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A Role of T Cell Receptor Specificity in the Thymic Regulatory

T Cell Development

by

Hyang-Mi Lee

A dissertation presented to the
Graduate School of Arts and Sciences
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

May 2012

Saint Louis, Missouri

Abstract

The development of regulatory T (Treg) cells is essential for the maintenance of immune tolerance and homeostasis. In the thymus, TCR-specificity to self-antigen appears to be a primary determinant for Treg cell lineage commitment, imprinting both self and foreign antigens in the peripheral Treg cell population to provide dominant tolerance. The degree of T cell self-reactivity considered dangerous by the immune system, thereby requiring thymic education to prevent autoimmunity, is unknown. Here, I analyzed a panel of TCRs with a broad range of reactivity to ovalbumin ($OVA_{323-339}$) in the RIP-mOVA self-antigen model for their ability to induce mechanisms of thymic tolerance. Thymic Treg cell generation in vivo was directly correlated with reactivity to OVA-peptide in a broad ~1,000-fold range, and its developmental "niche size" was unexpectedly dependent on TCR affinity. The threshold for Treg cell differentiation was almost 100-fold lower than that required for eliciting thymic negative selection and peripheral T cell responses. Thus, these data suggest that Treg cell differentiation is a default outcome of self-antigen encounter for $CD4^+$ thymocytes, and that thymic tolerance mechanisms are tuned to limit the escape of self-reactive effectors without Treg cell chaperones into the periphery. In addition, in the study of developmental stage of Treg cells, I demonstrated that differentiation of most Treg cells occurs at immature CD4SP subset, suggesting that medullary APCs may facilitate maturation of thymocyte after positive selection for efficient induction of Foxp3. In summary, this study suggest that Treg cell development is driven by self-antigen encounter of CD4SP cells, and TCR reactivity for self-antigen plays an instructive role in Treg cell differentiation, thereby

thymic tolerance mechanisms prevent autoimmunity by restraining the escape of self-reactive effector T cells into the periphery.

Acknowledgements

It is my great pleasure to thank those who helped me complete my thesis works. They made my life and this thesis possible with their collaborations, advices and support.

First, I am thankful to my advisor, Chyi-Song Hsieh for his understanding, guidance and support throughout my graduate study. Whenever I faced obstacles in my works, his advices helped me leap the huddles, and provided me directions. I also could learn valuable lessons from his enthusiasm and ways to address scientific questions, which will influence my attitude to future research. My thanks go to the past and present Hsieh lab people who supported me in various respects of science. Research themes and techniques that they have established formed the frameworks of the lab, and my works could be started and finished on the basis of them.

My thesis work could not have been progressed without help from colleagues in Washington University. I would like to thank to Dr. Ted Hansen, a chair of my thesis committee, for his kind guidance throughout all the process for graduation. I would also like to thank to Dr. Paul Allen and Dr. Daved Fremont for their help for my trial to measure affinity. I am very grateful to David Donermeyer and William McCoy for their help for purification of tetramer and OVA specific TCRs, which I could not even try without their support and effort. I would like to thank to Dr. Kenneth Murphy and Dr. Wataru Ise for providing reagents that were essential for starting my thesis work. I am also grateful to my thesis committee members, Dr. Yina Huang and Dr. Wojciech Swat, for their constrictive advices throughout my thesis work. I would also like to thank to Dr. Takeshi Egawa for his advices for my career.

I am grateful to my friends for their friendship that supported me to go through long time of thesis work. Joey became my first friend that I made in this country, and her friendship supported my life in and out of the lab. I would like to thank to my old friend, Soo-Yoen, for her long lasting friendship and trust. My thanks also go to my college friend for the memories and friendship that have emotionally supported me over years. I am also thankful to my classmate for sharing good and bad days in St. Louis.

Last, I would like to thank to my husband, Sang-Yong Hong, for his support for me even in his struggling graduate studies. I would like to express my deepest respect and love to my parents, sisters and brother for their support and dedication throughout my whole life.

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Chapter 1

Background and Significance

It has become well established that the naturally occurring regulatory subset (Treg) of CD4$^+$ T cells plays an important role in maintaining immune tolerance [1-3]. This is clearly illustrated by the observation that mutations in the forkhead transcription factor Foxp3, important for Treg cell function and development, results in lethal multi-organ autoimmunity. In humans, children are afflicted by the IPEX syndrome (immunodysregulation polyendocrinopathy and enteropathy) [4], whereas the murine equivalent is termed scurfy [5, 6]. Moreover, the acute depletion of Foxp3$^+$ Treg cell from normal mice unleashes a rapid diffuse lymphoproliferative syndrome leading to death within 2 weeks [7, 8]. Treg cells have also been shown to prevent excessive responses to pathogens, participate in immune surveillance on tumor cells, and control a balance between host and microbial flora [9-11]. In order to understand how Treg cells impact on these multiple facets of immunity, it is important to determine how Treg cells are generated.

Brief history of thymic Treg cell development

In order to recognize a wide variety of pathogens, the adaptive immune system generates an enormously diverse repertoire of antigen receptors by somatic gene rearrangement. One consequence of this diversity, however, is that the repertoire includes receptors that recognize self-antigen with the potential to cause autoimmunity. For T cells, the immune system utilizes an intricate system of development in a specialized organ, the thymus, to educate the immature T cell population to self prior to their release into the

periphery. One mechanism of education is the elimination of immature self-reactive cells, a process also known as negative selection [12-14] . However, it has become clear that this culling is not sufficient to achieve tolerance, and some potentially pathogenic cells escape into the periphery [15, 16].

The notion that a specialized regulatory T cell population is required to inhibit the cells that escape negative selection was suggested by the classic thymectomy experiment, in which T cell dependent autoimmunity occurred if thymectomy occurred at day 3, but not day 7 [17], resulting from what is now thought to be a delay in thymic Treg cell export [18, 19]. Follow up studies showed that depletion of the CD25 subset revealed the autoimmune propensity in CD4 single-positive (SP) thymocytes [20]. Thus, it was suggested that another important educational function of the thymus is to select regulatory T (Treg) cells to prevent spontaneous autoimmunity [21].

TCR specificity: Self-reactivity of thymic Treg cells

Since Foxp3$^+$ Treg cells comprise only a small fraction of CD4$^+$ T cells, an important question is whether there are defining features that determine selection into the Treg cell subset. Early studies inferred that Treg cells may be reactive to self antigens, as the presence of an organ was required for the maintenance or generation of peripheral T cells with the ability to suppress autoimmunity to that tissue [22-24]. Subsequent studies were more direct. For example, it was observed that superantigen increased the frequency of Treg cells with the appropriate TCRβ chain, suggesting that TCR stimulation was involved in thymic Treg cell development [25]. Moreover, TCR transgenic mice on a Rag-deficient background were not found to contain thymic Treg cells, whereas Rag-

sufficient mice, able to rearrange endogenous TCRα chains, could generate thymic Treg cells [20, 26]. These data therefore suggested that only certain TCR specificities could facilitate thymic Treg cell development.

Studies of TCR and cognate antigen transgenic mice provided direct support for the hypothesis that self-antigen recognition in the thymus was important for directing thymic Treg cell selection. For example, hemagglutinin (HA) specific TCR transgenic cells were selected to become Treg cells only when HA was transgenically expressed in the thymus [27]. Other TCR transgenic models including ovalbumin (OVA) -specific DO11.10 yielded similar results [28, 29]. From these data, the notion arose that Treg cell development occurred in the avidity window for self-antigen between positive and negative selection [30].

However, subsequent data were conflicted regarding whether intrinsic properties of TCR, such as self-reactivity, specify Treg lineage commitment. In hen egg lysozyme (HEL)-specific 3A9 TCR transgenic mice, analyses of the absolute number of Treg cells revealed no significant increase in Treg cell generation when transgenic TCR was coexpressed with HEL [31]. Similarly, it was found that the proportion of Treg cells increased without an increase of the absolute numbers when pigeon cytochrome c (PCC) - reactive AND TCR encountered varying amount of antigen expressed under the control of tetracycline-dependent transgene system [32] . This observation suggested that agonist TCR interaction resulted in deletion, rather than Treg cell selection. However, TCR specificities induced by alternative TCRα chain usage could induce Treg cells and protect them from agonist mediated deletion, resulting in an overall increase in the frequency of

Treg cells with increasing agonist ligands. Thus, not all TCR transgenic studies supported an agonist model of Treg cell selection.

Analyses of polyclonal TCR repertoire also reached conflicting conclusions. It was reported that Treg cells utilize a distinct, but overlapping, TCR repertoire from that of non-Treg cells, in favor of a model in which Treg lineage commitment is instructed by TCR specificity [33-35]. Moreover, T cells transduced with Treg TCRs often rapidly expanded after adoptive transfer into either normal or lymphopenic hosts, suggesting that Treg-derived TCRs confer reactivity to self-antigens in vivo [33, 36]. In contrast, another study argued that self-reactivity is dispensable for Treg cell selection, based on the observations that TCR repertoire of Treg and non-Treg cell subset were significantly overlapped, and hybridoma cells expressing Treg TCRs failed to respond to self-antigens presented by splenocytes or dendritic cells in vitro [37].

Finally, it was suggested that early thymic developmental events condition thymocytes for differentiation into Treg cell lineage before TCR rearrangement, implying that TCR specificity is not a specifying factor for thymic Treg cell induction, although post-differentiation selection based on antigen-specificity is possible [38]. Thus, there was considerable controversy regarding the role of TCR specificity in thymic Treg cell selection.

Data from the past several years, however, have been in favor of TCR-mediated Treg cell selection. First, TCRs from natural Treg, but not naive, cells, are able to facilitate thymic Treg cell development in TCR transgenic models [39, 40]. Second, experimental attenuation of MHC II expression on mTECs resulted in preferential Treg development, rather than negative selection, in a TCR transgenic model involving an

Aire-promoter driven antigen [41]. Third, intravenous peptide could stimulate Treg cell development if given within certain dose ranges [42]. Finally, direct assessment of TCR activation using a new Nur77-GFP reporter revealed that thymic Treg cells were recently exposed to enhanced levels of TCR stimulation [43]. Thus, the preponderance of current evidence, discussed in greater detail below, supports an important role for TCR specificity in thymic Treg cell differentiation.

TCR transgenic mice with natural Treg TCRs

The aforementioned TCR transgenic studies utilized TCRs that were not known to be normally found in the natural Treg cell population. It was therefore possible that the experimental manipulation of expressing the cognate antigen using various promoters may not mimic natural Treg cell development. Recently, several groups independently reported their experiences with TCR transgenic mice expressing natural Treg TCRs. One group isolated the natural Treg TCR from OTII αβ-TCR transgenic cells, and only observed negative selection after generating the Treg TCR transgenic line [44]. It is possible that this particular TCR was expressed as a secondary TCRα chain at low levels in OTII cells, and therefore had higher affinity than usual for self antigens leading to negative selection when expressed in a normal context. Two other groups used TCRs isolated from polyclonal fixed TCRβ repertoires in which the skewing of the TCR to the Treg cell subset was known [39, 40]. Intriguingly, the frequency of Foxp3^{+} thymocytes was surprisingly low in the TCR transgenic mice on a Rag-deficient background, which would not have been predicted based on previous studies of TCR by cognate antigen

transgenic mice [27-29]. In fact, the initial interpretation by both groups was that this observation resulted from an experimental artifact of TCR transgenesis.

Substantial thymic Treg cell development was eventually observed only when the clonal frequency of the TCR transgenic cells was diminished using either mixed bone marrow chimeras or intrathymic injection. In other words, the clonal frequency of TCR transgenic cells was inversely correlated with the frequency of Foxp3$^+$ cells. Moreover, the number of Treg cells generated reached plateau at high clonal frequency, and was much smaller than the total number of CD4$^+$CD8$^-$ (CD4SP) cells that could be generated by positive selection. These observations led to the notion that thymocytes with a given TCR specificity undergo intraclonal competition for a small niche with limited microenvironmental factor(s) important for thymic Treg cell development. Although the factors defining the Treg cell selection niche are unclear, the most compelling hypothesis is that the niche represents a limited amount of antigen for which developing thymocytes must compete in order to undergo Treg cell differentiation.

It is important to note, however, that the limitations imposed by niche size resulting in intra-(or inter) clonal competition may be more applicable to experimental immunology rather than normal T cell development in a fully polyclonal population, where the clonal frequency is likely to be extremely low–on the order of a few to tens of cells per antigen specificity [45]. In addition to TCR transgenic mice, restricted TCR repertoires may be susceptible to niche size issues depending on the extent of the limitation [35], and may provide an explanation regarding the increased overlap between Treg and non-Treg cell subsets in some studies [37] compared with others [33] .

The more physiologic implication from the observation of a small Treg cell developmental niche, at least based on the natural Treg TCRs reported to date, is that the antigens that drive thymic Treg cell development are likely to be uncommon and likely tissue-specific antigens, rather than ubiquitous antigens. Moreover, the size of the niche varies per TCR, suggesting that some Treg TCR ligands are more abundant than others. The notion of rare Treg cell-inducing ligands would be consistent with the notion that Aire-dependent tissue specific antigens presented by medullary thymic epithelial cells (mTECs) can facilitate thymic Treg cell development [46]. This is also supported by an observation of preferred Treg cell development compared to deletion in low amount of antigen expression [47]. Finally, uncommon self-ligands would explain why self-reactivity could not be easily detected by direct hybridoma assays [37], but required assessment of in vivo proliferation in lymphopenic hosts [33, 36]. Direct proof of this hypothesis will require isolation of natural Treg TCR ligands, which has not yet been reported to our knowledge. Thus, the preponderance of current data suggests that thymic Treg cell selection is driven by TCR encounter with rare self-antigens.

Affinity and avidity

As TCR specificity is a primary determinant for thymic Treg cell selection, an important question is to understand the parameters of TCR:ligand engagement required for this process. Initial experiments with TCR transgenic models suggested that a high affinity TCR interaction is required for thymic Treg cell selection. Comparison of two HA-specific TCR transgenic lines with 1000 fold differences in sensitivity to HA revealed that Treg cell development only occurred with the high affinity TCR [27].

Increasing the amount of antigen, i.e. avidity, could not compensate for the difference in affinity, as levels of antigen sufficient to induce negative selection were still unable to generate Foxp3$^+$ cells. A different study of Treg cell differentiation in thymic organ cultures used altered peptide ligands (APL) with varying affinities to the hemoglobin-specific N3.L2 TCR [48]. They observed that strong and weak agonist, but not antagonist, peptides could induce Treg cell differentiation. Thus, these data suggest that there may be an affinity threshold, akin to negative selection [13], for which TCR recognition of agonist ligands is required for thymic Treg cell differentiation.

The role of the avidity in Treg cell differentiation has been also addressed using transgenic mice and in vitro thymic organ cultures. Using a panel of transgenic lines with different expression levels of HA in the thymus, it was observed that Treg cell number was enhanced at low expression levels, whereas high levels resulting predominantly in negative selection [47]. A similar monophasic response was observed in vitro using a titration of agonist peptides into fetal thymic organ cultures of NOD TCR transgenic cells, in which Treg cell numbers were positively related to peptide dose at low concentration, but diminished at high antigen concentration due to extensive negative selection [49]. This phenomenon was also replicated in vivo using intravenous peptide administration [42]. Finally, a recent study using transgenic mice in which the level of antigen presentation was decreased by the downmodulation of MHC class II expression in Aire-expressing mTEC cells also showed a shift from negative selection to Treg cell development, which utilized a newly described transgenic of an Aire-promoter driven shRNA against C2TA, a master regulator of MHC class II expression (C2TA^{kd}) [41].

Thus, the experimental evidence favors a model by which Treg cell development is most efficient within an avidity window below that of negative selection.

While the current data provide a framework for understanding the role of avidity and affinity in thymic Treg cell selection, a number of questions remain. One potential issue is the use of transgenic lines in which there is only a single TCR clonotype. As discussed above, the efficiency of Treg cell development can be heavily influenced by the clonal frequency of the T cell [39, 40]. This complicates the interpretation of experiments that increase the amount of antigen level as a means to understand avidity. The traditional interpretation is that the increased number of ligands per APC leads to enhanced TCR signaling and Treg cell selection. However, it may be possible that the increased amount of antigen results in more APCs with sufficient ligand, enhancing the frequency of Foxp3$^+$ cells generated via an enlarged niche. Given that intraclonal competition also affects positive selection [50, 51], albeit to a lesser degree, it is reasonable to expect that negative selection is also affected by an antigenic niche. Thus, the analysis of Treg cell differentiation versus negative selection may be quite different at high versus low clonal frequencies.

Another issue is the use of antigen-transgenic lines driven by ubiquitous promoters, peptide injection into mice, or addition of peptides to fetal thymic organ cultures (FTOC), which presumes that Treg cell selecting ligands are fairly ubiquitous and the APC environment of the thymus is monolithic. Experimental evidence suggests that Treg cell selection utilizes a limited antigen niche [39], making ubiquitous antigens less likely to model thymic Treg cell development. Moreover, the distribution of selecting ligand on the various thymic APC subsets may or may not represent that of natural Treg

cell ligands. For example, expression of antigen in the cortex might favor negative selection rather than Treg cell development. However, these data may still be relevant as the range of antigens that select thymic Treg cell development has yet to be fully explored. Thus, although the current data show that affinity and avidity play important roles in thymic Treg cell selection as proof of principle, future experiments will be required to understand in a quantitative fashion how reactivity to self-antigens dictates cell-fate choice between Treg cell differentiation, negative selection, and release from the thymus as an effector cell with autoimmune potential.

A signaling threshold for thymic Treg cell development

One reason that it has been difficult to address the role of self-antigen reactivity in Treg cell selection is the lack of effective in vivo assessment for TCR stimulation. While T cell activation markers such as CD69 and CD25 are associated with the process of Treg cell development [52], they did not provide compelling evidence due to their transient expression or their upregulation as a Treg cell marker, respectively. Recently, a new marker for TCR activation was reported using a Nur77 promoter driven GFP which revealed several interesting aspects of thymic Treg cell differentiation [43]. In a polyclonal population, they found that GFP expression was high on Foxp3$^+$ compared with Foxp3$^-$ cells, confirming the notion that Treg cells underwent TCR mediated selection to agonist ligand.

More instructive, however, was the analysis of Nur77-GFP in G113 Treg TCR transgenic cells that were known to be restricted by a small developmental niche [39]. One prediction was that intraclonal competition would result in a downward shift of a

monophasic TCR signal measured by Nur77-GFP, thereby decreasing the proportion in the "avidity" window of Treg cell development. However, what was observed was that G113 cells existed in a biphasic plot with distinguishable Nur77-GFP[hi] and [lo] populations, which shifted in proportion depending on the clonal frequency of G113 thymocytes. Assuming that the Treg cell differentiation program itself does not affect Nur77-GFP levels, these data suggest that intraclonal competition operates by limiting the number of T cells obtaining a high level TCR signal, rather than decreasing the probability that a lesser TCR signal leads to Foxp3 induction. Niche size would then reflect the number of APCs that have sufficient antigen in terms of both affinity and avidity to induce "high level" TCR signaling, which remains to be biophysically defined in the context of Nur77-GFP. Thus, this suggests that Treg cell differentiation is dependent on a certain threshold of TCR signaling, arguing for a "digital," rather than "analog" interpretation of TCR signals for thymic Treg cell development.

Timing of thymic Treg cell differentiation: APC subsets

The notion that self-antigen specificity selects the thymic Treg cell population engenders the question of which APCs are presenting what self-antigens. For example, it is known that cTEC and mTEC present different antigenic repertoires due to the use of distinct antigen processing enzymes [53]. Moreover, Aire expression in mTECs has been shown to induce the expression of tissue-specific antigens in the thymus [54]. Other differences between APCs such as co-stimulation may also affect thymic Treg cell differentiation. Thus, it is important to understand the contribution of the various thymic APC subsets in the generation of the thymic Treg cell population.

Initial studies suggested that a normal frequency of CD25$^+$ Treg cells can be generated when MHC class II is exclusively expressed in the cortex of the thymus [55]. Consistent with cortical Treg cell development, some studies showed a considerable frequency of Treg cells at CD4$^+$CD8$^+$ (DP) stage [56, 57]. However, other studies favor the notion that Treg cell differentiation mainly occurs at the CD4SP stage [58, 59]. The temporal analysis of Treg cell generation during the neonatal period as well as in young adults using neonatal bone marrow injection demonstrated that DP cells expressing Foxp3 are rare and the development of Foxp3$^+$ cells mostly starts at immature CD4SP stage. Although a quantitative assessment of the relative contribution of cortical versus medullary APCs to thymic Treg cell differentiation is not established, it appears that most Treg cells are generated in the medulla.

In this thesis, I examined intrinsic and extrinsic factors for Treg cell development in the thymus using TCR repertoire analysis, the affinity of TCR for self-antigen and APC environment determining the developmental stage of Treg cells respectively. First, in the study of TCR affinity, I demonstrated that thymic Treg cell development is directly correlated with TCR affinity to self-antigen in a broad range. Second, I observed that foreign antigen specific Treg cells can be generated by recognition of self-antigen via presumably promiscuous antigen presentation by thymic APCs. Third, it was suggested that high TCR affinity expands the number of APC delivering a Treg cell inducing signal. Last, I found that the threshold for peripheral T cell responses is well above that for thymic Treg cell development and correspond to that for negative selection, suggesting that thymic tolerance mechanisms are developed to accompany escaping self-reactive effectors with Treg cells to prevent autoimmune responses in the periphery. Also, in the

study of developmental period of Treg cells, I demonstrated that the frequency of Treg cells generated at DP stage is much smaller than previously suggested, and most Treg cells differentiate at immature CD4SP stage, implying an important role of medullary APC in facilitating efficient induction of Foxp3. Taken together, this dissertation study suggests that Treg cell development is a default outcome of self-antigen encounter of CD4 SP thymocytes, and TCR affinity for self-antigen instructs Treg cell development.

Chapter 2

Tuning of thymic Treg cell selection to the self-reactive peripheral immune response

Introduction

The adaptive immune system generates a diverse array of antigen receptors to allow recognition of a variety of pathogens. However, a consequence of this diversity is that some receptors will recognize self with the potential to cause autoimmunity. For T cells, the issue of self-reactivity is mitigated as development occurs in a specialized organ, the thymus, where the immature T cell population is educated to the self-antigenic repertoire prior to their release into the periphery as mature T cells with the ability to cause autoimmunity. One important mechanism of education is the deletion of self-reactive T cells, also known as negative selection [12, 13]. However, not all self-reactive thymocytes are eliminated, and some escape the thymus as effector cells with the potential to cause autoimmunity [15, 16]. A second mechanism of education to self-antigens is now recognized to be the differentiation of self-reactive thymocytes to become Treg cells that suppress, rather than induce, inflammatory responses.

TCR specificity was initially thought to be important for thymic Treg cell development as it was observed that TCR transgenic mice on a Rag-deficient background do not have Treg cells [20, 60], implying that only certain TCRs can facilitate Treg cell differentiation. Subsequent studies of TCR and antigen double transgenic mice suggested that recognition of self-antigen was the pertinent requirement for thymic Treg cell induction [60, 61]. Other reports using fetal thymic organ cultures (FTOC) and in vivo

peptide injection have supported this model [42, 49]. Taken together with other studies[36, 39, 40], the preponderance of the data suggests that self-recognition is an important requirement for thymic Treg cell selection.

While these studies provide proof of principle that self-reactivity is required to trigger thymic education via Treg cell differentiation and negative selection, a number of questions remain. First, many studies modeled interactions with ubiquitous antigens via transgenic expression or peptide administration in vivo or in vitro [42, 49]. However, recent data suggests that thymic Treg cell development utilizes a limited antigenic niche [39, 40], implying that ubiquitous antigen presentation may not be an appropriate model. Thus, the level of self-reactivity that elicits thymic education mechanisms for CD4^{+} T cells to tissue-specific antigens is unknown.

Second, the study of TCR transgenic cells at high clonal frequencies may not represent what happens during normal thymic development, which occurs at very low clonal frequencies. For example, high clonal frequencies have recently been shown to markedly decrease the efficiency of thymic Treg cell development, presumably due to intraclonal competition [39, 40]. Thus, it may be possible that Treg cell development or negative selection may be different at high versus low clonal frequencies.

Finally, while there have been studies of MHC class I restricted T cells regarding thresholds of negative selection [16, 62], the range of TCR self-reactivities that elicit thymic tolerance mechanisms in CD4^{+} T cells in vivo has not been examined. In some studies, the level of self-reactivity required to trigger thymic Treg cell differentiation has been suggested to be quite high [41, 61], being at or near the level of self-reactivity that induces negative selection. Moreover, thymic Treg cell differentiation may be dependent

on a certain threshold of TCR affinity for antigen, as Treg cell development was not observed with low affinity interactions, even if the antigen was expressed at high enough levels to induce negative selection [63]. On the other hand, it was reported that there was great overlap between the Treg and non-Treg TCR repertoires [37], suggesting that a broad range of self-reactivity, perhaps including TCRs that recognize self at the level of positive selection, was sufficient for Treg cell development. Also, direct demonstration of natural Treg TCR recognition of self-antigens presented on thymic APCs has not been successful [37], suggesting that the antigen is rare or that the TCR affinity for antigen is low. Determining the self-reactivity thresholds for thymic Treg cell induction and negative selection would be useful for understanding how the immune system perceives the problem of self-recognition and utilizes thymic education to control it. Thus, the quantitative level of self-reactivity that triggers thymic education mechanisms, and how that relates to level of self-reactivity required for peripheral immune responses, are not known.

To address this question, I decided to fix the level of self-antigen and vary the efficiency of TCR recognition. Since natural Treg ligands are currently unknown, I utilized as a well-characterized model of a tissue-specific antigen, the RIP-mOVA transgenic line, in which the rat insulin promoter drives the expression of membrane bound ovalbumin (OVA) [64]. The developmental effects of thymic encounter with a relatively rare "self-antigen" can then be assessed at low clonal frequencies of T cells expressing a panel of naturally occurring TCRs with varying reactivity to $OVA_{323-339}$ identified from DO11β TCR transgenic mice. Remarkably, I observed a direct correlation between the degree of antigen-reactivity and thymic Treg cell generation over a broad

~1,000-fold range. Negative selection was apparent with the more self-reactive TCRs. Finally, peripheral responses as measured by proliferation of naive T cells after transfer into irradiated RIP-mOVA mice required a degree of OVA-reactivity that induces both thymic negative selection and Treg cell development. Thus, these data demonstrate that the level of self-reactivity plays a crucial instructive role in thymic Treg cell differentiation, which is tuned to be substantially below the threshold for peripheral immune responses to self-antigen.

Materials and Methods

1. Mice

Foxp3^{gfp} mice [65] were a kind gift from A. Rudensky (MSKCC). RIP-mOVA [64] mice Were obtained from Jackson Labs. DO11.10 TCRβ transgenic mouse line [66] was kindly provided by K. Murphy (Washington U.). All mice were backcrossed onto B6.C (B6 background congenic at the MHC locus with BALB/c) to facilitate breeding to mutant mice of B6 background, and were bred to generate DO11.10 TCRβ transgenic Foxp3^{gfp} TCRα$^{+/-}$ RIP-mOVA mice for TCR analysis. CD45.1 RIP-mOVA mice were also generated on the B6.C background. Mice were housed in a specific pathogen–free animal facility and were used according to protocols approved by the Institutional Animal Care and Use Committee at Washington University.

2. Reagents

The following monoclonal antibodies were from eBioscience, Biolegend and BD Pharmingen: antibody to CD4 (RM4-5), anti-CD8 (53-6.7), anti-CD45.1 (A20), anti-CD45.2 (104), anti-Thy-1.1 (HIS51), anti-CD25 (PC61), anti-HSA (M1/69), and anti–TCR Vβ8 (F23.1). Chicken ovalbumin (OVA$_{323\text{-}339}$) peptide (ISQAVHAAHAEINEAGR) was purchased from GenScript Corp. CFSE, Cell-Trace Far-Red DDAO dye and Cell-Trace Violet Proliferation kit were purchased from Invitrogen. Recombinant mouse IL-3, recombinant human IL-6, and recombinant mouse stem cell factor for the bone marrow culture were obtained from Peprotech.

3. Culture of T cells and TCR sequencing

Peripheral spleen and lymph node cells from DO11.10 TCRβ x Foxp3^{gfp} *Tcra*$^{+/-}$ RIP-mOVA$^-$ or $^+$ mice were labeled with DDAO and cultured with 1μM of OVA$_{323-339}$ for 5 days. DDAO low-Vβ8$^+$ CD4 T cells were purified from Foxp3$^-$ and Foxp3$^+$ subsets by FACS (FACSAria, Becton Dickenson). Sequencing and analysis of TRAV14 TCRα (Vα2) chains was performed as previously described [33].

4. Measurement of EC$_{50}$.

For NFAT activation assay, T cell hybridomas expressing DO11 TCRβ, mCD4, and an NFAT-GFP reporter [66], were retrovirally transduced with TCRα. FACS sorted mCD4$^+$Vβ8$^+$Vα2$^+$ cells were cultured with flt3 ligand-activated CD11$^+$ APC and various concentrations of OVA $_{323-339}$. GFP expression was measured by flow cytometry 40 hrs post stimulation. The NF-κB reporter cell line was generated by retroviral transduction of NF-κB-GFP construct [67] provided by B. Sleckman, Washington U., into DO11 TCRβ-mCD4 T cell hybridomas, and then transduced with TCRα chains. In these assays, the TA3 B cell hybridoma was used for APC to decrease background. To measure IL-2 production, 5KC T cell hybridomas expressing DO11β were transduced with a TCRα chain, and cultured with TA3 APCs. The supernatants were assessed for IL-2 using a CTLL-2 bioassay with Alamar blue (Arcus biologicals).

Thymic and peripheral T cells expressing an individual OVA peptide-specific TCR were generated by retroviral bone marrow chimeras as described below. Thymocytes were separated into CD4SP and DP subsets by magnetic cell purification using biotinylated anti-CD8 antibody and anti-biotin microbeads (AutoMACS, Miltenyi Biotec). CD4SP Were cultured with irradiated splenic APC pulsed with OVA$_{323-339}$ to

measure CD25 induction at 24 hours by flow cytometry. DP cells were also cultured with APCs to measure down regulation of CD4 and CD8 at 48hrs by flow cytometry. Pooled spleen and lymph node cells were labeled with CFSE and cultured with OVA$_{323-339}$. Cell division of retrovirally transduced cells (CD45.2$^+$ CD45.1$^-$ TCRβ$^+$ Vα2$^+$ CD4$^+$) was assessed by flow cytometry after 72 hours. To generate the dose response curve of each TCR, the activation/expression level at each concentration of peptide was normalized to that of anti-CD3 antibody (1µg/ml) stimulation, and EC$_{50}$ was determined by nonlinear regression using GraphPad software.

5. Tetramer binding

T cell hybridomas (2x10^5) expressing individual TCRs were stained with 20µg/ml of PE labeled tetramers of I-A^d protein with linked DO11 OVA epitope [68] at 37℃ for 90 min in round bottom 96-Well plates, and were further incubated with 2µg/ml of anti-TCRβ antibody at 4℃ for 30 min. Tetramer binding to TCRs was measured by MFI of PE using flow cytometry. 3K:I-A^b tetramer was used as a control.

6. Retroviral transduction of TCR into CD4$^-$CD8$^-$ thymocytes

Thymocytes from *Foxp3^{gfp}Rag1$^{-/-}$* mice were transduced with MigR1-derived retroviruses expressing TCRα-P2A-DO11 TCRβ IRES-Thy1.1 as described [69, 70]. One million cells in 10µl PBS was injected into the thymic lobe of sublethally irradiated (600 rads) wild-type and RIP-mOVA recipients. Development of Foxp3$^+$ thymocytes was analyzed by flow cytometry 2 weeks later.

7. Retroviral bone marrow chimeras

Generation of retroviral bone marrow chimeras have been previously described [39]. Here, I used $Foxp3^{gfp}Rag1^{-/-}$ B6.C mice as bone marrow donors, which were at times mixed with bone marrow of CD45.1 $Foxp3^{gfp}$ mice at ratios of 1:0, 1:1 or 1:4, prior to injection (5×10^6 total cells) into lethally irradiated (1,000 rads) CD45.1 wild-type or RIP-mOVA hosts. Thymic and peripheral T cells were used for experiments or analyzed by flow cytometry 6 weeks later.

8. Assessment of negative selection *in vivo*

Thymocytes expressing individual TCRs were generated by retroviral bone marrow chimeras (not mixed) in wild-type hosts. CD4SP cells were FACS sorted, and mixed with Cell Trace Violet-labeled polyclonal thymocytes at a 1:1 ratio. CD4SP cells were injected into the thymus of either CD45.1 wild-type or RIP-mOVA recipients, and analyzed by flow cytometry on day 3 post transfer.

9. Assessment of peripheral responses to antigen *in vivo*

Peripheral CD4$^+$ T cells expressing individual TCRs were generated by retroviral bone marrow chimeras in wild-type hosts, and labeled with Cell Trace Violet as a CFSE analog to follow cell division. Foxp3$^-$ CD4$^+$ T cells (10^5) were intravenously transferred into irradiated (500 rads) CD45.1 wild-type or RIP-mOVA recipients. Splenocytes were analyzed for proliferation and Treg cell conversion 14 days later by flow cytometry.

Chapter 2.1

Identification of OVA peptide-specific TCRs in a broad range of affinity

Results

Identification of naturally occurring OVA$_{323-339}$ peptide-specific TCRs

The degree of self-reactivity that triggers thymic Treg cell differentiation of CD4$^+$ T cells is unknown, with studies favoring high [63] versus low [37] thresholds of self-reactivity. As different thresholds for thymic Treg cell development may be predicted to generate different models regarding how self-reactive Treg and effector cells participate in immune responses to self- and foreign antigens in the periphery (Figure 2.1A,B), I sought to define this threshold using a panel of TCRs with varying self-reactivity. Although mutagenesis has been used to generate TCRs with different affinity to antigen, I decided to identify naturally rearranged OVA-reactive TCRs from the peripheral CD4$^+$ T cell population to avoid non-physiologic TCR interactions with peptide:MHC molecules. As the frequency of OVA-specific T cells in the normal T cell population is very low, I reasoned that using a TCR β-transgenic line from an OVA-specific TCR would increase the precursor frequency based on observations from the MHC class I restricted OT-I transgenic line [71]. Another advantage of fixing the TCR β-chain is that the physical framework for TCR recognition of peptide:MHC molecules will be similar between different TCR α-chains. I therefore obtained a TCR β transgenic line based on the DO11.10αβ TCR [66], which recognizes OVA$_{323-339}$ peptide at agonist level affinities [72, 73]. To ensure that only one TCR α chain is expressed per cell, the mice were bred to be

Tcra$^{+/-}$. Thus, I used DO11β *Foxp3*^{gfp} B6.C (H-2^d) *Tcra*$^{+/-}$ RIP-mOVA$^{+ or -}$ mice as a source of T cells to identify a panel of OVA-specific TCRs (Figure. 2.2A).

To enrich for OVA-specific TCRs amongst the TCR β transgenic polyclonal repertoire, I identified T cells that proliferated after in vitro stimulation with OVA peptide using dilution of DDAO, a cellular dye similar to CFSE but compatible with GFP (Figure 2.2B). However, there appeared to be substantial OVA-independent proliferation. To identify OVA-specific TCRs, I looked for TCR sequences that were enriched in the OVA-stimulated versus un-stimulated T cell populations. I obtained 3,785 TCRα sequences from Foxp3$^+$ and Foxp3$^-$ cells from RIP-mOVA$^+$ or $^-$ mice stimulated in vitro with or without OVA-peptide (Figure 2.2C). I restricted the initial analysis to the TRAV14 (Vα2) repertoire to limit the possible structural configurations of TCR interaction with OVA peptide:MHC II molecules. As expected, there was substantial overlap between the OVA stimulated and un-stimulated repertoires (Figure 2.3). In particular, the 9 most frequent Foxp3$^-$ TCRs on T cells from RIP-mOVA$^+$ mice were found at comparable frequencies irrespective of OVA-peptide stimulation, suggesting that the precursor frequency of OVA-specific cells is much lower in these mice due to negative selection.

In order to confirm OVA-peptide specificity, I selected the most frequent TCRs found in the OVA but not control cultures (highlighted in grey, Figure 2.3) and expressed them on DO11β T cell hybridoma lines that contain GFP driven by NFAT as a reporter for TCR stimulation [66]. Since the parent line prior to retroviral transduction was the same, the hybridoma cells should differ only by the TCRα sequence, allowing direct comparison of antigen recognition regardless of the cellular source of the TCR. However,

only a subset of these TCRs showed reactivity to OVA peptide presented by Flt3L induced dendritic cells (Figure 2.4), suggesting that low frequencies and well to well variability limited the effectiveness of this subtraction approach.

In these screening experiments, I noted that the only TCRs which exhibited OVA-reactivity, regardless of T cell subset or RIP-mOVA status, utilize TRAJ21 (Jα21), which happens to be the same J-region utilized by the TRAV5D (Vα13) DO11$\alpha\beta$-chain. This suggests that Jα21 provides structural features that facilitate recognition of OVA-peptide in the context of DO11β. I therefore identified all naturally rearranged Jα21 containing TCRs in our data set irrespective of frequency, T cell subset, or genotype, and tested them for OVA-reactivity (Figure 2.5). Interestingly, only TCRs with a CDR3 length of 10 showed reactivity to OVA. Thus, I have identified a panel of 8 naturally arising TCRs all with Jα21 and the same CDR3 length that recognize OVA-peptide:MHC class II I-A^d (Figure 2.6), suggesting that these TCRs all recognize in a very similar manner OVA$_{323-339}$ bound to I-A^d in a single register.

The panel of TCRs exhibit a broad range of reactivity to OVA

To calculate the efficiency of OVA recognition, I determined the effective concentration of OVA peptide that elicited half-maximal responses (EC$_{50}$) for each TCR using non-linear regression analysis (Figure 2.7A). As an inter-experiment control, the peptide responses were normalized to that of anti-CD3 antibody treated cells. Since the EC$_{50}$ is dependent on the functional characteristics of the assay, I decided to use DO11$\alpha\beta$ as a reference TCR for a typical agonist level response to foreign antigen. Thus, the

relative efficiency by which a TCR recognizes OVA peptide in comparison with DO11 is denoted as $\Delta Log(EC_{50}) = Log(EC_{50} \ DO11) - Log(EC_{50} \ TCR)$.

The first quantification of the efficiency of OVA recognition utilized the NFAT-GFP reporter hybridoma cells generated for the initial functional screening above (Figure 2.6). I observed that our TCRs spanned over 3 logs in relative EC_{50} to our reference TCR DO11, with TCRs N7 and N9 within 1 log of DO11, R1/R4 within the 2nd log, and R2, N12, N13 and P1 with even lower reactivity to OVA. Note that changes in one or two amino acids greatly affected the recognition of OVA, such that P1 exhibits greater than a thousand fold-lower sensitivity to peptide antigen than DO11.10, although there may also be effects in the CDR1/2 regions for R2 and N9 (Figure 2.6A). Thus, I have identified a panel of 8 TCRs that recognize OVA with a broad range of efficiencies.

Thymic Treg cell selection of TCRs with foreign antigen-reactivity

I first wanted to establish that our panel of OVA-reactive TCRs does not facilitate thymic Treg cell development in the absence of OVA, which would preclude straightforward interpretation of OVA-dependent Treg cell generation. I utilized retroviral transduction of DN thymocytes followed by intrathymic transfer into congenically marked hosts [69, 70] to assess the development of Foxp3^{+} cells 2 weeks after transfer. I did not observe Treg cell development in T cells expressing the DO11 TCR, as well as five OVA-reactive TCRs (Figure 2.7B). Interestingly, there were three TCRs which facilitated thymic Treg cell development in the absence of OVA, suggesting that these TCRs may recognize unknown self-antigens for Treg cell selection. This is further supported by the observation of R1 and R2 in the Treg TCR repertoires from

wild-type mice (Figure 2.3). It appears that both TRAV14 subtype and CDR3 sequence affects this presumed self-reactivity, as R2 and N9 use the same TRAV14 subtype but different CDR3 sequence, whereas R1 and R2 utilize the same CDR3 sequence, but have different TRAV regions. Thus, these data demonstrate that it is possible to generate Treg cells in the thymus that also recognize foreign antigens, presumably due to the promiscuity of TCR recognition.

Figure Legends

Figure 2.1. Hypothetical models on the relationship between thresholds for thymic Treg cell selection and peripheral tolerance and immunity.

(A) High threshold of self-reactivity model for thymic Treg cell selection. Self-reactive TCRs above a high threshold are selected into Treg cells, which prevent autoimmunity elicited by lower affinity T cells. In this model, there is no selection of Treg cells to foreign antigens, allowing effector T cells to efficiently respond to foreign antigens. Blue circles represent self-reactivity at the level of positive selection, and maroon circles self-reactivity at varying levels as indicted by the darkness of the fill color. Treg cells are indicated in shades of green fill color. (B) Low threshold of self-reactivity model. In this model, a broad repertoire of Treg cells is generated with low correlation to self-reactivity. Tolerance to self occurs as Treg cells are generated to most antigen-specificities. However, Treg cells will also be generated to foreign antigens, and has been proposed to prevent excess immunopathology that can be occurred during immune responses to pathogens. (C) Our data suggest that thymic Treg cell generation is best represented by an intermediate model as it is directly driven by self-reactivity over a broad range, but is above the level of positive selection. In this model, foreign antigen-reactive thymic Treg cells arise due to incidental self-reactivity of the TCR, presumably related to promiscuous antigen recognition. For negative selection, the shape of the curve is not known (indicated by dashed line and ?). For positive selection, the range of affinities is not well established (indicated by dashed line). Int., intermediate.

Figure 2.2. TCR sequencing of OVA peptide-reactive T cells

(A) Flow cytometry of the thymic and splenic CD4$^+$ T cells from DO11β *Foxp*^{gfp} *Tcra*$^{+/-}$ RIP-mOVA$^{-\text{ or }+}$ mice. (B) Gating scheme for sorting of proliferating cells after in vitro activation with OVA-peptide. DDAO is a dye analogous to CFSE that is used to determine cell division. The percentage of cells in each quadrant is indicated by the number. Boxes with dashed lines represent the gates used to sort proliferating Foxp3$^-$ or Foxp3$^+$ cells. (C) Numbers of TCRs sequenced from the sorted cell populations indicated in (B). Each experiment represents pooled spleen and lymph nodes from 2 mice. TCR sequence database was generated by pooling sequences from all experiments.

Figure 2.3. Selection of possible OVA peptide-reactive TCRs using repertoire analysis

The 20 most frequent TCRs are shown for Foxp3$^+$ and Foxp3$^-$ cell subsets from RIP-mOVA$^{-\text{ or }+}$ mice (numbers in blue). TCRs found only in the OVA peptide culture were presumed to be OVA reactive and cloned for functional screening using hybridoma cells (highlighted in grey). TRAJ21 TCRs are shown in red. Note that R1* and R2** TCRs of the same CDR amino acid sequence are distinguished by usage of polymorphic TRAV14-3*01 and TRAV14D-1*01, respectively. Numbers in the table represent the frequencies of the TCR in each subset.

Figure 2.4. Confirmation of OVA peptide-reactivity

TCRs selected from the repertoire analysis in Figure 3 were retrovirally expressed on the NFAT-GFP reporter hybridoma line. TCRs selected from Foxp3⁻ RIP-mOVA⁻ cells were also tested, but did not have any OVA-reactive TCRs not shown above. TCRs that contain TRAJ21 sequence in their CDR3 are shown in red as Supplementary Figure 3. One TRAJ21 TCR did not show reactivity to 1 mM OVA peptide in vitro (†), and one did not express in vivo in the TCRα-P2A-DO11β vector for unknown reasons (‡). These two TCRs were therefore not studied further. Data are representative plots from two independent experiments.

Figure 2.5. Screening of TRAJ21 containing TCRs

(A) Summary of all TRAJ21 TCRs found in our TCR database. TCRs that induced NFAT-GFP expression are highlighted in grey with differences in amino acid sequence marked in red. (B) Flow cytometry of NFAT-GFP expression by TCRs in (A). Data are representative plots from two independent experiments.

Figure 2.6. TCRs exhibit a broad range of reactivity to OVA peptide.

(A) Summary of sensitivity to OVA-peptide for 9 TCRs including DO11. Sensitivity to OVA-peptide was determined using an NFAT-GFP hybridoma as discussed in Figure 7A. (B) Flow cytometric plots of NFAT-GFP expression by hybridoma cells expressing OVA-reactive TCRs in (A) after stimulation with Flt3L-induced DCs and 1 mM OVA peptide. (C) Expression levels of retrovirally transduced TCRs on hybridoma cells. Data are representative from three independent experiments.

Figure 2.7. In vitro and in vivo analysis of OVA-reactive TCRs

(A) In vitro assessment of TCR sensitivity to OVA peptide. NFAT-GFP reporter hybridomas expressing each of 9 TCRs, including DO11 (red), was generated via retroviral transduction of TCRα chains, and tested against a range of OVA peptide concentrations presented by Flt3L induced dendritic cells. Data shown are the frequency of NFAT-GFP$^+$ cells normalized to that of αCD3 antibody treated cells for each hybridoma line. Data are representative of three independent experiments. (B) In vivo assessment of thymic Treg cell development. Double-negative (DN) thymocytes from *Foxp3*^{gfp}*Rag1*$^{-/-}$ were retrovirally transduced with each OVA-specific TCR and transferred into the thymus of wild-type mice, and analyzed for Treg cell generation 14-days post transfer. Data are representative of 3-4 mice for each TCR from at 2 independent experiments.
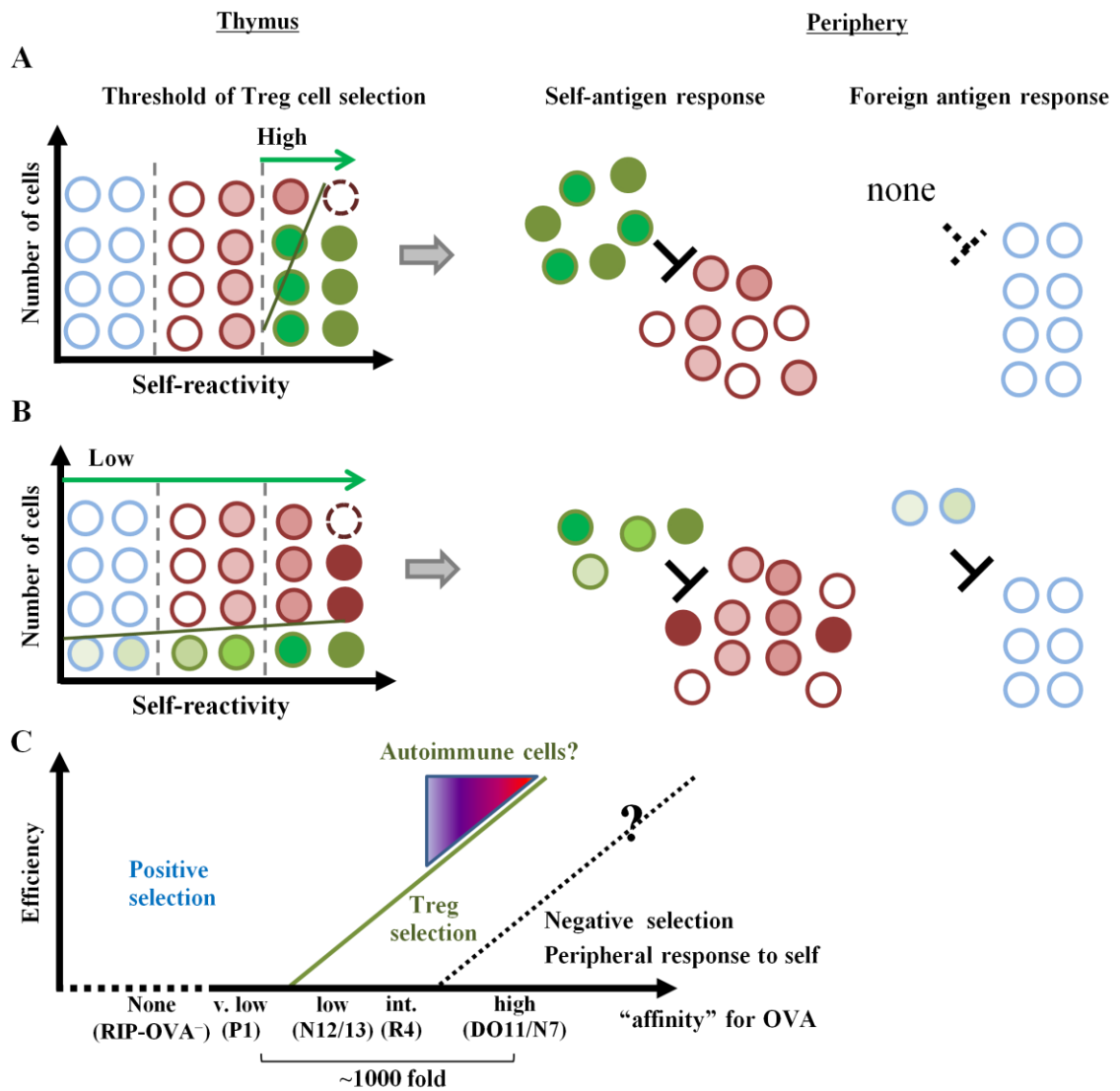
Figure 2.1. Hypothetical models on the relationship between thresholds for thymic Treg cell selection and peripheral tolerance and immunity.

A

B

C
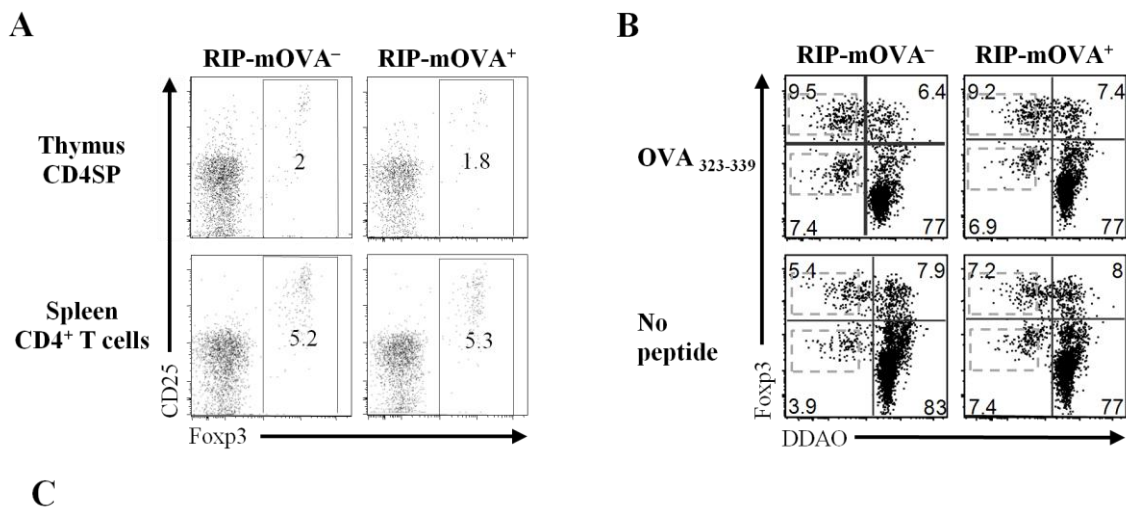
	RIP-mOVA⁻				RIP-mOVA⁺			
	no peptide		OVA$_{323\text{-}339}$		no peptide		OVA$_{323\text{-}339}$	
	Foxp3⁻	Foxp3⁺	Foxp3⁻	Foxp3⁺	Foxp3⁻	Foxp3⁺	Foxp3⁻	Foxp3⁺
Expt. 1	50	127	114	72	101	132	120	124
Expt. 2	55	100	108	129	135	117	105	101
Expt. 3	117	84	122	162	120	163	208	131
Expt. 4	119	142	99	121	128	137	112	130
Total No. Sequences	341	453	443	484	484	549	545	486

Figure 2.2. TCR sequencing of OVA peptide-reactive T cells

Foxp3+

RIP-mOVA−

clone	CDR a.a. sequence	no peptide Foxp3−	no peptide Foxp3+	OVA$_{323\text{-}339}$ Foxp3−	OVA$_{323\text{-}339}$ Foxp3+
R1	AASPNYNVLY*			15.3	11.8
R2	AASPNYNVLY**				7.2
	AATNSGTYQR		1.1		3.7
	AASRGGRALI	0.9	24.7		3.5
	AARDRGSALGRLH		8.2		3.5
	AASAGNYQLI		0.9		3.3
	AAGNTNTGKLT			1.8	2.7
	AAAGNKLT		5.5		2.5
	AANTGNTGKLI		0.2		2.3
	AAKGTNAYKVI				2.3
	AASDRHMGYKLT		0.4		1.9
	AAKSGSFNKLT		0.2		1.7
	AARWNYNVLY				1.4
	AARDPYANKMI				1.4
	ATGSFNKLT				1.2
	AARRGNTGYQNFY				1.2
	AARPGNMGYKLT				1.2
	AARGGAGSWQLI		0.4		1.2
	AASAQPGTGSNRLT		0.7	3.2	1.0
	AARRVGSSGNKLI		0.4		1.0

RIP-mOVA+

clone	CDR a.a. sequence	no peptide Foxp3−	no peptide Foxp3+	OVA$_{323\text{-}339}$ Foxp3−	OVA$_{323\text{-}339}$ Foxp3+
R1	AASPNYNVLY*			0.6	50.8
R2	AASPNYNVLY**				14.4
	AASRGGRALI	0.4	25.1	2.0	6.0
	AATPNYNVLY				3.3
	AAAGNKLT		3.8		2.5
	AARQGSGSWQLI		2.7		1.2
	AATNSGTYQR		4.0		0.8
	AASKGGRALI		0.7		0.8
	AARARNTNKVV		4.6		0.8
	AARKWYNQGKLI		1.8		0.8
	AVPEGHQGGRALI				0.8
	AARGGAGSWQLI		1.1		0.6
R4	AAVPNYNVLY				0.6
	AANRNYKPT				0.6
	AAHRNYKPT				0.6
	AASAGNYQLI		0.7		0.4
	AAKSGSFNKLT		0.2		0.4
	AAGAYQGGRALI				0.4
	AARGYGSSGNKLI	0.2			0.4
	AAKGGSFNKLT		0.2		0.4

Foxp3−

RIP-mOVA−

clone	CDR a.a. sequence	no peptide Foxp3−	no peptide Foxp3+	OVA$_{323\text{-}339}$ Foxp3−	OVA$_{323\text{-}339}$ Foxp3+
R1	AASPNYNVLY*			15.3	11.8
	AASDYSNNRLT	29.3	0.2	11.5	
	AAIHQGGRALI	35.8	0.2	7.9	0.2
	AALYPSNTNKVV			4.5	
	AAKNNYAQGLT			4.1	0.2
	AASQGSGTYQR	6.7		4.1	
P1	AAPPNYNVLY			3.4	
	AASAQPGTGSNRLT		0.7	3.2	1.0
	AAGNTNTGKLT			1.8	2.7
	AATTNYAQGLT			1.8	
	AAFYPSNTNKVV			1.8	
	AAHYPPNTNKVV			1.6	
	AAFLTGNTGKLI			1.6	
	AARYNQGKLI	0.3		1.4	
	AASSQGTGSKLS			1.1	
	AASRRAGNYKYV			1.1	
	AASGLAGTYQR			1.1	
	AASGGSNYKLT		0.4	1.1	
	AALYPTNTNKVV			1.1	
	AAKWTGANTGKLT			1.1	

RIP-mOVA+

clone	CDR a.a. sequence	no peptide Foxp3−	no peptide Foxp3+	OVA$_{323\text{-}339}$ Foxp3−	OVA$_{323\text{-}339}$ Foxp3+
	AAIHQGGRALI	52.7	0.2	43.7	
	AASQGSGTYQR	7.0	0.5	11.4	
	AAMDMGYKLT	2.9	0.2	6.2	
	AAGRGGRALI	3.3		5.7	
	AATRGGRALI	4.1		5.5	
	AAMHQGGRALI	2.1		3.7	
	AASRGGRALI	0.4	25.1	2.0	6.0
	AAKQGGRALI	2.1		1.5	
	AAKWVGGADRLT	1.4	0.2	1.5	
	AAKKTGGYKVV			1.3	
	AALGNSGTYQR	1.2		1.3	
	AHPPPGTGSNRLT	0.4		1.1	
	AATYNTNTGKLT	1.0	0.5	0.9	0.2
	AAGNTNTGKLT	0.2		0.7	
	AANGGYKPT			0.7	
P1	AAPPNYNVLY			0.7	
	AASGGGGNYKYV	0.6		0.7	
	AASRRAGNYKYV			0.7	
	AASSQGTGSKLS	0.4		0.7	
R1	AASPNYNVLY*			0.6	50.8

Figure 2.3. Selection of possible OVA peptide-reactive TCRs using repertoire analysis

33
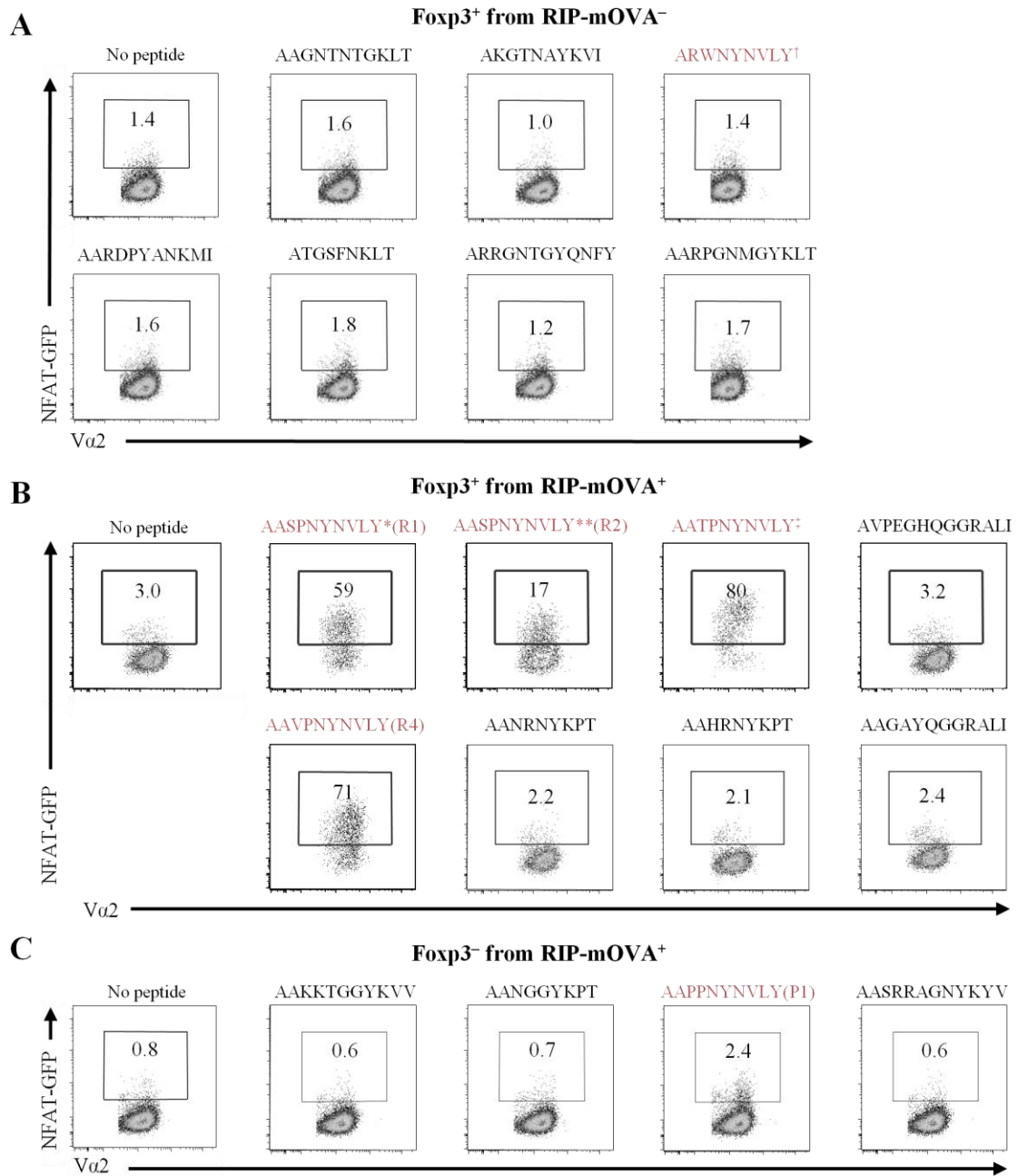
Figure 2.4. Confirmation of OVA peptide-reactivity

A

clone	CDR3 a.a. sequence	RIP-mOVA⁻		RIP-mOVA⁺	
		Foxp3⁻	Foxp3⁺	Foxp3⁻	Foxp3⁺
N6	AASRNYNVLY	0.5			
N7	AACPNYNVLY	0.2			
N8	AARWNYNVLY		1.4		
N9	AAAPNYNVLY		1.0		
N10	AARWPNYNVLY		0.4		
N11	AARSPNYNVLY		0.4		
N12	ATTPNYNVLY	0.5			
N13	AASKNYNVLY	0.2	0.2		
N15	AASGAGNYNVLY		0.2		
N16	AARVPNYNVLY		0.2		
N17	AASPLMSNYNVLY		0.2		
N18	AASKGLYNVLY		0.2		
N19	ARGANYNVLY		0.2		
R7	ATSPNYNVLY				0.4
R8	AASGPLSNYNVLY				0.4
R12	AASRDYNVLY				0.2
	Total number of sequences	443	484	545	486

B
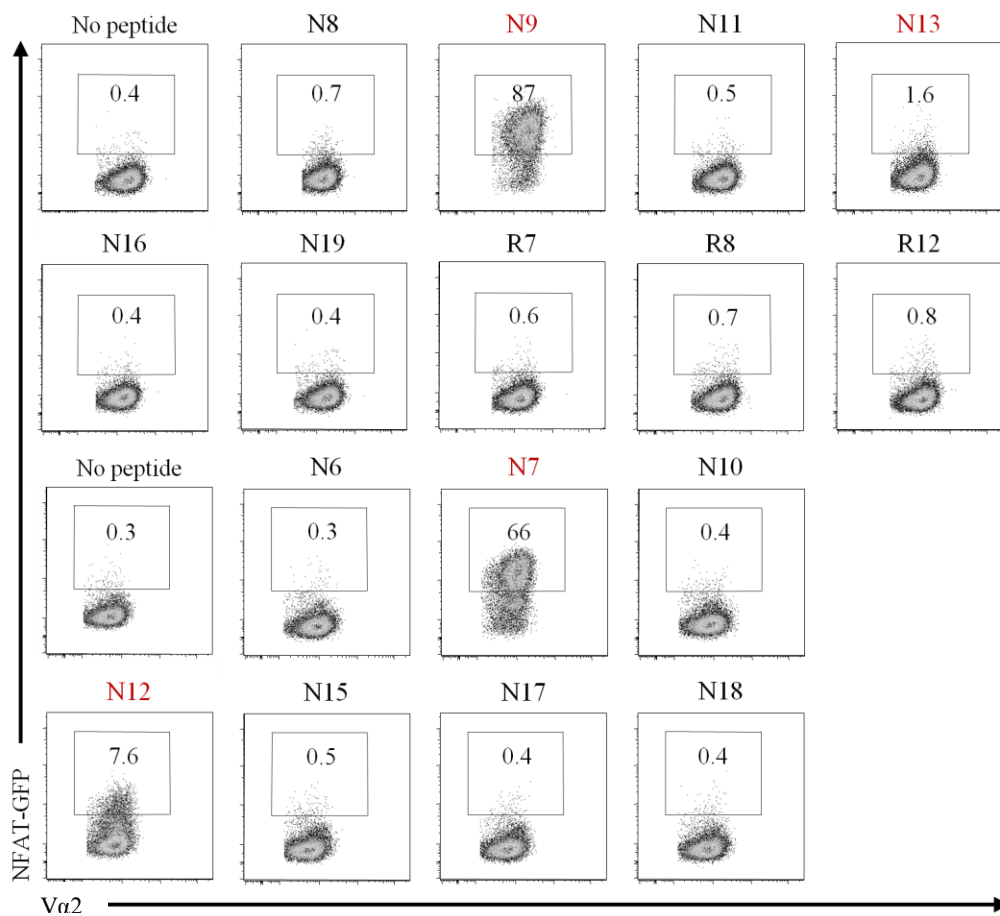
Figure 2.5. Screening of TRAJ21 containing TCRs

A

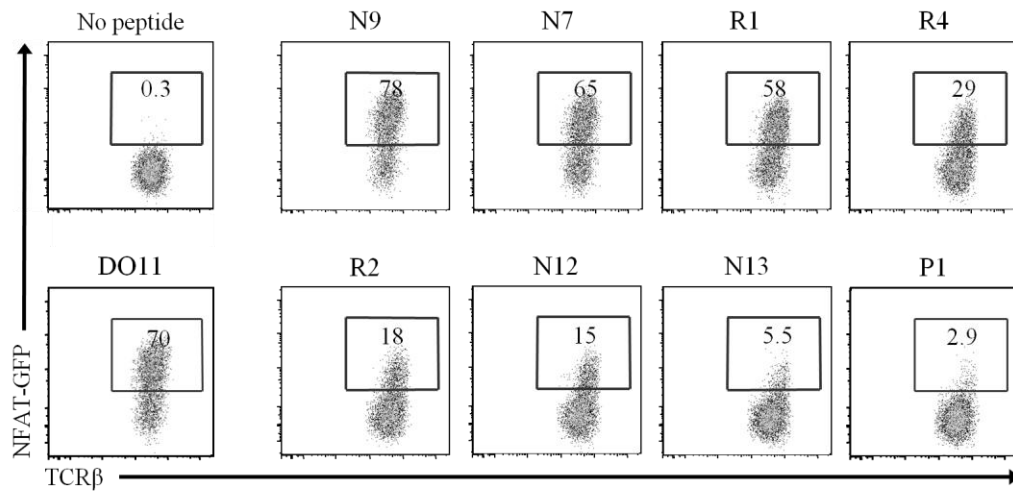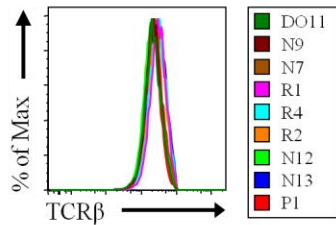| Clone	CDR3 a.a. Sequence	Vα usage	Δ Log (EC50) to DO11
N9	AA**A**PNYNVLY	TRAV14D-1*01	0.02 ± 0.1
DO11	AA**S**PNYNVLY	TRAV5D-4*01	0
N7	AA**C**PNYNVLY	TRAV14-3*01	-0.2 ± 0.2
R4	AA**V**PNYNVLY	TRAV14-3*01	-1.2 ± 0.5
R1	AA**S**PNYNVLY	TRAV14-3*01	-1.6 ± 0.1
N12	A**TT**PNYNVLY	TRAV14-3*01	-2.6 ± 0.2
R2	AA**S**PNYNVLY	TRAV14D-1*01	-2.6 ± 0.1
N13	AA**SK**NYNVLY	TRAV14-3*01	-3.5 ± 0.2
P1	AA**P**PNYNVLY	TRAV14-3*01	-3.7 ± 0.3

B

C

Figure 2.6. TCRs exhibit a broad range of reactivity to OVA peptide.
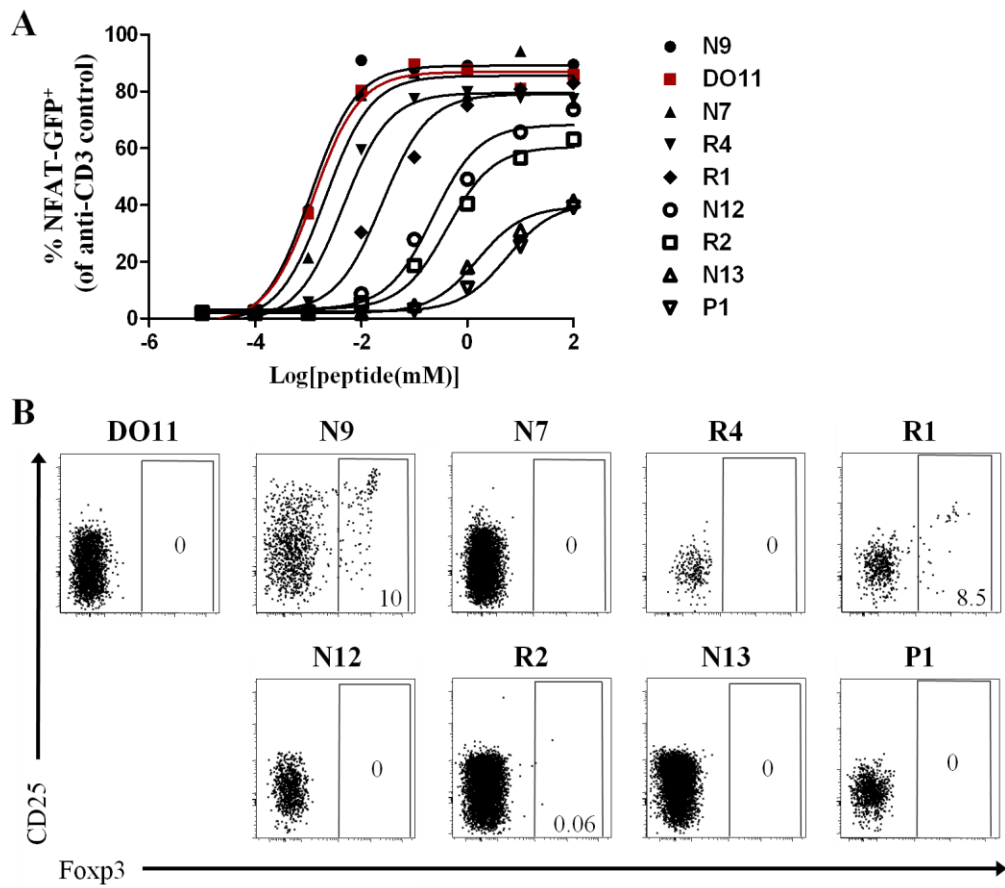
Figure 2.7. In vitro and in vivo analysis of OVA-reactive TCRs

Chapter 2.2

A role of TCR affinity in thymic tolerance mechanisms and self-reactive peripheral immune response

Results

TCR affinity is a primary determinant for thymic Treg cell development

To avoid the complication of OVA-independent Treg cell generation, I decided to focus on the 6 TCRs, including DO11, which did not facilitate thymic Treg cell development in wild-type mice. The differences between the 5 TCRs are only in the CDR3 amino acid sequence, as all of these TCRs use the same TRAV14-3*01 subtype. Because measurement of OVA reactivity so far was based a single readout using the NFAT-GFP reporter, I sought to expand the analysis of functional responses to TCR stimulation. I utilized another marker, NF-κB-GFP, which was introduced into the same parent hybridoma line used by NFAT-GFP [66]. Since this parent hybridoma line produced little IL-2, I tested IL-2 production after re-expressing these TCRs in a different TCR-deficient hybridoma line, 5KC-73.8.20 [74]. Although the range of measured EC_{50}s varied from ~50-5,000 fold depending on the assay, the ordering of TCRs in terms of efficiency of OVA recognition remained mostly constant (Figure 2.8A, Table 2.1).

Since TCR signaling may differ between hybridoma cells and primary T cells, I also assessed peptide reactivity using thymocytes and peripheral T cells. T cells expressing individual OVA peptide-reactive TCRs were generated using retroviral bone marrow chimeras using $Rag1^{-/-}$ donors. The EC_{50} for each TCR was determined in vitro

by assessing down-regulation of co-receptors CD4 and CD8 on $CD4^+CD8^+$ (double positive, DP) thymocytes, CD25 upregulation on $CD4^+CD8^-$ (CD4 single positive, CD4SP) thymocytes, and proliferation of peripheral $CD4^+$ T cells (Figure 2.8B). Similar to the results obtained using hybridoma assays, the OVA-specific TCRs showed a broad range of sensitivity such that the least reactive TCR P1 was ~300-6,000 folds less efficient than DO11.10 at recognizing OVA (Table 2.1). Thus, the data from 6 different hybridoma and primary T cell assays agree that these 6 TCRs exhibit a broad range of sensitivity for OVA recognition.

To estimate the TCR affinity for peptide:MHC, I measured the mean fluorescence intensity (MFI) of equilibrium tetramer binding, which provides a relative assessment of monomeric TCR:pMHC affinity [75-77]. Consistent with the functional assessment of OVA-reactivity, tetramer binding classified N7 and R4 as higher affinity TCRs (Figure 2.8C) in comparison with N12, N13, and P1, which showed little to no binding. Although the latter 3 TCRs can elicit in vitro responses to OVA-peptide, these TCRs are below the affinity range that can be quantified using this tetramer binding assay. Thus, these tetramer data suggest that this panel of TCRs recognize OVA peptide:MHC with a broad range of affinities.

I next assessed the ability of thymic OVA expression via RIP-mOVA to induce thymic Treg cell development using retroviral transduction of TCRs into $Rag1^{-/-}$ thymocytes followed by intrathymic transfer as described above. As expected, DO11 facilitated thymic Treg cell development in the presence of RIP-mOVA [78]. Interestingly, all of the other TCRs except for P1, the lowest-affinity TCR, induced Treg cells to a degree that varied with the efficiency of OVA recognition (Figure 2.9 A,B).

Treg cell generation could even be detected in cells expressing the low affinity TCRs N12 and N13, which could not be reliably measured by equilibrium tetramer binding, and require ~1200 fold (average of responses in 6 assays between P1 and DO11 in Table 2.1) more antigen to achieve the same response in comparison to DO11. However, I was unable to observe Treg cells generated by P1, which is very close to N13 in its sensitivity to OVA. Although stochastic expression of RIP-mOVA in thymic mTEC cells may contribute to mouse to mouse variability [79], differences between TCRs were readily observed. Thus, these data show that TCRs which are on average one hundred to one thousand fold less sensitive than DO11 can generate thymic Treg cells, demonstrating that Treg cell differentiation can occur within a broad range of self-reactivity.

Linear regression analyses for all assays revealed that the ability of TCRs to facilitate Treg cell generation is directly proportion to their affinity for OVA peptide as provided by RIP-mOVA (Figure 2.9C). The efficiency of self-recognition was also directly correlated with the generation of $Foxp3^-CD25^+$ cells (Figure 2.9D,E), consistent with the proposal that the process of thymic Treg cell generation occurs via TCR-dependent generation of $Foxp3^-$ Treg precursor cells [52]. Thus, these data demonstrate that the degree of self-recognition is the primary determinant for Treg cell differentiation in the thymus.

Treg cell selection niche is dependent on TCR affinity for self-antigen

Thymic Treg cell selection utilizes a small "niche" based on the observation of intraclonal competition in studies of transgenic mice using naturally arising Treg TCRs [39, 40]. Since the natural ligands for these Treg TCRs are unknown, the factors that

affect this developmental niche are unknown. I therefore asked whether affinity to self-antigen affects the size of the Treg cell developmental niche. Since the intrathymic transfer of retrovirally transduced thymocytes does not assess Treg cell generation at steady state, I decided to analyze retroviral bone marrow chimeras using the two highest affinity TCRs along with DO11. Various ratios of congenically marked wild-type bone marrow was added to achieve varying clonal frequencies. I confirmed that these OVA-reactive TCRs did not facilitate Treg cell generation in wild-type mice, even at low clonal frequencies (Figure 2.10A). Consistent with previous reports [39, 40], I observed that Treg cell development with all 3 TCRs was inversely correlated with clonal frequency in the presence of OVA peptide presentation (Figure 2.4A). Moreover, the efficiency of Treg cell generation was directly correlated with affinity, as evidenced by the ability of higher affinity TCRs to generate a higher frequency of Treg cells at a given clonal frequency (Figure 2.10B). The affinity of the TCR also correlated with the absolute number of Treg cells that are generated in the thymus, implying that higher affinity TCRs have a larger niche size for thymic Treg cell development (Figure 2.10C,D). Since the amount of antigen generated by the RIP-mOVA transgene should be equal between different bone marrow chimeras, I hypothesize that niche size in this case represents the number of APCs that present sufficient antigen to trigger Treg cell selection for a given TCR affinity.

Threshold for negative selection

Some studies using TCR transgenic models have reported negative selection to be coincident with Treg cell generation [32, 61], whereas other studies have suggested that

Treg cell selection may occur without obvious negative selection [39]. Thus, the level of self-reactivity requwered to elicit Treg cell differentiation versus negative selection remains poorly defined.

An interesting observation from our studies of clonal frequency and Treg cell development was that the frequency of Treg cells using the higher affinity TCRs, DO11 and N7, actually Int down again at clonal frequencies below 0.1% (red box, Figure 2.10A). This was not apparent for the intermediate affinity TCR R4, nor for other TCRs tested in previous reports [39], although it remains possible that this could be observed if loIr clonal frequencies could be achieved experimentally. One possible explanation for this observation is that TCRs with high affinity to antigen can induce negative selection rather than Treg cell differentation at low clonal frequencies. In this case, the remaining Foxp3$^-$ CD4SP cells have presumably have not yet encountered antigen.

To test the hypothesis that negative selection to RIP-mOVA occurs with T cells expressing TCRs N7 and DO11, but not R4, I assessed whether purified CD4SP thymocytes from retroviral bone marrow chimeras in wild-type hosts would be deleted after intrathymic transfer into RIP-mOVA$^+$ recipients. At day 3 post-transfer, I observed that the relative ratio of thymocytes expressing the higher affinity TCRs DO11 and N7, but not R4, to the co-injected polyclonal thymocytes Were decreased in RIP-mOVA hosts, indicative of negative selection (Figure 2.11). Consistent with previous reports [63], these data suggest that the propensity to induce Treg cell differentiation does not always rescue the cells from negative selection. Since R4 is approximately 15-fold (average of 6 assays, Table 2.1) less efficient at recognizing OVA than DO11, these data

suggest that detectable negative selection occurs at a substantially higher affinity threshold than Treg cell selection.

Differential affinity thresholds for thymic Treg cell development and peripheral responses to self

These studies in the thymus suggest that a broad range of TCR affinity can elicit Treg cell development, albeit at varying degrees. As the affinity for self-antigen decreases, the percentage of Foxp3$^-$ conventional T cells emigrating from the thymus increases. Thus, understanding the relationship between the range of affinities that facilitate thymic Treg cell generation versus peripheral responses is important for clarifying the how self-reactive Treg cells may mediate self-tolerance in the periphery.

To address this question, I adoptively transferred peripheral Foxp3$^-$ CD4 T cells bearing OVA-reactive TCRs into sub-lethally irradiated RIP-mOVA recipients, as no proliferative responses were observed in non-irradiated mice. Intriguingly, the intermediate affinity TCR R4 or low affinity TCR N12 did not respond to OVA in vivo under these conditions (Figure 2.12 A, B). By contrast, the higher affinity TCRs DO11 and N7 induced peripheral expansion of Foxp3$^-$ cells as well as a small amount of Treg cell differentiation (Figure 2.12 A, B). Consistent with previous observations [80], peripheral Treg cell generation occurred on cells with less proliferation (Figure 2.12 C). Interestingly, the affinity threshold for response to this peripheral self-antigen is similar to that of thymic negative selection, whereas the threshold for thymic Treg cell development is almost 100 fold lower (average R4 versus P1 sensitivity to OVA, Table 2.1). Thus, these data suggest a model where thymic Treg cell differentiation is tuned

such that T cells with sufficient self-reactivity to elicit peripheral immune responses cannot wholly escape the thymus as effector cells, and will always be accompanied by thymically generated Treg cells (Figure 2.1C).

Figure Legends

Figure 2.8. Defining efficiency of OVA recognition using hybridoma and primary T cells.

(A) Hybridoma assays. Hybridoma cells expressing OVA-specific TCRs were assessed for NF-κB-GFP expression (left) and IL-2 production (right) in response to varying amounts of OVA peptide presented by TA3 APCs. (B) Assays of TCR reactivity using primary thymocytes and peripheral T cells. T cells were obtained from retroviral bone marrow chimeras using wild-type hosts. DP thymocytes were assessed for down-regulation of CD4 and CD8 coreceptors in response to Flt3L DCs and varying concentrations of OVA peptide (left). CD4SP thymocytes were tested for CD25 upregulation (middle). Finally, peripheral CD4$^+$ T cells were tested for in vitro proliferation (right). Non-linear regression analysis and calculation of EC$_{50}$ was performed as per Figure 7a, and summarized in Table 2.1 as ΔEC$_{50}$. Graphs shown are representative of three independent experiments. (C) Binding of OVA:I-A^d tetramer to hybridoma cells expressing OVA-reactive TCRs. Representative plots are shown on the left, and summarized on the right (mean ± s.d., n=3 independent experiments). 3K:I-A^b tetramer was used as a control.

Figure 2.9. Thymic Treg cell generation is instructed by the level of TCR reactivity to self-antigen.

(A) Flow cytometry of Treg cell generation by OVA-reactive TCRs in the presence of RIP-mOVA. Retrovirally transduced *Rag1*$^{-/-}$ DN cells were transferred into thymus of RIP-mOVA mice, and were analyzed at 2 weeks as in Figure 2.7 B. (B) Data

in (A) are summarized, with each dot representing the frequency (left) and absolute number (right) of Foxp3$^+$ cells from an individual recipient. The plot in the middle shows the mean Foxp3$^+$ percentage ($\pm$ S.E.M.) correlated with the ΔLog(EC_{50}) of the TCR for NFAT-GFP activation as compared with DO11. Foxp3$^+$ cells were not detected (n.d.) with the P1 TCR. (C) Correlation of Treg cell generation with in vitro sensitivity to OVA. To determine whether TCR affinity is directly correlated with the efficiency of Treg cell selection in vivo, I plotted the in vivo efficiency of Treg cell generation versus the ΔLog(EC_{50}) of the TCR as compared with DO11, and analyzed it by linear regression. For tetramer binding, I used the Log(MFI) as compared with DO11. Each symbol represents an individual TCR indicated in the legend. (D) The frequency of Foxp3$^-$ CD25^{hi} CD4SP cells in the experiments described in (A) are shown (mean $\pm$ s.d., 5 independent experiments). (E) Correlation of Foxp3$^-$CD25^{hi} CD4SP cells with sensitivity of TCR to OVA peptide. Frequencies of Foxp3$^-$CD25^{hi} cells shown in (D) were plotted as per (C).

Figure 2.10. A role for TCR affinity in the thymic Treg cell selection "niche"

(A) Inverse relationship between clonal frequency and thymic Treg cell development. Thymic Treg cell development was assessed in mixed bone marrow chimeras with varying ratios of wild-type to retrovirally transduced bone marrow. Data shown are the percentage of Foxp3$^+$CD4SP cells versus the clonal frequency in the CD4SP subset for the indicated TCR. Each symbol represents data from an individual recipient from 2-3 independent experiments for each TCR. Data points in the dashed red boxes fall outside of the previously described inverse relationship. (B) Data from the

experiment shown in (A) are plotted log-log to illustrate the similar slopes, with differences in the intercept. Note that the points in the red boxes are not shown in this plot. (C) Absolute numbers of Treg cells from the data shown in (B) are plotted versus clonal frequency. (D) Correlation of Treg cell selection niche size to TCR affinity. The number of Foxp3$^+$ cells was analyzed by linear regression with OVA-reactivity measured by NFAT activation (left) and tetramer binding (right) as per Figure 2.9. Each symbol represents an individual TCR as indicated in the legend.

Figure 2.11. Treg cell development coincident with negative selection by high affinity TCRs

(A) Assessment of negative selection of CD4SP thymocytes. OVA-reactive CD4SP thymocytes and Cell-tracker violet labeled WT cells were intrathymically injected into wild-type and RIP-mOVA mice. Flow cytometry was performed 3 days later. Representative FACS plots are shown discriminating the injected wild-type and OVA-specific CD4SP cells marked as blue numbers (left), and the TCR transduced population (right). No Treg cells were observed in wild-type recipients for all TCRs tested (not shown). (B) Negative selection as assessed by the ratio of OVA-specific CD4SP cells to WT cells added as an injection control. Each dot represents an individual recipient, with 2 independent experiments per TCR. Statistical differences were accessed by unpaired t test. (C) Summary of negative selection experiment in (B). The percent difference in the mean values from wild-type hosts compared with RIP-mOVA hosts is shown for each TCR.

Figure 2.12. High affinity TCR recognition of peripheral self-antigen is required to elicit peripheral T cell responses.

(A) Assessment of peripheral T cells responses. Naïve peripheral Foxp3$^-$CD4$^+$ T cells were intravenously transferred into sublethally irradiated RIP-mOVA hosts. Preliminary experiments did not reveal proliferation in non-irradiated hosts. Representative flow cytometry plots are shown of the transferred splenic T cells after 14 days to determine proliferation via dilution of cell-tracker violet dye. (B) Summaries of frequencies of proliferated (left) and Foxp3$^+$ (right) cells–see gating in (A). Each dot represents data from a single recipient, with 2 independent experiments per TCR. (C) Peripheral conversion is inversely correlated with proliferation. Dilution of cell-tracker violet is shown for Foxp3$^+$ (dark line) and Foxp3$^-$ (grey shading) cells.

Clone	CDR3 a.a Sequence	Hybridoma[†]			T cells[†]			Tetramer binding[‡]	Thymic Selection
		NFAT activation	NFκB activation	IL-2 production	CD4/8 on DP	CD25 on CD4SP	CD4 T cell proliferation		
DO	AA**S**PNYNVLY								Neg./Treg
N7	AA**C**PNYNVLY	-0.2±0.2	-0.3±0.1	-0.1±0.1	-0.8±0.2	-0.9±0.6	-0.7±0.2	-0.30±0.03	Neg./Treg
R4	AA**V**PNYNVLY	-1.2±0.5	-0.5±0.2	-0.7±0.2	-0.9±0.1	-1.6±0.6	-1.3±0.5	-0.84±0.04	Treg
N12	A**TT**PNYNVLY	-2.6±0.2	-1.3±0.5	-1.5±0.3	-1.7±0.2	-2.3±0.7	-2.1±0.6	≤-1.30±0.05	Treg
N13	AA**SK**NYNVLY	-3.5±0.2	-1.6±0.3	-2.2±0.4	-2.0±0.2	-3.0±0.9	-3.8±0.3	≤-1.35±0.04	Treg
P1	AA**P**PNYNVLY	-3.7±0.3	-1.7±0.3	-2.7±0.2	-2.5±0.3	-2.1±0.5	-3.0±0.4	≤-1.34±0.05	Tconv

[†] The relative sensitivity of OVA recognition is shown as the difference of Log (EC_{50}) from that of DO11.

[‡] The relative affinities of TCRs are shown as the difference of Log (MFI) from that of DO11.

Data shown are mean ± s.e.m. (n=3).

Thymic selection is based on in vivo assays presented throughout the paper. Neg., negative selection. Treg, Treg cell selection is observed.

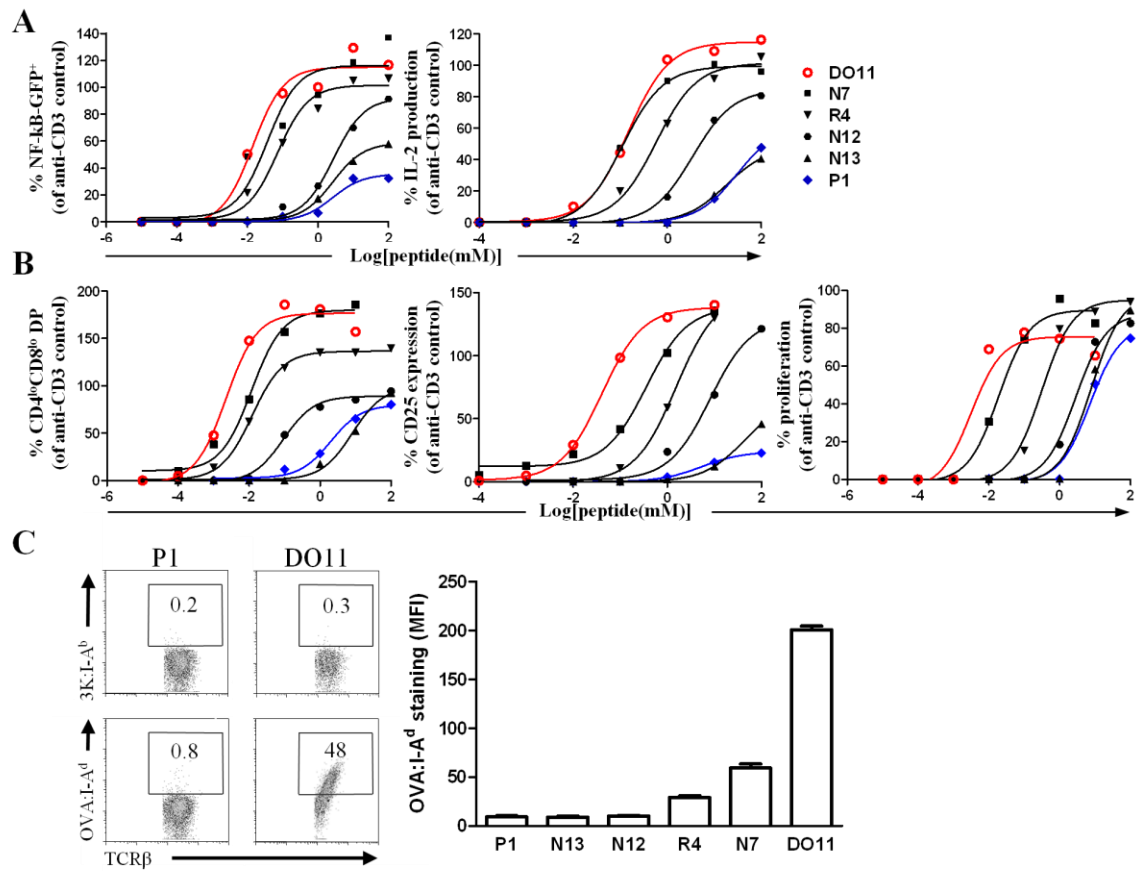**Table 2.1. Summary of assessments of TCR reactivity**

Figure 2.8. Defining efficiency of OVA recognition using hybridoma and primary T cells
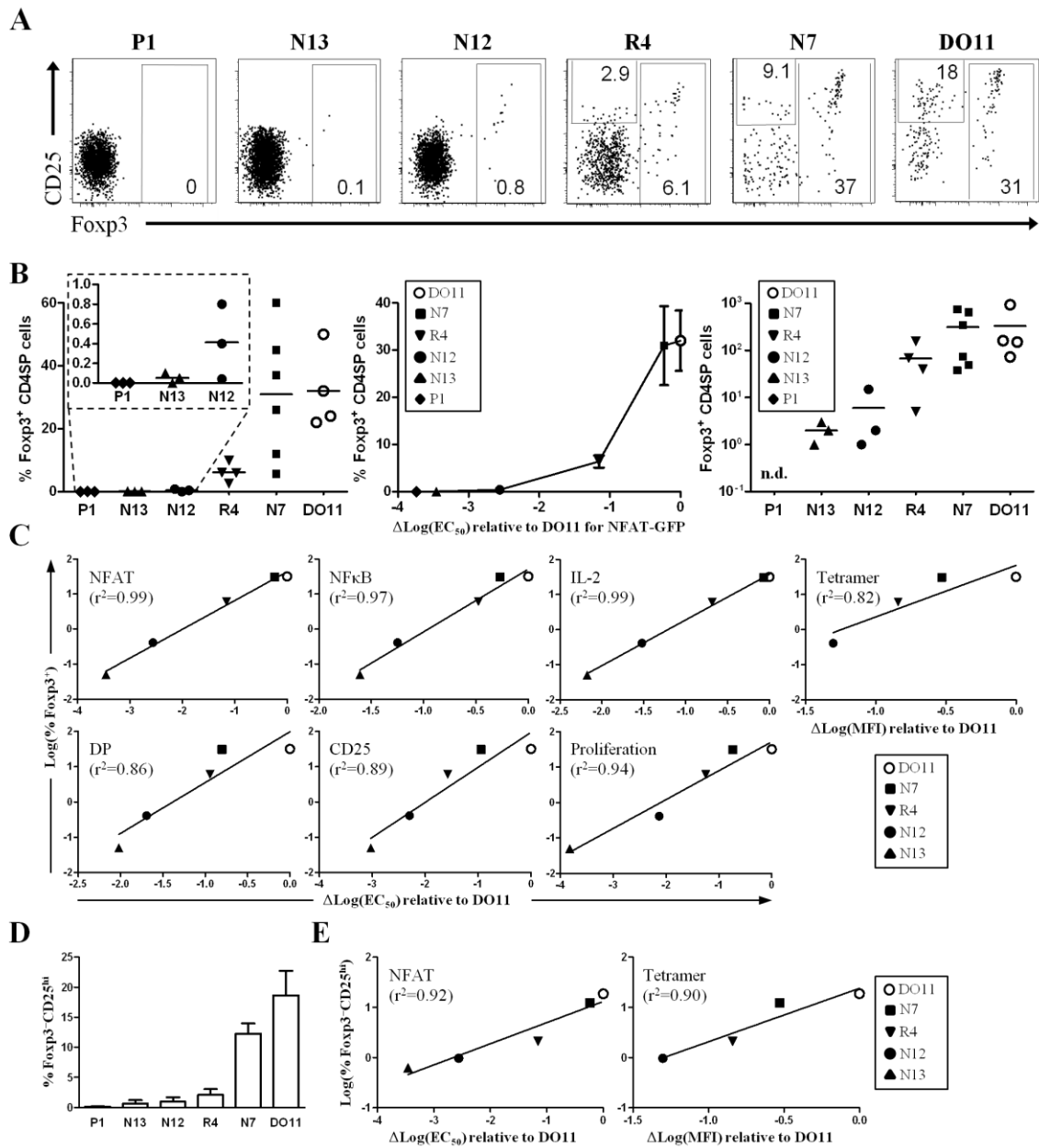
Figure 2.9. Thymic Treg cell generation is instructed by the level of TCR reactivity to self-antigen.
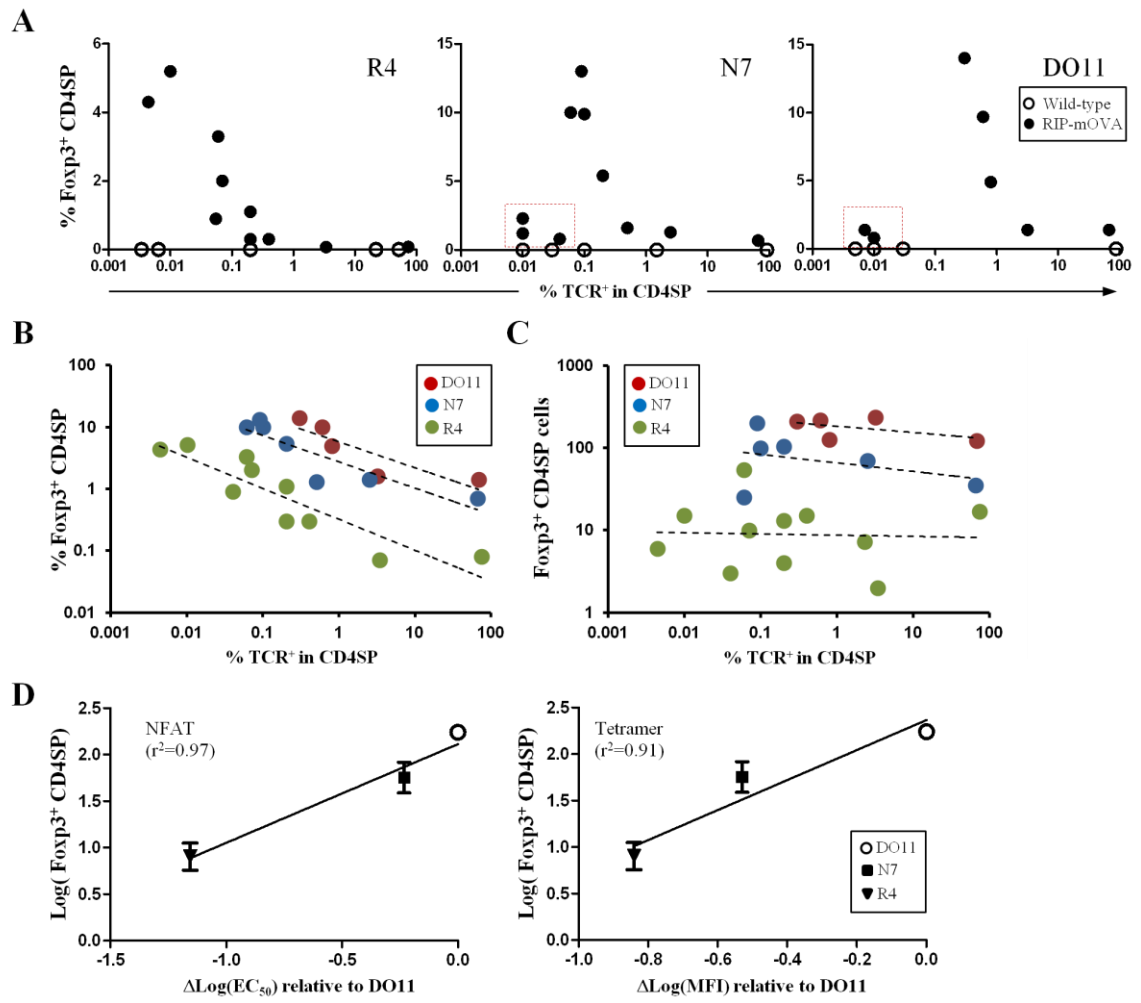
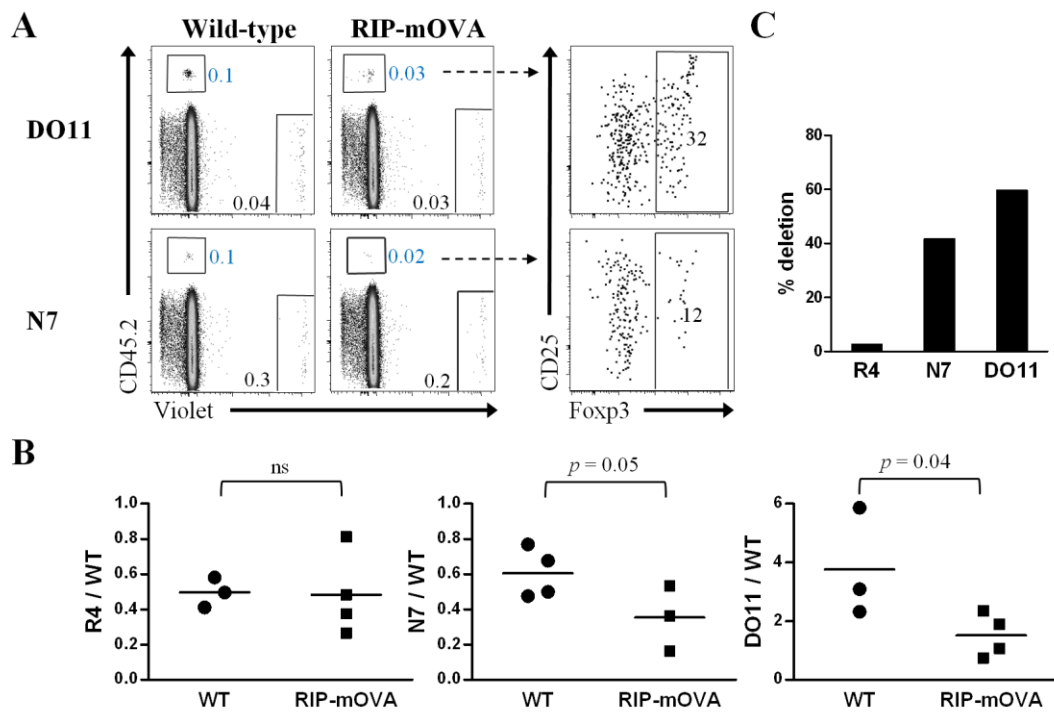**Figure 2.10. A role for TCR affinity in the thymic Treg cell selection "niche"**

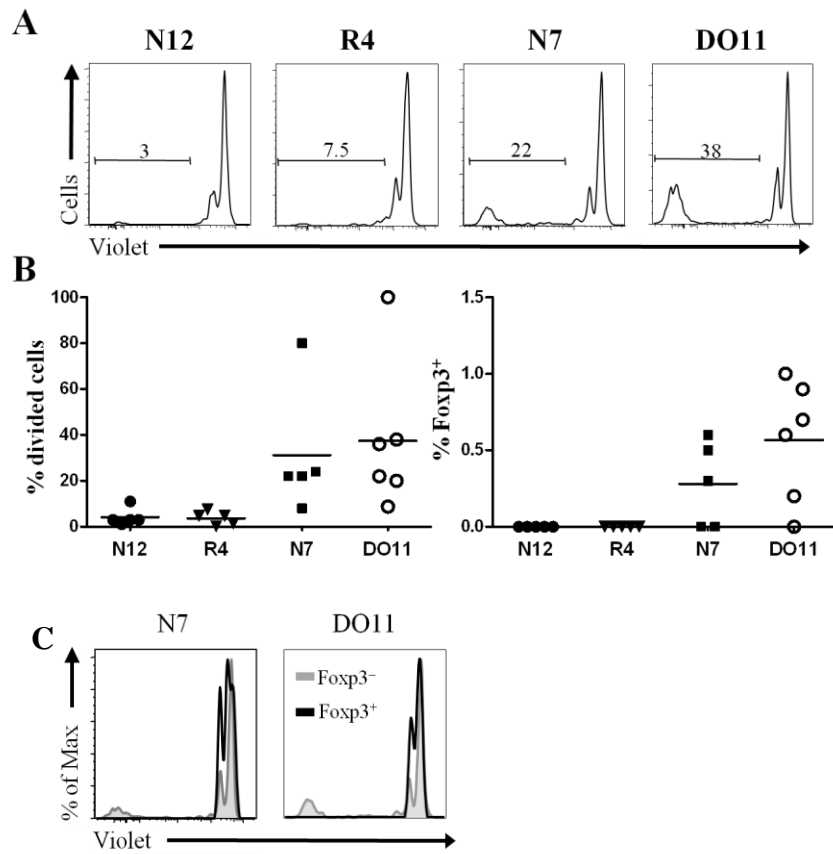**Figure 2.11. Treg cell development coincident with negative selection by high affinity TCRs**

Figure 2.12. High affinity TCR recognition of peripheral self-antigen is required to elicit peripheral T cell responses.

Discussion

Although previous studies provided proof of principle that self-reactivity is an important factor for thymic Treg cell differentiation and negative selection, there was little sense of how much self-reactivity is required at low clonal frequencies. Quantification of the relationship between self-reactivity and thymic Treg cell selection is important for understanding how thymic Treg cells may provide tolerance in the periphery. A high threshold of self-reactivity for Treg cell differentiation [63] would predict that many low-affinity self-reactive T cells would escape the thymus as effector cells, relying primarily on the presence of high affinity self-reactive Treg cells to provide tolerance. By contrast, a low threshold of self-reactivity for Treg cell differentiation would broaden the Treg cell repertoire such that it may almost approximate that of the effector cell repertoire [37], increasing the likelihood that thymic Treg cells will participate in not only immune responses to self, but also non-self antigens. Thus, understanding the relationship between self-reactivity and thymic education mechanisms would be useful to discriminate between these potential models for establishing self-tolerance.

I addressed this question by analyzing a panel of TCRs with different affinities for OVA peptide:MHC molecules for their in vivo response to the RIP-mOVA model antigen. While using a single antigen transgenic line has technical advantages as it should fix the amount and distribution of the antigen, another important consideration is that it models a tissue specific, rather than ubiquitous, antigen. Whereas T cell autoimmunity to ubiquitous antigens is not well described, tissue specific antigens are likely targets in a number of human autoimmune diseases such as Hashimoto's thyroiditis and type 1

diabetes. Thus, understanding tolerance to a model tissue specific antigen may be clinically relevant.

These data suggests that tolerance mechanisms to tissue specific antigens expressed in the thymus are broad and robust. I was surprised to observe that Treg cell development to this model tissue-specific self-antigen still occurs with TCRs which are ~ 1000 fold less sensitive to OVA peptide in comparison with the reference TCR DO11, which represents a typical agonist interaction with foreign antigen. Although I did not perform a direct measurement of affinity, the steady state tetramer binding studies in conjunction with the broad range of functional assessments of TCR reactivity provide a clear estimate of relative affinity for this panel of TCRs compared with the DO11 TCR. It is interesting to contrast these results from analysis of negative selection of a $CD8^+$ TCR, in which a narrow affinity range was observed [13]. Whether this is explained by differences in the assays used, or whether this reflects a fundamental difference between T cells at different stages of development or MHC restriction, will need to be clarified by future studies. None-the-less, in contrast with CD8 negative selection, the range of affinity that the immune system considers of sufficient self-reactivity to warrant thymic Treg cell generation is extremely broad.

I also observed that the level of self-reactivity is directly correlated with the efficiency of thymic Treg cell generation. In fact, thymic Treg cell development appeared indistinguishable from any other in vitro or in vivo TCR-dependent process. These data therefore support the notion that one of the primary purposes of TCR activation this particular stage in development is Treg cell generation, which appears to

wane during T cell maturation [59, 81]. Thus, the TCR's autoimmune potential dictates its ability to generate thymic Treg cells.

The relationship between TCR affinity and the efficiency of thymic Treg cell generation is also manifest in the apparent "niche" size, e.g. the number of Treg cells generated, even though the physical antigen niche is likely stable due to the use of single transgenic line to express OVA as "self-antigen." This may arise via two possible mechanisms. One is that the stronger the TCR signal is interpreted in an analog manner increasing the likelihood that Foxp3 will be induced. Alternatively, the TCR signal is interpreted in a digital manner such that only APCs that present sufficient antigen can induce Foxp3 by exceeding a threshold of TCR signaling. Increased affinity would therefore expand the number of APCs that could generate sufficient TCR signals. Although direct evidences to test these hypotheses are required, the former model would predict that TCR affinity would affect the slope of Treg cell generation as clonal frequency decreases. Rather, I observed that TCR affinity shifted the entire curve with almost same slope, such that higher TCR affinities resulted in enhanced Treg cell generation at higher clonal frequencies, suggestive of a larger developmental "niche." Moreover, a recent study of TCR activation using a Nur77-GFP reporter is consistent with a signaling threshold for Treg cell development [43]. Thus, I favor the interpretation that higher TCR affinity increases the number of APCs that can deliver a threshold level of TCR stimulation.

Although the affinity of the TCR for self-antigen and the Treg cell niche size is directly correlated, this may only be true at higher clonal frequencies for TCRs with agonist level affinity for self-antigen. It appears that negative selection likely perturbs

this linear relationship at lower clonal frequencies, as T cells encountering self-antigen are often deleted instead of becoming Treg cells. While it has been difficult to quantify the role of intraclonal competition for negative selection, this may be expected based on studies of Treg cell development [39, 40] and positive selection [50].

The threshold of observable negative selection appears to be substantially higher than that of Treg cell differentiation. However, it must be noted that the sensitivity of detecting Foxp3 induction as determined by the induction of Foxp3^{gfp} is excellent, whereas measuring the loss of cells is comparatively insensitive. It may be possible that negative selection occurs at lower affinity, but cannot be measured. However, trace levels of negative selection is unlikely to have any impact on immune tolerance, as essentially the same number of self-reactive T cells escapes the thymus, whereas a small amount of Treg cell development may have a substantial impact due to the importance of Treg cells in dominant tolerance.

Thymic Treg cell generation may be considered a window into how the immune system perceives the need to combat self-reactivity during development prior to the export of cells into the periphery. In comparison with thymic responses, I found that the TCR affinities requwered for peripheral T cell responses were much higher than that requwered for thymic Treg cell selection, and rather more similar to that requwered for negative selection. While these data wwell need to be validated for other model self-antigens, and if possible, true self-antigens if they can be genetically changed without affecting function, this suggests that the range of self-reactivity requwered for Treg cell selection is substantially below that requwered for peripheral T cell responses.

In summary, these data using a model tissue specific antigen suggest that the immune system utilizes straighforward rules for determining the thymic Treg cell population. Self-reactivity within a broad range, but above that for positive selection, selects for Treg cells in a graded manner depending on the extent of self-reactivity. Cognate, or agonist levels of self-reactivity also result in negative selection. In this manner, the immune system selects for a Treg cell population which is highly tuned towards high affinity self-antigen recognition while including some low affinity TCRs. Together with deletion of high affinity TCRs, these rules imprint the self-antigen repertoire on the Treg cell population. Interestingly, I found that incidental self-reactivity can also clearly generate Treg cells to foreign antigens, which is consistent with studies showing that a small fraction of cells binding to tetramers of foreign antigens are Treg cells [82]. Thus, self:non-self discrimination likely revolves around the quantitative bias of the TCRs amongst the Treg and naïve T cell population.

These data clarify how self-reactivity in the CD4$^+$ T cell subset is constrained by the immune system. Based on these data, I would predict that the bulk of the TCRs that cause autoimmune disease would be of higher affinity than R4, which is ~ 15 fold less sensitive than DO11. These TCRs are low enough affinity to avoid marked negative selection and incompletely drive thymic Treg cell selection, permiting the escape of self-reactive naïve T cells [16]. Under normal circumstances, it is difficult to imagine that these escaped self-reactive T cells could overcome the suppression by the thymic Treg cell population concomitantly generated in response to a broad range of self-reactivity. It seems that pro-inflammatory conditions that obviate or block Treg cell suppression would be requwered to release these potential autoimmune effector cells. Such an

inciting event is often not obvious in spontaneous human autoimmunity. One interesting future question is whether the B6 genotype studied here has an unusually low threshold for thymic Treg cell generation, as it is known to be resistant to spontaneous autoimmunity. Perhaps other autoimmune prone backgrounds wwell requwere a higher threshold of self-reactivity for thymic Treg cell generation. Alternatively, these robust thymic mechanisms may hint at the possibility that spontaneous autoimmunity may not result from a failure of central tolerance, but rather occurs to antigens that are poorly expressed in the thymus. For example, no indication was found in mixed bone marrow chimeras that the self-reactive TCR 2D2 induces thymic Treg cell generation even at low clonal frequencies (not shown), suggesting that the MOG-epitope is either not presented in the thymus or the affinity of the 2D2 TCR for MHC-MOG peptide complex is too low relative to the amount of thymic MOG-peptide. Future studies wwell be required to test the hypothesis that sponatneous autoimmunity is primarily a failure of peripheral tolerance.

Chapter 3

Rare development of thymic Treg cells at CD4$^+$CD8$^+$ subset

Introduction

Natural CD4$^+$Foxp3$^+$ Treg cells are essential for preventing autoimmunity [83]. Although peripheral generation of Treg cells has been demonstrated [84], recent studies suggest that the majority of peripheral Treg cells arise via thymic Treg cell development [33-35, 85]. One popular model for thymic Treg cell development is that Treg cells are selected based on TCR avidity for self-antigens at a level between positive and negative selection [61], which is supported by studies demonstrating that the thymic Treg and non-Treg TCR repertoires differ [33-35, 52]. Thus, TCR specificity for self-antigens appears to play an important role in Treg cell development.

As the APCs encountered by thymocytes can differ substantially between certain steps in thymic development [86, 87], the stage at which self-reactive thymocytes become Treg cells may play an important role in determining the TCR repertoire of the natural Treg cell population. DP cells are typically found in the thymic cortex, in which the predominant APCs are cortical thymic epithelial cells (cTECs). By contrast, mature CD4SP cells are typically found in the medulla, where medullary thymic epithelial cells (mTECs) and bone marrow derived dendritic cells are commonly found. These different APC subsets appear to utilize different lysosomal proteases for antigen processing, as cTEC use cathepsin L, whereas thymic dendritic cells and mTEC dominantly use cathepsin S [53]. In addition, expression of the transcription factor Aire expands the array of tissue-specific antigens presented by mTECs [88], which may be important for

Treg cell selection [46]. These data therefore suggest that the antigenic environment likely differs for DP versus CD4SP cells.

Several studies have demonstrated that Foxp3$^+$ Treg cell lineage commitment can occur at the DP stage of thymic development. First, Foxp3$^+$ DP cells can be found in TCR transgenic mice in which the T cells encounter their cognate antigen in the thymus [61, 89]. Second, cTEC-restricted expression of MHC class II is sufficient to induce the generation of Foxp3$^+$ T cells [55, 56]. Finally, analysis of *Foxp3*-knockin reporter mice revealed that Foxp3$^+$ cells can be found in the DP subset [19, 57, 65, 90], which has been reported to represent precursors to Foxp3$^+$ CD4SP cells [57]. The fraction of total Foxp3$^+$ thymocytes in the DP stage ranges between ~5 – 30% depending on the gating scheme and report [57, 65]. Thus, a substantial fraction of Treg cells acquire Foxp3 expression at the DP stage of thymic development.

I reasoned that the TCR repertoire of Foxp3$^+$ DP cells would be different from that observed for Foxp3$^+$ CD4SP cells [33-35] due to differences in antigen presentation at these stages of thymic development. However, highly purified populations of Foxp3$^+$ DP cells could not be obtained by flow cytometric sorting. Here, I show that this difficulty arises primarily from Foxp3$^+$CD4SP:Foxp3$^-$DP doublets being recognized as Foxp3$^+$ DP cells. Thus, our data demonstrate that the proportion of Foxp3$^+$ Treg cells in the DP stage is much lower than previously reported, and argue for a minor role for Foxp3-induction at the DP stage in the generation of the thymic Treg cell subset.

Materials and Methods

1. Mice

   Foxp3[gfp] reporter mice was kindly provided by Dr. Alexander Rudensky [65]; MHC class II deficient mice [28] were purchased from the Jackson Laboratory (Bar Harbor, ME); and B6.SJL CD45.1 congenic mice were purchased from the National Cancer Institute (Frederick, MD). Mice were analyzed at 6-9 weeks of age, housed in specific pathogen-free facility at Washington University, and used under protocols approved by the Animals Studies Committee.

2. Flow cytometry and FACS cell sorting.

   Monoclonal antibodies were purchased from eBioscience (San Diego, CA) and Biolegend (San Diego, CA). Flow cytometric analysis and sorting were performed using a FACSAria (Becton Dickinson, San Jose, CA) and FlowJo software (Tree Star, Ashland, OR). For routine flow cytometry, thymocytes were stained at 40×10^6 /ml in Ca^{++}/Mg^{++} free DPBS supplemented with 2.5% FCS and 0.02% sodium azide for 20 minutes on ice. Free antibody was removed by washing after centrifugation. To reduce cell:cell interaction in certain experiments, I stained in the presence of 1mM EDTA at 4℃ with constant rotation; and instead of washing after centrifugation, the cells were simply diluted 50 fold in PBS immediately prior to flow cytometry. To assess DNA content, thymocytes were stained with antibodies under the condition for routine flow cytometry, and fixed by 0.1% paraformaldehyde in DPBS for at least 2 hours. Fixed thymocytes were permeablized using 70% ethanol for propidium iodide staining.

3. Neonatal bone marrow chimera.

Bone marrow cells from *Foxp3*^{gfp} mice were T-cell depleted using AutoMACS magnetic bead removal of CD4 and CD8 labeled cells (Miltenyi Biotech, Auburn, CA). Ten million bone marrow cells were intraperitoneally injected into 2-day old mice.

Results

Flow cytometric analysis of Foxp3$^+$ DP thymocytes

Previous studies have demonstrated Foxp3$^+$ DP cells in both TCR transgenic and polyclonal settings [55, 56, 65, 90], with up to ~30% of Foxp3$^+$ thymocytes reported to be present in the DP subset [57]. I reasoned that Foxp3$^+$ DP and CD4SP cells may represent Treg cells selected on different arrays of peptide:MHC class II complexes presented on cortical versus medullary APCs, which could be revealed by a comparison of the TCR repertoires between DP and CD4SP Foxp3$^+$ cells. However, initial attempts to purify Foxp3$^+$ DP cells by our usual flow cytometric sorting parameters were hampered by low purity in the 40-60% range (Figure 3.1). Even double FACS purification did not result in a high purity sort (data not shown). This did not appear to be a mechanical limitation of the sorter, as Foxp3$^+$ CD8SP cells could be sorted with reasonable purity, even though they are at comparable frequency to Foxp3$^+$ DP cells.

Post-sort analysis of Foxp3$^+$ DP cells showed that a large portion of the contaminants were Foxp3$^-$ DP cells (Figure 3.1). This was expected since Foxp3$^-$ DP cells are by far the most frequent cell-type in the thymus. Surprisingly, many of the contaminants were Foxp3$^+$ CD4SP cells. Since these cells represent only ~0.3% of total thymocytes, this suggested that contamination was occurring in a non-random fashion. Because these contaminants Were present at a 1:1 ratio (Figure 3.1, bottom), this hinted at the possibility that many of the Foxp3$^+$ DP cells recognized by flow cytometry may represent doublets of Foxp3$^-$ DP and Foxp3$^+$ CD4SP cells.

To test this hypothesis, I performed additional flow cytometric analysis of Foxp3$^+$ DP cells. An appreciable frequency of Foxp3$^+$ in the DP subset was only observed only

when generous forward scatter-area (FSC-A) and side scatter-area (SSC-A) gating parameters were used (Figure 3.2A, large gate). Area parameters are derived from the integration of height and width parameters (e.g. FSC-H and FSC-W) as the cell passes through the detector. However, I found that most Foxp3$^+$ DP cells exhibited an exaggerated FSC-W compared to FSC-H. The same was true for SSC (data not shown). This traditionally implies that two cells serially passed the detector so close together that they could not be discriminated by the cytometer as individual cells. Rather, this event appears as a single large cell with increased FSC-A/SSC-A due to integration. Consistent with this interpretation, a smaller FSC/SSC gating scheme (small gate) decreased the frequency Foxp3$^+$ cells in the DP subset by approximately 20 fold, and eliminated most of the doublets by FSC-H/FSC-W criteria (Figure 3.2A). Another interesting observation is that the MFI of CD4 is approximately two-fold greater on Foxp3$^+$ DP cells compared with Foxp3$^-$ DP cells (Figure 3.3A). Taken together, these additional flow cytometric analyses support the notion that most Foxp3$^+$ DP events are comprised of doublets.

To assess whether doublet formation occurred in vitro during cell-surface labeling with fluorescent antibodies, I attempted to decrease cell-cell interactions by avoiding centrifugation and staining in the presence of 1mM EDTA. I found that the frequency of singlets in the large gate improved considerably, with a corresponding decrease in the frequency of DP cells in the Foxp3$^+$ subset (Figure 3.3B, Figure 3.5B). Thus, these data suggest that the majority of doublets observed during routine cell staining and processing for flow cytometry occur ex vivo.

To confirm the hypothesis that Foxp3$^+$ DP events identified by routine flow cytometry are comprised of Foxp3$^-$ DP cells adhered with Foxp3$^+$ CD4SP cells, I mixed

CD45.1 *Foxp3*^{gfp} thymocytes with CD45.2 *Foxp3*^{WT} thymocytes prior to staining and flow cytometry. While Foxp3$^+$CD4SP and Foxp3$^+$CD8SP cells appropriately expressed only CD45.1, CD45.2 staining was readily apparent on many Foxp3$^+$DP events (Figure 3.4A). Assuming that doublet formation was similar between CD45.1 and CD45.2 cells, this would imply that over 80% of Foxp3$^+$DP events identified under these conditions were comprised of doublets generated ex vivo. The efficiency of doublet formation in vitro was also dependent on cell concentration during staining (Figure 3.5). In addition, Foxp3$^+$ flow cytometric events with high DNA content were preferentially found in the DP subset (Figure 3.6). Thus, these data directly demonstrate that doublets are formed ex vivo and are preferentially found in the Foxp3$^+$DP gate.

Using this mixing assay to assess doublet formation, I then tested the efficiency of height by width parameters for "doublet discrimination" gating. I observed that the frequency of doublets markedly increased as the FSC-H size of the gate increases even if the FSC-W gating remains unchanged (Figure 3.4B, Figure 3.7). Furthermore, the increase in the frequency of Foxp3$^+$ cells in the DP gate (Figure 3.4C) parallels the frequency of doublets associated with larger FSC-H gates (Figure 3.4B). Conversely, the frequency of Foxp3$^+$ cells in the CD4SP subset decreases (Figure 3.4C). Thus, while "doublet discrimination" gating is useful to eliminate doublets which pass through the detector in series, it can be defeated by a generous FSC-H (and SSC-H) gate, thereby including doublets which pass through in parallel. Taken together, these data demonstrate that the frequency of DP cells in the Foxp3$^+$ subset is substantially less than previously reported [57], representing approximately 1% of Foxp3$^+$ thymocytes (Table 3.1).

Characterization of Foxp3⁺ DP thymocytes

As it is unclear whether previous studies excluded doublets from the analysis of Foxp3$^+$ DP events, I performed a flow cytometric characterization of Foxp3$^+$ DP cells using a small gate. One observation was that the levels of CD4 and CD8 are lower than those found on typical Foxp3$^-$ DP cells (Figure 3.2, small gate), suggesting that Foxp3$^+$ DP cells Were transitioning out of the DP stage after undergoing positive selection. This is consistent with the observation that Foxp3$^+$ DP cells are TCR^{hi} and express lower levels of HSA than Foxp3$^-$ DP cells (Figure 3.7). However, the HSA levels on Foxp3$^+$ DP cells were higher than those on Foxp3$^+$ CD4SP cells. In addition, Foxp3$^+$ DP cells show higher level of CD69 expression than Foxp3$^-$ DP, indicating that Foxp3$^+$ DP cells may have recently received a positively selecting TCR signal. In other respects, DP and CD4SP cells were similar, expressing Treg surface markers such as CD25, GITR, OX-40 and CD103 (Figure 3.8). Thus, these data suggest that Foxp3$^+$ DP cells have undergone positive selection and express traditional Treg cell markers.

Since most Foxp3$^+$ thymocytes are presumably MHC class II restricted as most reside in the CD4SP subset, I asked whether Foxp3$^+$ DP cells require MHC class II for their development. Consistent with a previous report [65], I observed a marked decrease in Foxp3$^+$ CD4SP cells and a increase in Foxp3$^+$ DP and CD8SP cells in *Foxp3*^{gfp} MHC class II deficient mice (Figure 3.9A). This was also reflected in an overall decrease in frequency of Foxp3$^+$ thymocytes (Figure 3.9B). Curiously, many Foxp3$^+$ DP cells in MHC class II-deficient mice in the large gate by FSC-A/SSC-A parameters show enhanced levels of CD8 compared to their counterpart in wild-type mice. Since these cells are not present when the small gate is used, they may represent doublets of Foxp3$^+$

DP or CD8SP cells with Foxp3$^-$ DP cells in MHC class II-deficient mice. Nonetheless, the presence of Foxp3$^+$ CD4^{lo}CD8^{lo} DP cells in MHC class II-deficient mice suggest that Treg cell selection can occur on MHC class I.

Developmental kinetics of Foxp3$^+$ thymocytes

A previous study of neonatal thymic development showed that Foxp3$^+$ DP and CD4SP cells appeared coincidently [19]. Since this study examined development in a neonatal and potentially lymphopenic thymic setting, it was possible that the thymic medulla was not fully mature [91-93]. I therefore analyzed T cell development in lymphoreplete thymuses using neonatal bone marrow chimeras. It took approximately 2.5 weeks post transfer of congenically marked bone marrow cells to see appreciable numbers of developing donor thymocytes (Figure 3.10A). I did not observe a period in which DP cells contributed a substantial fraction to the total thymic Foxp3$^+$ population (Figure 3.10B). Curiously, the frequency of Foxp3$^+$ cells with the CD4SP subset increased over a period of approximately 1.5 weeks until it reached steady state, consistent with a delay in Foxp3$^+$ cell development at the CD4SP stage (Figure 3.10C) observed in neonatal mice [19]. These data therefore illustrate that the temporal delay in the generation of Foxp3$^+$ as compared with Foxp3$^-$ CD4SP cells does not result from conditions unique to the neonatal thymus.

I also examined the kinetics of Foxp3$^+$ Treg cell development in the CD4SP stage. Although this time course analysis cannot prove precursor:product relationships, it appears that Foxp3$^+$ cells arise from the HSA^{hi} CD4SP subset before downregulating HSA (Figure 3.10D). Furthermore, Foxp3$^-$CD25$^+$ CD4SP cells, which have been

suggested to be enriched in thymic Treg cell precursors [52], are found initially at much higher frequency than Foxp3$^+$ cells, but decrease over time as the frequency of Foxp3$^+$ correspondingly increases (Figure 3.10E). Although correlative, these data imply that Treg cell development occurs at the HSA^{hi} CD4SP stage through a Foxp3$^-$ CD25$^+$ intermediate.

Figure Legends

Table 3.1. Summary of CD4/8 distribution of Foxp3$^+$ thymocytes

Data shown are the mean frequency of Foxp3$^+$ thymocytes which fall into the DP, CD4SP, and CD8SP gates as per Figure 3.2. EDTA indicates whether 1mM EDTA and avoidance of centrifugation was used to diminish cell:cell interactions. Large and small gates represent those shown in Figure 3.2.

Figure 3.1. Contaminants in sorted Foxp3$^+$ DP thymocytes

Foxp3$^+$ DP thymocytes were sorted from *Foxp3*^{gfp} mice and analyzed by flow cytometry.   Representative plots of two independent experiments are shown. Mean percentage (± S.D, n=2) of contaminants in sorted cells is derived from the post-sort gating scheme (%CD4SP x %Foxp3$^+$ and %DP x %Foxp3$^-$).

Figure 3.2. Most Foxp3$^+$ DP events by flow cytometry appear to be doublets.

(A) Flow cytometric analysis of Foxp3$^+$ thymocytes. Thymocytes from *Foxp3*^{gfp} mice were stained using our normal protocol and analyzed by flow cytometry. The large (dotted line) and small (black line) gates use the area parameter, which is integrated based on the height and width of the signal from an individual event. FSC-H/FSC-W plots are shown on the right for the indicated Foxp3$^+$ populations. Using these parameters, a doublet often appears to have a larger width than height, unless the doublet crosses the beam in tandem. Numbers in the FACS plots represents the frequency of the cells in the indicated gate. (B) Reduction in Foxp3$^+$ DP events using EDTA and avoidance of

centrifugation. Thymocytes from *Foxp3*^{gfp} mouse were prepared as described in the Methods, and analyzed as above.

Figure 3.3. Most Foxp3$^+$ DP events are composed of doublets.

(A) Foxp3$^+$ DP events are two-fold brighter for CD4 than Foxp3$^-$ DP events. Thymocytes from *Foxp3*^{gfp} mouse were prepared as described in the Methods, and analyzed as per Figure 3.2. Mean fluorescence intensity (MFI) of CD4 is shown for events in the large gate above from 3 independent experiments ($\pm$ S.D.). (B) Reduction in Foxp3$^+$ DP events using EDTA and avoidance of centrifugation. Representative flow cytometric plots analogous to those in Figure 3.2 are shown. (C) Summary of singlet frequencies in Foxp3$^+$ DP, CD4SP and CD8SP subsets using the large FSC-A/SSC-A gate above. Each dot represents data from an independent experiment.

Figure 3.4. Doublets in the Foxp3$^+$ DP thymocyte subset

(A) Foxp3$^+$ DP doublets are formed during staining. CD45.2 *Foxp3*^{gfp} thymocytes were mixed with CD45.1 *Foxp3*^{WT} thymocytes at 1:1 ratio, stained using the normal protocol, and analyzed by flow cytometry using a large FSC-A/SSC-A gate (see Figure 3.2). Data shown are gated on Foxp3^{gfp+} cells and as indicated above the plots, and a representative of two independent experiments. The numbers represent the frequency of the cells in the quadrant. (B) "Doublet discrimination" gating is imperfect for eliminating doublets. Thymocytes in the large FSC-A/SSC-A gate as in (A) were further analyzed using gates with small FSC-W and different FSC-H for the frequency of CD45.1$^+$CD45.2$^+$ doublets. Representative plots and quantification of doublets are shown

according to FSC-H value. (C) Frequency of Foxp3$^+$ DP cells is proportional to doublet formation. The mean frequencies of DP and CD4SP in Foxp3$^+$ thymocytes from each FSC-H gate are plotted.

Figure 3.5. Increased cell concentration during staining enhances the frequency of Foxp3^{gfp+} DP events.

Thymocytes were stained as per Figure 3.4 at the indicated cell concentration and analyzed by flow cytometry. The mean percentages of Foxp3^{gfp+} DP cells which are CD45.1$^+$, and the frequency of Foxp3^{gfp+} cells which fall in the DP gate, are shown from 4 independent experiments (± S.D.).

Figure 3.6. Frequency of Foxp3$^+$ DP cells increases with DNA content.

Thymocytes were fixed and stained for DNA content as described in the Material and Methods section. DNA content was assessed by flow cytometry of PI staining using a large FSC-A/SSC-A and Foxp3$^+$ gate. Data shown are representative of 2 dependent experiments.

Figure 3.7. Doublet-discrimination gating decreases the frequency of Foxp3^{gfp+} DP events.

Thymocytes were stained as per Figure 3.4 and analyzed by flow cytometry. The plot on the left was gated using a large FSC-A/SSC-A gate. On the right, the mean

percentages of CD45.1$^+$CD45.2$^+$ doublets and DP subset in Foxp3^{gfp+} cells in the indicated FSC-H/FSC-W gates are shown from 3 independent experiments (± S.D.).

Figure 3.8. Foxp3$^+$ DP and CD4SP cells express similar cell surface markers.

Thymocytes from *Foxp3*^{gfp} mice were analyzed for cell surface expression of indicated markers on Foxp3$^-$ and Foxp3$^+$ DP cells; and Foxp3$^-$ and Foxp3$^+$ CD4SP cells as indicated using small FSC-A/SSC-A and doublet discrimination (width/height) gates as per Figure 3.2. Data are representative of 4 mice analyzed.

Figure 3.9. Foxp3$^+$ DP cells can be generated by MHC class I.

(A) Development of Foxp3$^+$ cells in MHC class II-deficient mice. Foxp3$^+$ thymocytes from wild-type and MHC class II-deficient mice were analyzed using FSC-A/SSC-A large (dotted line) and small gates (black line). Plots shown are representative of three independent experiments. (B) Reduction of Foxp3$^+$ thymocytes generation in the absence of MHC class II. The mean percentages of Foxp3$^+$ in thymocytes from wild-type and MHC class II-deficient mice are shown.

Figure 3.10. Kinetics of thymic regulatory T cell development

(A) Delay in generation of Foxp3$^+$ cells in lymphoreplete thymuses. CD45.2 *Foxp3*^{gfp} T cell-depleted bone marrow cells were intraperitoneally injected into 2-day old CD45.1 neonates, and donor-derived thymocytes were analyzed by flow cytometry at the indicated age. Data shown are gated on DP or CD4SP cells, and are representative of

three independent experiments with at least two mice per time point. (B) Foxp3$^+$ CD4SP and DP Treg cells are coincidentally observed. Mean percentage of cells in the various CD4/CD8 subsets amongst the Foxp3$^+$ thymocyte population are plotted. At 2.5 weeks, too few Foxp3$^+$ events were recorded to allow meaningful comparisons (N/A). (C) Summary of time course of Foxp3$^+$ cell generation. The frequency of Foxp3$^+$ cells within the DP or CD4SP subset is plotted with respect to time after bone marrow transfer. Data shown are mean ±S.D. (n = 3). (D) Appearance of HSA^{hi} Foxp3^{int} cells during the initial wave of Treg cell development after bone marrow transfer. Data shown are representative flow cytometric plots gated on CD45.1$^-$CD45.2$^+$ CD4SP cells. (E) Foxp3$^-$CD25$^+$ and Foxp3$^+$ CD4SP cell frequencies are inversely correlated. Data shown are the mean ± S.D., n=3.

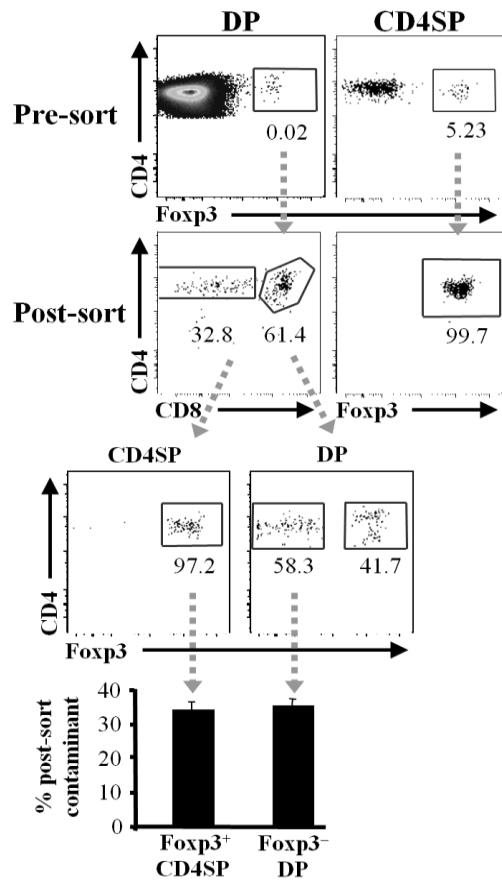|	Large gate		Small gate	
EDTA:	−	+	−	+
CD8SP	3.1 ± 0.5	3.2 ± 0.6	4.0 ± 0.1	3.4 ± 0.8
CD4SP	68.8 ± 10	86.8 ± 0.9	87.8 ± 1.5	91.4 ± 1.2
DP	21.5 ± 11	4.1 ± 0.2	1.27 ± 0.4	1.4 ± 0.2

Table 3.1. Summary of CD4/8 distribution of Foxp3^{+} thymocytes
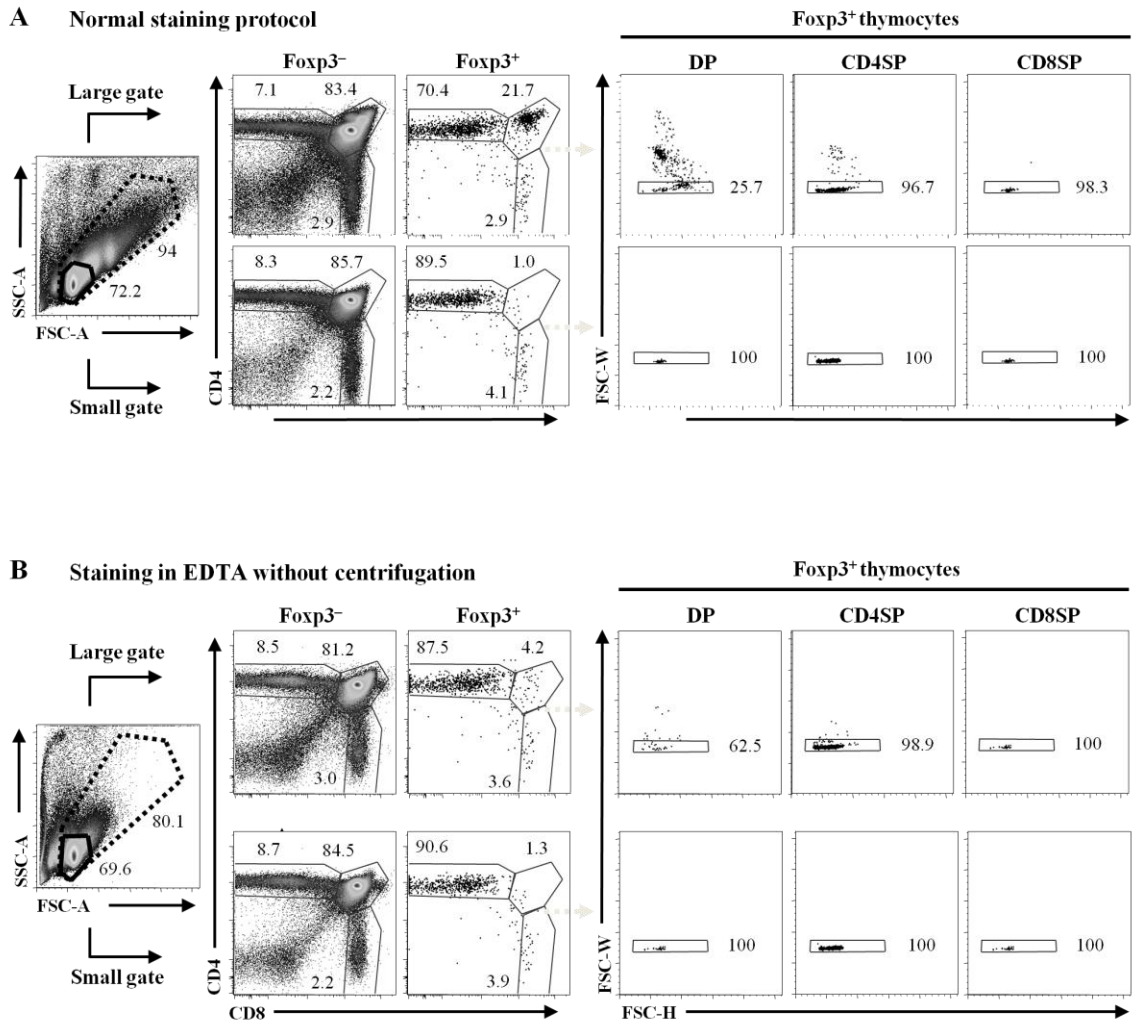
Figure 3.1. Contaminants in sorted Foxp3$^+$ DP thymocytes

A   **Normal staining protocol**

B   **Staining in EDTA without centrifugation**
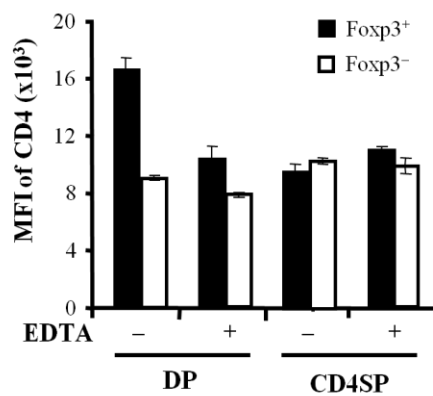
Figure 3.2. Most Foxp3$^+$ DP events by flow cytometry appear to be doublets.

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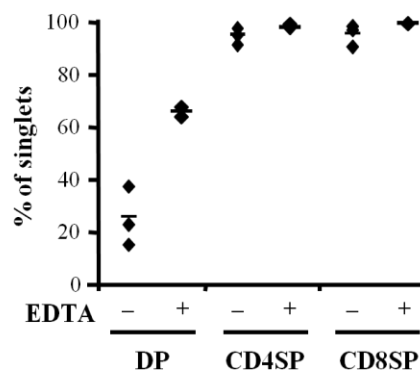**A** Thymocytes in the large gate

B Foxp3⁺ thymocytes in the large gate

Figure 3.3. Most Foxp3⁺ DP events are comprised of doublets.

A

Mixed *Foxp3*gfp CD45.1 + *Foxp3*WT CD45.2 staining

Foxp3+ DP   Foxp3+ CD4SP   Foxp3+ CD8SP
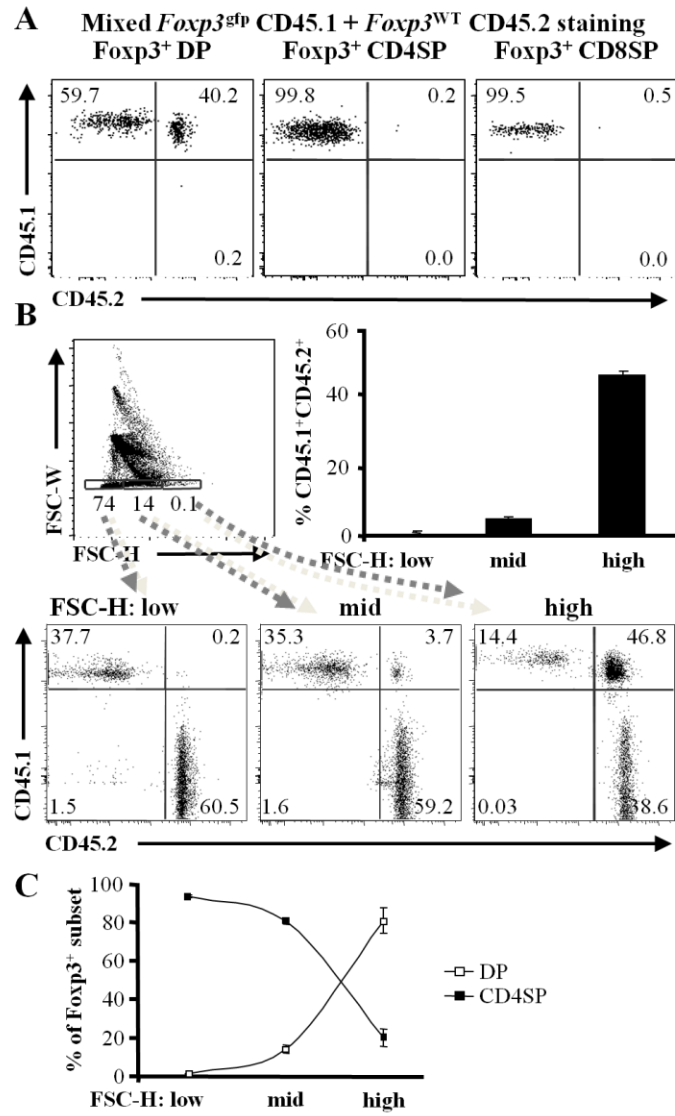
B

C

Figure 3.4. Doublets in the Foxp3+ DP thymocyte subset

Figure 3.5. Increased cell concentration during staining enhances the frequency of Foxp3^{gfp+} DP events.
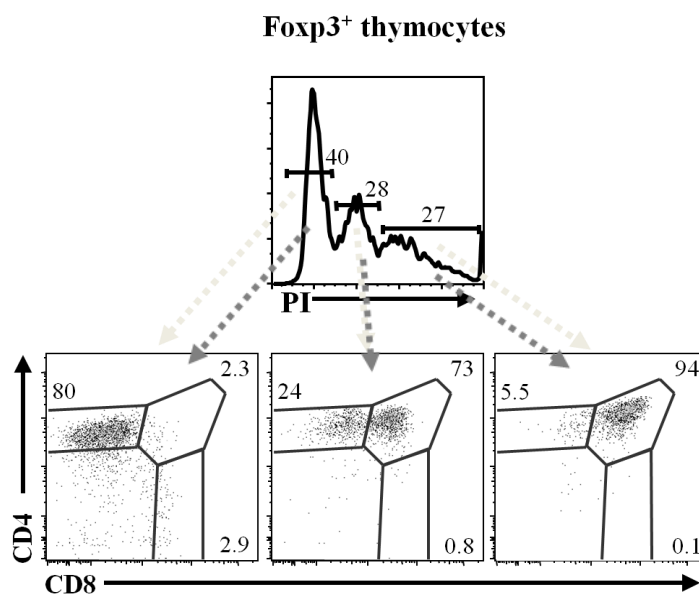
Foxp3+ thymocytes

Figure 3.6. Frequency of Foxp3+ DP cells increases with DNA content.
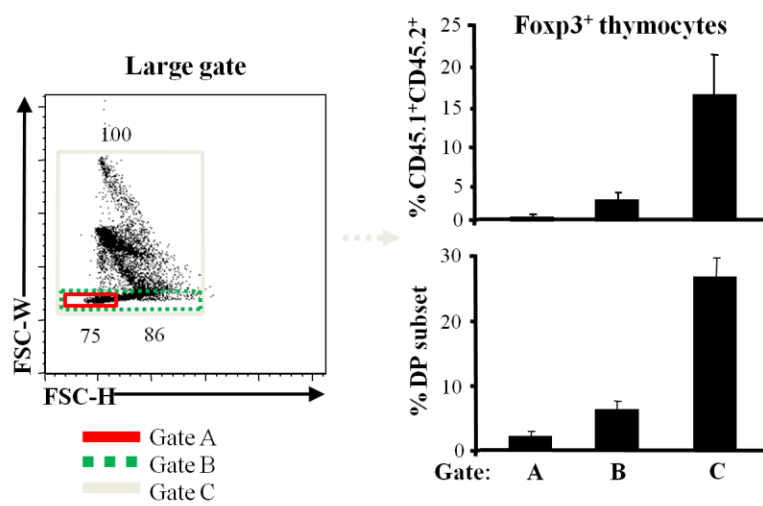
Figure 3.7. Doublet-discrimination gating decreases the frequency of Foxp3^{gfp+} DP events.
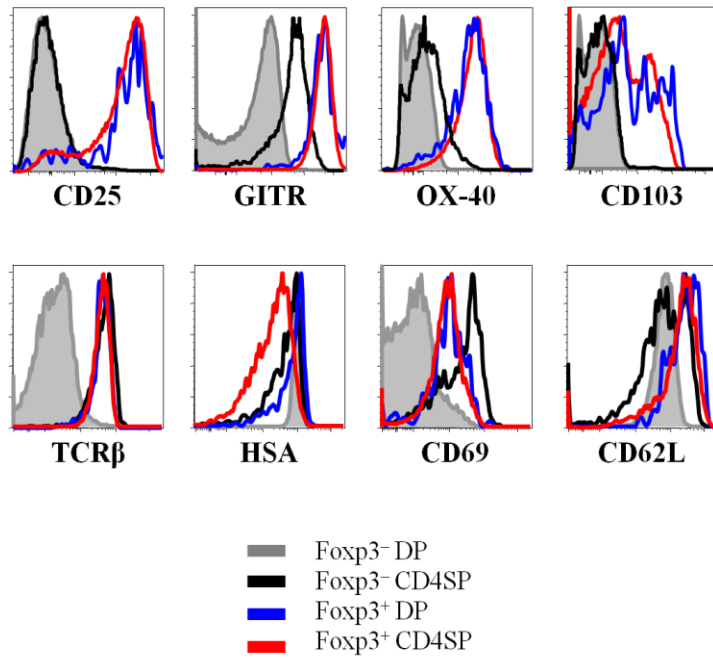
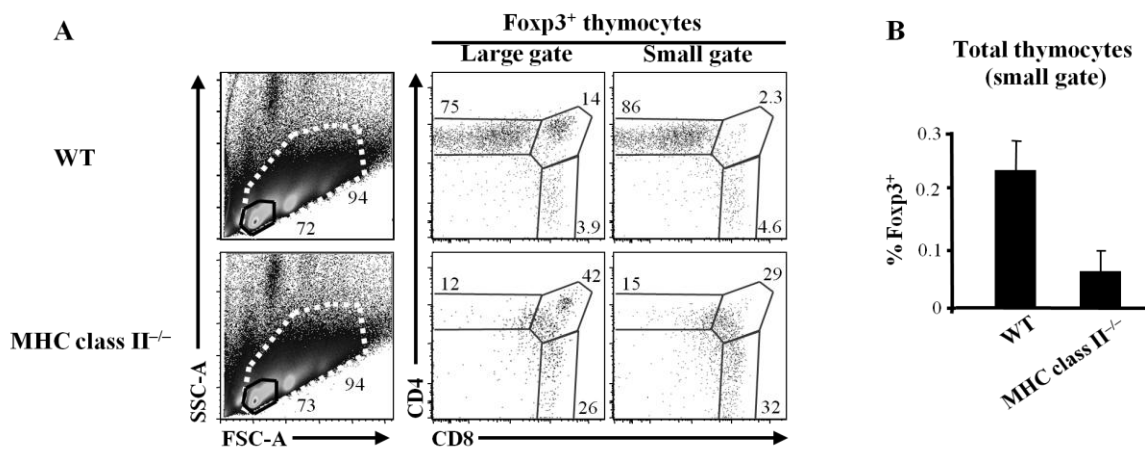**Figure 3.8. Foxp3^{+} DP and CD4SP cells express similar cell surface markers.**

Figure 3.9. Foxp3⁺ DP cells can be generated by MHC class I.
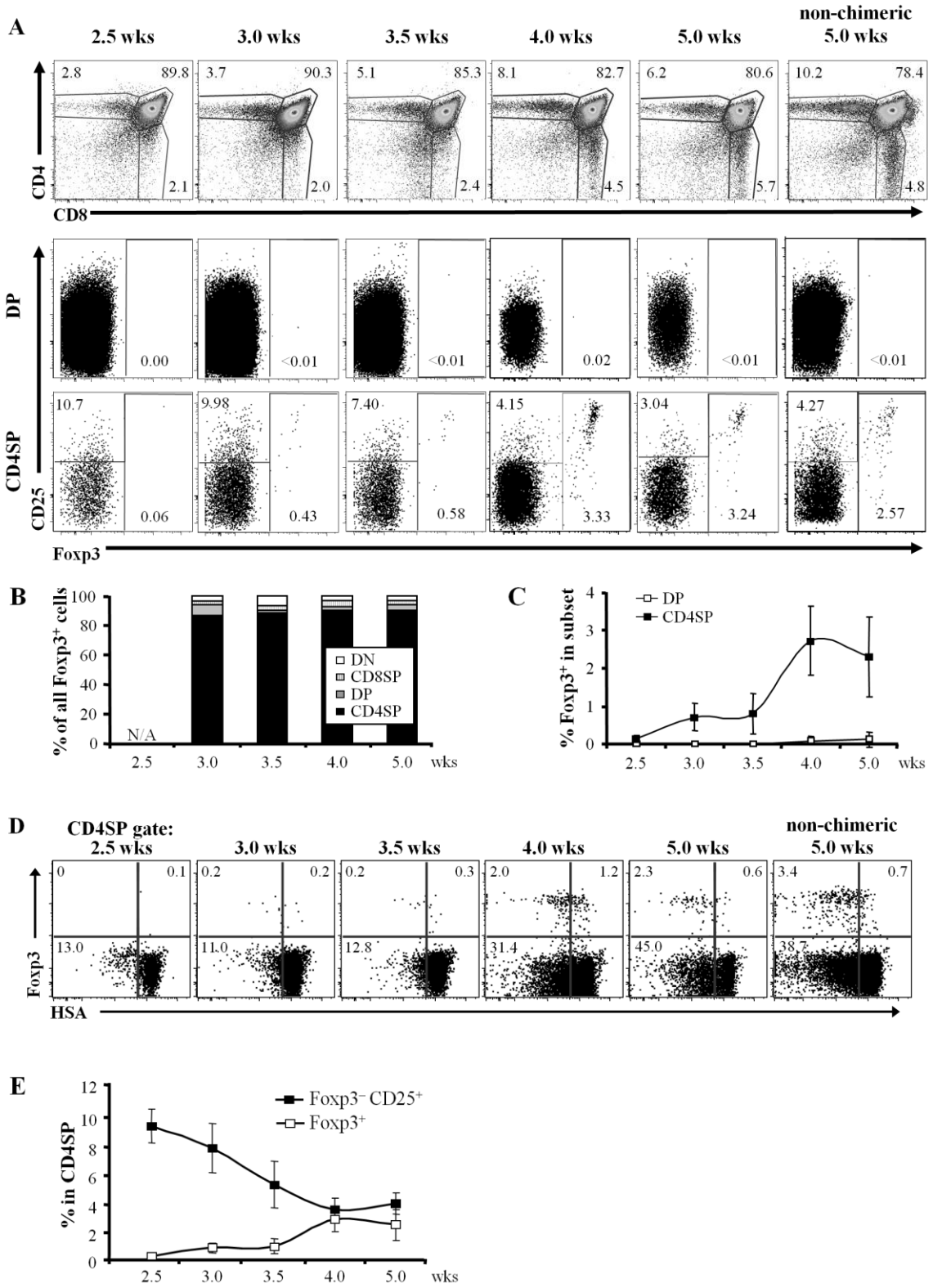
Figure 3.10. Kinetics of thymic regulatory T cell development

Discussion

These data highlight one circumstance in which identifying rare cell populations by flow cytometry is problematic due to the congruence of several factors. First, the gating criteria are based only on positive staining, facilitating the inclusion of doublets. By contrast, identifying rare Foxp3$^+$ CD8SP cells requires a CD4$^-$ gate, excluding doublets to everything but another CD8SP cell, which is statistically much less likely assuming random interactions. Second, Foxp3$^-$ DP cells represents the vast majority of thymocytes, making it likely that random doublet formation would include this subset, regardless of whether there are additional attractive forces between Foxp3$^+$ CD4SP and Foxp3$^-$ DP cells. Third, the actual Foxp3$^+$ DP cell population appears to be rare, allowing infrequent doublets of Foxp3$^+$CD4SP with Foxp3$^-$DP to be relatively more prominent. The low frequency of Foxp3$^+$ DP cells may further encourage staining under more concentrated cell conditions, facilitating doublet formation (Figure 3.5). Moreover, the rarity of the cells may prompt more relaxed gating to include as many events as possible, diminishing the effectiveness of strategies designed to improve "doublet discrimination" (Figure 3.4 B,C). I believe that relaxed gating resulted in our initial inability to sort these rare Foxp3$^+$ DP cells with high purity (Figure 3.7). The use of stringent gating criteria is therefore required for more specific identification of Foxp3$^+$ DP cells.

These data demonstrate that the induction of Foxp3 in the DP stage is a relatively rare occurrence. Although the use of a small gate may result in the exclusion of larger Foxp3$^+$ cells which are not doublets, I estimated the frequency of Foxp3$^+$ cells that are DP cells by flow cytometry to be approximately 1%, which is considerably lower than a recent estimate [57]. While I cannot exclude that TCR interactions with self-antigens at

the DP stage condition thymocytes to undergo Treg cell development in a multi-step process [52], these data suggest that the vast majority of Foxp3 acquisition occurs at the immature HSA[hi] stage of CD4SP development (Figure 3.10), and favor a model in which Treg cell development in the DP and CD4SP stage occur in parallel, rather than in serial [94].

In addition to the enumeration of Foxp3^{+} DP cells, these results illustrate the difficulty of studying with this rare Foxp3^{+} population. While a difference of 1% versus 3% Foxp3^{+} cells in the DP gate may be of little importance with regard to the role of Treg cell development in the DP stage, analysis of a cell population in which the majority was comprised of doublets would be problematic. For example, TCR sequences from Foxp3^{+} DP event would have been difficult to interpret due to contamination with CD4SP Treg cells. Thus, great care should be taken with functional, developmental, or phenotypic evaluation of Foxp3^{+} DP cells to avoid the potential for bias by inclusion of Foxp3^{+} CD4SP cells due to doublets.

Chapter 4

Concluding Remarks and Future Directions

As Foxp3$^+$ CD4$^+$ Treg cells plays an important role in the maintenance of tolerance to self, studying the development of Treg cells is essential to understand tolerance mechanism required to prevent autoimmunity. I have studied differentiation of thymic Treg cells using TCR repertoire analysis by addressing two questions. How does self-reactivity of TCR drive Treg cell development? (Chapter 2) When does differentiation of Treg cells occur in the thymus? (Chapter 3) In my dissertation study, I showed that differentiation of thymic Treg cells is instructed by TCR reactivity for self-antigen presented by mTEC, thereby most of Treg cells appear at immature CD4SP stage. Moreover, assessment of the effect of TCR affinity in negative selection and peripheral T cell response gave an insight of how self-reactivity of TCR operates to establish self tolerance and to prevent autoimmunity.

How much self-reactivity is required for thymic Treg cell development?

Although the notion that self-reactivity plays a critical role in thymic Treg cell development has been supported by many reports for a decade, the degree of T cell self-reactivity considered dangerous by the immune system, thereby requiring thymic education to prevent autoimmunity, is unknown. I analyzed a panel of TCRs with a broad range of reactivity to ovalbumin (OVA$_{323-339}$) in the RIP-mOVA self-antigen model for their ability to induce mechanisms of thymic tolerance. In Chapter 3, I quantified TCR

specificity and correlated it with thymic T cell selection, such as Treg development and negative selection. Thymic Treg cell generation in vivo was directly correlated with reactivity to OVA-peptide in a broad ~1,000-fold range, and its developmental "niche size" was unexpectedly dependent on TCR affinity. The threshold for Treg cell differentiation was almost 100-fold lower than that required for eliciting thymic negative selection and peripheral T cell responses. Thus, these data suggest that Treg cell differentiation is a default outcome of self-antigen encounter for CD4$^+$ thymocytes, and that thymic tolerance mechanisms are tuned to limit the escape of self-reactive effectors without Treg cell chaperones into the periphery.

Timing of Treg cell development in the thymus

The CD4$^+$CD8$^+$ (DP) stage of thymic development has been thought to be the earliest period which generates natural Treg cells important for the prevention of autoimmunity. However, I found that most Foxp3$^+$ DP cells identified by routine flow cytometry represent doublets comprised of Foxp3$^-$ DP and Foxp3$^+$ CD4$^+$CD8$^-$ (CD4SP) cells. This was determined using analysis of flow cytometric height and width parameters, post-sort contaminants, and thymocyte mixing studies. Temporal analysis of Treg cell development arising from bone marrow precursors in neonatal bone marrow chimeras suggested that Foxp3$^+$ DP cells are not a major percentage of Foxp3$^+$ thymocytes, and supported the notion that most Treg cell development occurred at the immature HSA^{hi} CD4SP stage. Thus, these data demonstrate that the frequency of Foxp3$^+$ cells generated at the DP stage is much smaller than previously recognized, suggesting that additional thymocyte maturation may be required to facilitate efficient

induction of Foxp3. In addition, this study highlights a potential bias which may happen in identifying very rare population among DP cells comprising most of thymocytes.

Questions to be addressed

Observations from my dissertation study, lead to interesting questions regarding the role of TCR affinity in mechanisms of establishing tolerance to self in the thymus and periphery. Does thymic Treg cell differentiation utilize continuous signaling mode or digital threshold mode? Does interclonal competition inhibit Treg cell development of lower affinity TCRs? How does TCR affinity affect effector versus Treg cell generation in the periphery? Does the level of self-reactivity control suppressor function of Treg cells? A panel of OVA-reactive TCRs obtained in this dissertation would be useful to address these questions.

TCR signaling mode for thymic Treg cell development

It was demonstrated that Treg cell differentiation is driven by self-reactivity to likely non-ubiquitous tissue-specific antigen, and that this is reflected in the size of the Treg cell developmental niche in my dissertation study. This leads to an important question. How is the level of TCR-antigen interaction translated into Foxp3 expression? This may be explained in two different models. One model is that there is a TCR stimulation threshold required for Foxp3 induction. Another model is that the level of TCR stimulation translated into the degree of Foxp3 expression in continuous manner, by which strong TCR stimulation increases the probability of Foxp3 induction. The approach to address this question would be using the panel of OVA-reactive TCRs to

examine whether TCR signals are interpreted in a digital or analog manner for Foxp3 expression. To evaluate the strength of TCR signaling in vivo, we obtained a new reporter mouse, Nur77-GFP [43] from Dr. Hogquist (U of Minnesota), and are breeding it to $Rag1^{-/-}$ in B6.C background to obtain donor DN thymocytes for retroviral bone marrow chimera or intrathymic transfer.

If Treg cell development occurs in digital threshold model, it would be expected to be observed that Treg cells will always show a certain level of GFP irrespective of TCR affinity, in which lower affinity TCRs results in less number of cells with the same GFP level, while the level of effector T cells would vary with TCR affinity. On the other hand, if Treg cell differentiation is based on continuous TCR signaling, the level of GFP in Treg cells would differ depending on TCR affinity, where intensity of GFP from Treg cells decreases with lower TCR affinity. Since c-Rel has been reported to play a primary role in Treg cell development by interpreting TCR stimulation into Foxp3 expression [95-98], it would be worth to examine whether NF-κB pathway is activated in digital manner.

Interclonal competition in thymic Treg cell development

Although it was shown that the size of Treg cell development niche is dependent on TCR affinity to self-antigen, it remains unclear how thymocytes with various affinity interact with the limited niche. Thus, it would be interesting to examine whether there is interclonal competition among self-reactive thymocytes for the same self-antigen, and whether it inhibits Treg cell differentiation of thymocytes with lower self-reactivity. The panel of OVA-reactive TCRs would be useful tool to test the hypothesis that interclonal competition may favor the differentiation of Treg cells of higher self-reactivity, while

cells with lower self-reactivity develop as effector cells. If interclonal competition is observed, it would suggest that TCRs with lower affinity for self-antigen is more likely to escape thymic tolerance mechanism as effector cells, potentially autoimmune T cells, whereas self-reactive TCRs have higher probabilities of undergoing thymic education to preserve tolerance.

To test whether high affinity TCR would affect Treg cell development of the other TCR, a TCR in the panel (e.g. N7) would be transferred with DO11, the highest affinity TCR in, into the of congenic hosts. One expected outcome from this experiment is that DO11 has no effect on development of the second TCR into Treg cells, suggesting that niche size is sufficient for Treg cell differentiation of both TCRs. This may suggest that Treg cell development occurs independently in terms of self-reactivity of thymocyte population. On the other hand, Treg cell differentiation may be hindered by higher affinity TCRs. This may imply that lower affinity TCRs are outcompeted by higher affinity, resulting in escape of thymocytes with lower affinity from thymic tolerance mechanism, becoming effector T cell subset.

Interclonal competition for peripheral antigenic niche

The degree of self-reactivity of TCR was demonstrated to play an instructive role in thymic Treg cell generation in this study. Self-reactivity is also known to drive peripheral autoimmune responses [85, 99]. However, the relationship of self-reactivity between thymic and peripheral tolerance is unknown. Moreover, the level of self-reactivity inducing peripheral Treg cell generation compared to that of autoimmune effector cells is unknown. Thus, it would be interesting to see how TCRs with varying

affinities compete for the same peripheral antigenic niche, and whether high affinity TCRs outcompete low affinity ones in competition peripheral self-antigen, resulting that the antigen specific Treg cell pool is mostly filled with Treg cells with high affinity TCRs. The panel of OVA-reactive TCRs will be used to study the quantitative role of self-reactivity in peripheral tolerance as well. Peripheral CD4 T cells expressing individual OVA-reactive TCR will be transferred into RIP-mOVA hosts and assessed for Treg versus effector T cell development by flow cytometry. One possible outcome is that self-reactivity of TCR will be correlated with peripheral generation of Treg cell. By contrast, TCR affinity may not contribute much to Treg cell generation unlikely its instructive role in the thymus. This would suggest that other factors besides TCR affinity control cell fate into Treg cells in the periphery.

Role of TCR affinity for Treg cell suppression of autoimmunity

Given that Treg cells need TCR stimulation to exert their suppressive function, it is unknown whether Treg cells recognize same self-antigen as effector T cells to protect autoimmunity. Moreover, it has not been studied which level of self-reactivity in the Treg cells is required to suppress autoimmune effector T cells. These questions would be directly addressed using OVA-reactive TCRs generating Treg cells with varying affinities. Initially, naïve T cells will be sorted and transferred into RIP-mOVA $Rag1^{-/-}$ hosts to determine the level of TCR affinity required to induce autoimmune diabetes. After establishing conditions for diabetes induction in RIP-mOVA $Rag1^{-/-}$ mice, Treg cells expressing individual TCR will be co-transferred with naïve T cells to determine the level of self-reactivity of Treg cells to prevent or mitigate diabetes. Results from this study

could address a question whether Treg cells need to have a higher or equal affinity to peripheral self-antigen for efficient suppression of effector T cells.

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