shown). Splenocytes were cultured at 5×10^5 cells/well in 200 mL of TCM and stimulated overnight with T cell media alone, anti-CD3 (1 mg/mL), or PMA (100 ng/mL) and ionomcyin (1 mg/mL**)** before staining.

Statistical analyses

All values were graphed and analyzed for statistical significance using Prism (GraphPad). The statistical test used to calculate each *p* value is indicated in the figure legends. *p* values < 0.05 were considered significant.

Mice. WT C57BL/6, WT Foxp3-RFP reporter, and CD28 KO mice were purchased from The Jackson Laboratory. WT B6.SJL mice were purchased from The Jackson Laboratory or the National Cancer Institute. WT Foxp3-GFP mice were a generous gift from Dr. Vijay Kuchroo through Dr. Laurence Turka. c-Rel KO mice were a generous gift from Dr. Hsiou-Chi Liou through Dr. Youhai Chen. Germline DGKζ KO mice were generated as described (Zhong et al., 2008) and intercrossed to WT Foxp3-RFP reporter, WT Foxp3-GFP reporter, CD28 KO, and c-Rel KO mice. Mice were 6-16 weeks of age at time of use and were housed in pathogen-free conditions and treated in strict compliance with Institutional Animal Care and Use Committee regulations of the University of Pennsylvania.

Reagents and antibodies

Recombinant human IL-2 was purchased from Peprotech (Rocky Hill, NJ)**.** Anti-CD3 (2C11) was purchased from BD Pharmingen (San Diego, CA). Antibodies for flow cytometry were purchased from either BD Pharmingen: anti-CD3 (500A2), anti-CD4 (RM4-5), anti-CD19 (1D3), anti-CD25 (PC61), anti-B220 (RA3-6B2), anti-CD69 (H1.2F3), anti-CD117 (2B8), anti-Sca-1 (E13-161.7), and streptavidin-PE-Cy7; Biolegend (San Diego, CA): anti-CD8α (53-6.7), anti-CD11c (N418), anti-CD11b (M1/70), anti-CD44 (IM7), anti-CD45.2 (104), anti-FcεRI (MAR-1), anti-Gr-1 (RB6-8C5), anti-NK1.1 (PK136), anti-TCRβ (H57-597), anti-TCRγδ (UC7-13D5), anti-Ter110 (TER-119); eBioscience: anti-BrdU (PRB-1), anti-Foxp3 (FJK-16s), anti-CD45.1 (A20); or Molecular Probes, Invitrogen (Carlsbad, CA): CFSE, DAPI, LIVE/DEAD Fixable Dead Cell stain. BrdU and mouse and rat IgG were purchased from Sigma-Aldrich.

Flow cytometry and cell sorting

Cells were stained in FACS buffer (2% FBS (HyClone), 0.05% sodium azide in PBS) on ice for 30 minutes with antibodies against surface antigens, followed by two washes with FACS buffer. For cell sorting, T cells were purified with CD90.2 magnetic beads using MACS columns (Miltenyi Biotec) according to manufacturer's protocol prior to cell surface staining. Intracellular Foxp3

(eBioscience) and BrdU (BD Pharmingen) staining were performed with kits according to manufacturer's protocol. To preserve GFP fluorescence in MIGR transduced cells, cells were fixed with 1% paraformaldehyde for 10 minutes after surface staining and prior to Foxp3 staining. An anti-GFP Ab was also added along with the Foxp3 stain. Flow cytometry was performed with an LSR II or FACSCanto (BD Biosciences). FACS was performed with a FACSAria cell sorter (BD Biosciences) at the UPenn Flow Cytometry and Cell Sorting Core. FACS-sorted populations were typically of 92-99% purity.

Mixed BM chimeras

Donor BM was depleted of T cells by incubation with anti-CD4 and anti-CD8 antibodies, followed by labeling and depletion with goat anti-rat magnetic beads (Qiagen). T cell-depleted BM from $CD45.1^+$ WT donor mice were mixed at a 1:1 ratio with BM from $CD45.2^+$ WT donor or DGKζ KO donor mice and injected i.v. into $CD45.1^+$ recipient mice that were lethally irradiated (9.5 Gy) at least 4 hours prior to injection. Recipient mice were kept on antibiotic (trimethoprim/sulfamethoxazole) drinking water for 3 weeks after BM transplantation. After eight to nine weeks, recipient mice were bled to detect donor BM engraftment.

Retroviral transduction of BM

MIGR retrovirus was produced and transduced as described (Jordan, 2006). Briefly, five days prior to BM harvest, each donor mouse was given an i.p. injection of 5 mg of 5-fluorouracil. On the fifth day after injection, BM was harvested from the femur, tibia, and hip bones and placed in single cell suspension. BM cells were then cultured overnight at 37 degrees C in the presence of pre-stimulation media containing 10 ng/mL of IL-3 and IL-6 and 50 ng/mL of stem cell factor to induce stem cell cycling. On the sixth day, BM cells were placed in a six well plate along with the appropriate MIGR virus in 4 mL of pre-stimulation media along with 4 μg/mL of polybrene at a final concentration of 2-5 × 10^6 cells/mL. The six-well plate was then centrifuged for 90 minutes at 1300*g* at room temperature to induce MIGR infection of the BM cells. After this spin infection, the plate was placed at 37 degree C. Two to three hours later, the BM cells were gently resuspended

to disrupt cell pellets and rested overnight at 37 degrees C. On the seventh day, the spin infection was repeated as on the sixth day and the transduced BM cells were allowed to rest for at least four hours prior to i.v. injection into lethally irradiated (9.5 Gy) CD45.1$^+$ recipient mice. Recipient mice were allowed to reconstitute for eight to nine weeks.

In vitro Treg differentiation assay from CD4$^+$ SP thymocytes

Immature Foxp3$^-$CD69$^+$CD44^{low}CD4$^+$ SP thymocytes were FACS-purified from CD8α MACS-depleted thymuses from WT Foxp3-GFP reporter and DGKζ KO Foxp3-GFP reporter mice. 5 × 10^4 Foxp3$^-$CD69$^+$CD44^{low}CD4$^+$ SP thymocytes were cultured in 96 well flat bottom plates in the presence of 10 μg/mL of plate bound anti-CD3 alone or together with 100 U/mL of human recombinant IL-2 for three days. On the third day, cells were harvested, stained, and acquired by flow cytometry.

Statistical analyses

All values were graphed and analyzed for statistical significance using Prism (GraphPad). The statistical test used to calculate each *p* value is indicated in the figure legends. *p* values < 0.05 were considered significant.

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