TUMOR-NECROSIS FACTOR RECEPTOR SUPERFAMILY COSTIMULATION DRIVES THYMIC REGULATORY T CELL DEVELOPMENT

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Dedication

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Abstract

$CD4^+Foxp3^+$ regulatory T cells (Tregs) develop in the thymus and are critical for maintaining immune tolerance and preventing autoimmune disease. Previous work has demonstrated that thymic Tregs develop by an IL-2 and STAT5-dependent mechanism, and that the T cell antigen receptors (TCRs) collectively expressed by thymic-derived Tregs react with self-antigen with high-affinity. In this regard, Tregs are similar to autoreactive T cells undergoing negative selection. Relatively little is known about how the selection bias in the Treg TCR repertoire is imposed and how the decision to become a Treg as opposed to undergoing apoptosis is made.

This thesis demonstrates that progenitors of thymic Tregs, which are $CD25^+$ and $Foxp3^-$, highly upregulate three family members of the TNF receptor superfamily (TNFRSF): GITR, OX40, and TNFR2. The expression level of these receptors directly mirrors perceived TCR signal strength by developing Tregs. Stimulating Treg progenitors with the ligands for these receptors enhances the expression of the IL-2 receptor alpha chain, CD25, resulting in increased sensitivity to IL-2, and ultimately increasing the likelihood of maturation into the $Foxp3^+$ thymic Treg lineage. Loss of TNFRSF expression in Treg progenitors blocks Treg development *in vivo*. Finally, we demonstrate that TNFRSF costimulation directly shapes the Treg TCR repertoire.

A second line of investigation in this thesis research is focused on uncovering the developmental and functional potential of a second, more recently

described population of Treg progenitors which are $CD25^{-}$ and $Foxp3^{lo}$. We find that despite lacking CD25, these cells do efficiently respond to IL-2, and that one potential explanation for this initially confusing finding is that $CD25^{-}Foxp3^{lo}$ Treg progenitors express higher levels of CD122, which is the beta chain of the IL-2 receptor that mediates signaling upon ligand binding. We also demonstrate that $CD25^{-}Foxp3^{lo}$ Treg progenitors are phenotypically 'older' and have resided in the thymus longer than $CD25^{+}Foxp3^{-}$ Treg progenitors, and this may also explain their ability to enter the Treg lineage in conjunction with the finding that they also express elevated levels of CD122. Finally, we demonstrate that both populations of Treg progenitors are responsive to TNFRSF costimulation and that ligation of GITR in these cells shapes the resultant Treg TCR repertoire. Further studies involving TCR sequencing and *in vivo* competition experiments are pending, and will be required to fully understand this alternative population of Treg progenitors.

These studies shed important light on previously unappreciated aspects of thymic Treg differentiation. It is my hope that ultimately knowledge gained from this thesis research may aid in the development of novel therapeutics for treating autoimmune disorders and other chronic inflammatory conditions.

Table of Contents

List of Figures

Chapter 1

Introduction

Evolutionary pressure for adaptive immunity

Early eukaryotes were the first creatures to evolve innate immune defenses[1], and these characteristics were pivotal for fueling the evolution of complex and sustained life. These early forms of immunity were encoded in a genetic context that was limiting. A limited set of genes encoded a limited set of proteins that allowed for responses to a limited set of potential pathogens[2]. While these heritable defense mechanisms were and still are critical for survival, the constant co-evolution of microbial life imposed the need to recognize an ever-growing number of antigens (peptides derived from pathogens). This would require that an ever-growing number of antigen receptors be encoded and expressed on the surfaces of immune cells. This problem is difficult to resolve given the finitude of space and energy available for maintenance of the genome.

Approximately half a billion years ago, an abrupt event spurred a change in the genetic "format" in which antigen receptors are encoded, allowing for a vastly larger set of shapes to be recognized by the products of only a few genes. It is now understood that the genome of an ancestor of jawed vertebrates was invaded by a transposon[3]. This mobile DNA element encoded a recombinase enzyme capable of randomly recombining specially flanked gene segments in primitive antigen receptor loci, vastly increasing the diversity of the protein products that could be expressed[4]. Susumu Tonegawa performed the seminal experiments that uncovered the molecular basis of this genetic diversification,

showing that somatic rearrangement of antigen receptor genes was required to produce functional gene products from the immunoglobulin locus[5]. We now appreciate the somatic rearrangement of antigen receptor loci as a required checkpoint in lymphocyte development. Each cell must randomly rearrange these gene segments to yield a functional antigen receptor, and diversity is maintained by a polyclonal repertoire of millions of lymphocytes each bearing their own unique antigen receptor[6].

Generation of T cell receptors (TCRs) and positive selection of thymocytes

There are numerous parallels in the development of B and T lymphocytes, but for relevance to the work presented in this thesis, only T cell development will be discussed further.

T cells arise from hematopoietic precursors, called thymic progenitor cells, which are generated in the bone marrow and travel via the blood to the thymus. These cells enter the thymus at the cortico-medullary junction, and their immigration is first initiated when a ligand they express, called PSGL-1, binds the adhesion molecule P-selectin expressed on the surface of thymic endothelial cells[7]. These interactions slow down thymic progenitor cells and hold them near the endothelium. Transmigration through the endothelium and into the tissue of the thymus depends on the expression of the chemokine receptor CCR9, which allows these progenitors to traffic along a gradient of CCL25—a chemokine

secreted by the thymic stroma[8]. CCR7 is also expressed by thymic progenitor cells and also drives thymic immigration via CCL19 and CCL21 (ref [9]). The recruitment of thymic progenitor cells is recognized to be a controlled, or "gated" phenomenon that is increased or decreased in proportion to the abundance of T cells in peripheral lymphoid organs via a S1P/S1PR1 dependent mechanism[10].

Newly immigrating thymic progenitor cells arrive with the potential to commit to several different lineages, including T, B, NKT, and dendritic cells, but signals transduced to these precursors from the thymic stroma via the receptor Notch1 lead them to commit to the T cell lineage[11, 12]. The zinc-finger transcription factor Bc11b is also required for early T-lineage commitment[13]. Expression of either an α:β or a γ:δ TCR is a decision made later in T cell development, and Notch signaling continues to play an instructive role in this process as well[14]. For relevance, the remaining discussion focuses on the selection of α:β T cells.

Early T lineage-committed cells lack surface expression of the co-receptors CD4 and CD8 and are called double-negative (DN) thymocytes. DN thymocytes can be subdivided into four stages, named DN1-DN4, on the basis of their express of the adhesion molecule CD44, and the high affinity α chain component of the IL2 receptor, CD25. DN1 cells are CD44+CD25- and they proliferate in response to further interaction with the thymic stroma mediated via c-Kit, Notch1, and the IL7 receptor[15, 16]. Their T cell receptor (TCR) loci have not yet been rearranged[3].

The upregulation of CD25 marks DN2 cells, and it is at this stage that thymocytes begin to rearrange the TCR β-chain locus through the process of somatic recombination. DN2 cells are the first thymocytes to begin expressing the recombinases RAG1 and RAG2. These enzymes recognize so-called recombination signal sequences (RSS), which are highly conserved heptameric and nonameric sequences flanking numerous gene segments within the TCR loci. RAG recombinases, which are expressed only by lymphoid cells, align the RSS sequences, and together with several other DNA modifying enzymes, mediate the random enzymatic rearrangement and linkage of DNA segments found in germline-configured TCR gene loci into "recombined" gene products that code for functional receptors. This process has been termed V(D)J recombination. The initial D_β to J_β rearrangements occur at the DN2 stage. These recombination events progress in the form of V_β to DJ_β rearrangements in DN3 cells, which can be identified by their reduction in expression of CD44[3].

DN3 thymocytes that successfully perform V(D)J recombination and form a β chain express those molecules on their surfaces paired initially with a surrogate α chain (which is not rearranged by V(D)J recombination) called pre-T cell α, or pTα[17]. Once assembled, this β:pTα complex associates with subunits of the T cell co-receptor molecule, CD3. Expression of these pre-TCR complexes represents a key checkpoint in T cell development. DN3 cells which do not receive a survival signal via β:pTα complexes cease to differentiate. Those which

express functional β chains receive a pro-survival signal and thus progress to the DN4 stage, where further rearrangements at the β chain locus are halted via allelic exclusion. DN4 cells undergo intense proliferation, and are marked on the cell surface by downregulation of CD25 (refs [18, 19, 20]).

After this second major wave of proliferation, DN4 cells upregulate both CD4 and CD8 co-receptors and are referred to as double-positive (DP) thymocytes. These cells begin trafficking back to the cortico-medullary junction. VJ recombination begins to occur at the TCR α chain loci in DP thymocytes, and functional products of these rearrangements are then expressed on the cell surface as heterodimers with β chains, producing mature α:β TCRs. If these TCRs fail to recognize pMHC molecules, additional attempts at α chain rearrangements are made[21]. Successful recognition of pMHC complexes with low affinity by α:β TCRs delivers a survival signal allowing for continued development. This process is known as positive selection[22, 23, 24, 25].

Positively selected thymocytes bearing functional TCRs must complete an additional lineage commitment—whether to become a CD4 or CD8 single positive (CD4SP, CD8SP) thymocyte recognizing class II or class I MHC restricted antigens, respectively. Commitment to the helper T (CD4+) lineage or the cytotoxic T (CD8+) lineage is thought to be determined at least in part by the cumulative strength of TCR signaling[26]. The binding of pMHC complexes to

functional TCRs initially down-regulates the expression of CD4 and CD8, resulting in an immature single positive (ISP) population that expresses low levels of both co-receptors. This event is followed by re-expression of CD4, which is known to more strongly recruit the kinase Lck[27]. Lck initiates the intracellular TCR signal after pMHC binding by phosphorylating key tyrosine residues on the intracellular domains of the CD3 epsilon and zeta chains[3]. If a DP thymocyte bears an MHC class II-restricted TCR, a stronger signal is propagated at this stage than if it is MHC class I-restricted[27]. Stronger cumulative TCR signals in the former scenario are thought to inactivate a distal response element that normally silences expression of a transcription factor called Th-POK[28]. Th-POK drives expression of CD4 and completes commitment in the case of MHC class II-restricted thymocytes[29]. In the absence of Th-POK expression, and in the presence of IL7, Runx3 expression leads to CD8 lineage commitment[28, 30].

Negative selection of self-reactive T cells

Somatic diversification of a heritable "template" for antigen recognition within individual cells has afforded a great survival advantage, but as a consequence of the random nature of TCR gene rearrangement, some cells will bear receptors that recognize self-antigens and may provoke autoimmunity. Not surprisingly, mechanisms to edit the antigen receptor repertoire to remove self-reactive cells have evolved[31].

Maturation to the single-positive stage coincides with migration to the thymic medulla, where several key thymic antigen presenting cell (APC) populations are located. These include medullary thymic epithelial cells (mTEC) and multiple subsets of dendritic cells (DCs) including SIRPα+ and CD8α+ conventional DCs, and plasmacytoid DCs[32]. A transcription factor called AIRE is expressed in mTEC that allows these cells to "promiscuously" express genes normally expressed in only specific cell types throughout the body[33]. AIRE-dependent expression of these proteins followed by their degradation by specific proteosomal subunits leads to the generation of a diverse repertoire of self-antigens that are presented on class I or class II MHC molecules. Dendritic cells do not express AIRE, but they capture self-peptide:MHC complexes from the surface of mTECs and present them to single positive thymocytes entering the medulla[34].

Through this process, medullary APCs test each TCR for reactivity to self-antigens. TCRs that bind self-peptide:MHC complexes with very high affinity are killed by apoptosis. This is termed negative selection, and it is responsible for culling the majority of self-reactive T cells prior to their emigration to the peripheral lymphoid organs[31]. By preferentially allowing the survival of cells expressing TCRs that bind self-peptide:MHC complexes with low to intermediate affinity, the process of negative selection biases the repertoire to contain more T

cells capable of binding to foreign antigens and eliciting a productive immune response, rather than autoimmunity[35, 36].

Dominant tolerance via regulatory T cells

Central tolerance is an imperfect process, because some self-reactive T cells escape negative selection and appear in peripheral lymphoid organs[37, 38, 39]. Fortunately, another mechanism has evolved in the thymus to control self-reactivity, and this is the differentiation—and not death—of a portion of self-reactive CD4SP thymocytes into Foxp3+ regulatory T cells, or Tregs[35, 40, 41]. Expression of Foxp3 by Tregs drives a transcriptome yielding multiple mechanisms for suppressing the activation and effector function of other immune cells[41]. This "dominant tolerance" is critically required for the regulation of immune responses to foreign antigens and to prevent immune responses against self. In humans (and mice) that lack a functional *FOXP3* gene, systemic autoimmune disease is present in virtually all organ systems at a very young age, and death will occur within the first few years of life unless these patients are transplanted with normal bone marrow[42, 43, 44]. More subtle defects in Treg development and/or function have been associated with human autoimmune/inflammatory diseases such as Crohn's disease and type 1 diabetes[45, 46, 47, 48, 49, 50].

The process by which a fraction of self-reactive T cells avoid negative selection, but instead differentiate into these potent immuno-suppressive cells is not clear, and has been the focus of my thesis research.

Disclaimer: portions of the remaining introductory text are reproduced from a review article written by the author [51]**.**

Interleukin-2 (IL2) drives Treg development in the thymus

It has now been twenty years since the first reports appeared demonstrating that interleukin-2 (IL2) is required to prevent the development of systemic autoimmune disease[52]. Subsequent studies by Sakaguchi and colleagues identified CD25, α chain of the IL2 receptor, as one of the first useful markers for the identification Tregs[53]. This led to the initial hypothesis that IL2 is required for the development or function of Tregs and more recently the implementation in the clinic of agonist IL2:anti-IL2 complexes for the treatment of autoimmune and inflammatory conditions[54, 55].

Supporting the initial hypothesis that IL2 is involved in Treg development, work by Malek and colleagues demonstrated that the autoimmune disease that developed in *Il2rb*$^{-/-}$ mice could be prevented by the transfer of CD25^{+} Tregs from WT mice into *Il2rb*$^{-/-}$ host mice. These studies demonstrated that *Il2rb*$^{-/-}$ mice lacked a functional population of Tregs[56]. Additional work by this same group

demonstrated that expression of an *Il2rb* transgene that was expressed solely in the thymus was sufficient to rescue the defect in Treg development suggesting that the defect in *Il2rb*$^{-/-}$ mice is due to a failure of Treg development in the absence of IL2 (ref [57]). In contrast, Lafaille and colleagues found that transfer of CD4^{+} T cells from *Il2*$^{-/-}$ mice into a wild type (IL2-sufficient) mouse model of experimental autoimmune encephalomyelitis prevented disease, while CD4^{+} T cells from *Il2ra*$^{-/-}$ mice did not. These results indicate that the phenotype seen in *Il2*$^{-/-}$ mice is due to a defect in Treg function and not development[58]. Supporting this observation, two other groups used either Foxp3-GFP reporter mice, or the ability to stain for intracellular FOXP3, to demonstrate that young *Il2*$^{-/-}$ mice have FOXP3^{+} Tregs and that the defect in these mice had to do with reduced function or "fitness" of these cells[59, 60]. Finally, work from the Farrar lab and Steve Ziegler's lab reconciled these findings by demonstrating that while young *Il2*$^{-/-}$ mice do not lack FOXP3^{+} Tregs, comparable *Il2rb*$^{-/-}$ mice have a substantial defect in Treg development[61, 62]. This latter result reflects redundancy between IL2 and IL15 as *Il2*$^{-/-}$ *x Il15*$^{-/-}$ mice mimic the defect in Treg development observed in *Il2rb*$^{-/-}$ mice[61]. It is important to point out that under physiological circumstances IL15 does not play a role in Treg development or function as IL2 signaling in Tregs leads to downregulation of the IL15Rα chain, thereby rendering these cells much less responsive to IL15 (ref [63]). Thus, subsequent studies have demonstrated that the original experiments by Malek and Lafaille

and colleagues were both correct as IL2 plays an important role in both Treg development and function.

STAT5 Activation Drives Thymic Treg Lineage Commitment

$CD4^+CD25^+FOXP3^+$ Tregs that develop in the thymus (also known as 'natural Tregs') constitute 2-4% of CD4SP thymocytes, yet this relatively small population plays a critical role in maintaining peripheral tolerance and preventing autoimmunity. The TCR repertoire of these natural Tregs overlaps with that of non-regulatory T cell populations but is skewed to favor TCRs that interact with higher affinity to self-antigens in the thymus[40, 64, 65, 66, 67, 68]. The molecular mechanisms that drive Treg development have been tied to three primary signaling modules. First, TCR signaling plays a key role as TCRs with higher affinity for self-antigen are preferentially selected into the Treg lineage[65, 69]. Second, the costimulatory receptor CD28 also plays an important role as *Cd28*$^{-/-}$ and *B7-1/B7-2*$^{-/-}$ mice both show clear defects in Treg development[70, 71, 72, 73]. Third, signals emanating from the interleukin-2 receptor are also required for Treg differentiation in the thymus[61, 62]. These observations culminated in the development of a two-step model of thymic Treg development, in which a TCR- and CD28-dependent, but cytokine-independent first step generates an IL2-responsive intermediate "Treg progenitor" that lacks FOXP3 expression. Subsequently, a TCR-independent, IL2/STAT5-dependent second step results in

the rapid conversion of Treg progenitors into mature FOXP3^{+} Tregs[74, 75]. This model is discussed in further detail below.

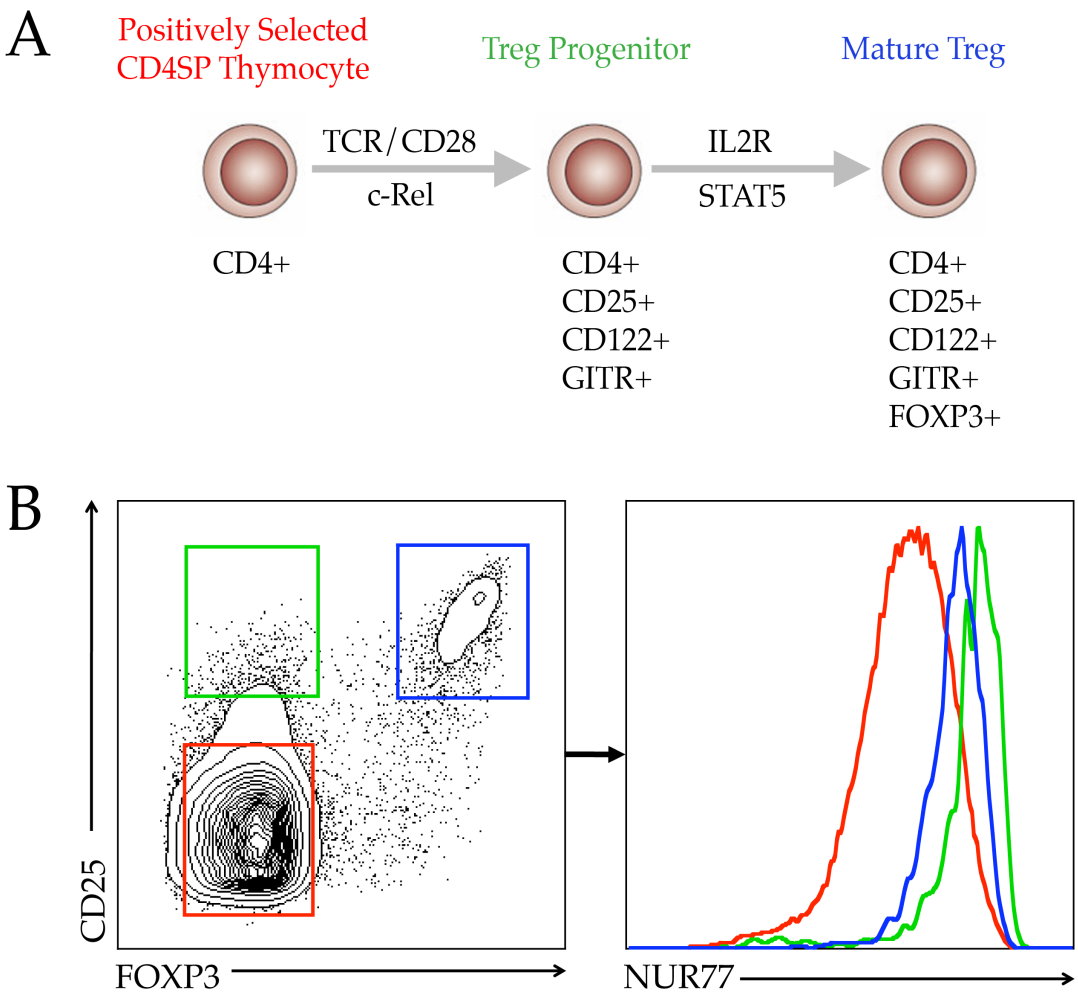


Figure 1-1. Two-step model of thymic Treg development. (a) CD4SP thymocytes perceiving high affinity/avidity signals emanating from TCR/CD28 are first programmed via the NF-κB pathway to express IL2Rα and IL2Rβ, rendering them highly responsive to IL2. A second step, which is TCR-independent, but cytokine-dependent, is completed when Treg progenitors receive IL2 signals transmitted via STAT5 to subsequently drive expression of *Foxp3*. This second step yields mature, fully functional FOXP3^{+} Tregs. (b) CD4SP thymocytes plotted on the basis of CD25 and FOXP3 expression can be categorized into (1)

conventional or non-Treg cells which are $CD4^{+}CD25^{-}FOXP3^{-}$ (gated in red), (2) $CD4^{+}CD25^{+}FOXP3^{-}$ Treg progenitors, which are also $CD122^{hi}$ and $GITR^{hi}$ (gated in green), and (3) $CD4^{+}CD25^{+}FOXP3^{+}$ mature Tregs (gated in blue). The representative TCR signal strength of each of these populations, reported via NUR77-GFP expression, is shown in the histogram on the right. Drs. Amy Moran and Kristin Hogquist initially reported these findings in 2011 (reference 65).

Upon interacting with medullary antigen presenting cells (APC) presenting self-peptide:MHC II complexes, strong TCR signals in a fraction of CD4SP thymocytes cause them to differentiate into Treg progenitors, marked by elevated expression of the high-affinity IL2Rα chain (CD25), the IL2Rβ chain (CD122), and the costimulatory TNF receptor superfamily member, glucocorticoid-induced TNF-related protein (GITR)[74, 75]. The emergence of this $CD4^{+}CD25^{+}CD122^{hi}GITR^{hi}FOXP3^{-}$ Treg progenitor population requires canonical activation of the NF-κB pathway downstream of TCR and CD28 ligation. Activation of LCK from these receptors signals through the canonical NF-κB pathway to ultimately promote nuclear translocation of c-REL and REL-A[72, 76, 77, 78, 79]. The requirement for NF-κB activation in Treg differentiation is demonstrated by the absence of thymic Tregs—and importantly, Treg progenitors—in animals deficient in *Cd28, Prkcq, Carma1, Bcl10,* and *Rel*[71, 72, 80, 81, 82]. Further studies revealed that c-REL binds the conserved non-coding sequence 3 (CNS3) located in the *Foxp3* gene to promote epigenetic modification of *Foxp3* rendering it permissive for subsequent transcription initiation[83].

The conversion of $FOXP3^{-}$ Treg progenitors into mature $FOXP3^{+}$ Tregs in the thymus occurs via a TCR-independent but IL2/STAT5-dependent process[74, 75]. Ligand binding by the high affinity IL2R complex leads to phosphorylation of three key tyrosine residues located in the cytoplasmic domain of IL2Rβ by the

kinases JAK1 and JAK3. Phosphorylation of Tyr-338 recruits the SH2-containing adaptor molecule, SHC, facilitating activation of the RAS/MAPK/ERK and PI3K/AKT pathways via GRB2 and GAB2, respectively. Phosphorylation of IL2Rβ at Tyr-510 (and to a lesser degree Tyr-392) is critical for recruiting and activating STAT5[84]. The importance of IL2R signaling in thymic Treg differentiation is clearly demonstrated by the fact that the lethal autoimmunity in mice lacking *Il2rb* is due to a failure to generate thymic Tregs, and this phenotype is completely restored by adoptive transfer of small numbers of wild type Tregs[56]. Moreover, retroviral transduction of *Il2rb*$^{-/-}$ bone marrow with wild type *Il2rb*, or a mutant construct capable of activating only STAT5 via Tyr-510, restored thymic Treg generation in bone marrow chimeric mice. In contrast, restoration of Treg development did not occur when mutant constructs capable of activating RAS/PI3K, but not STAT5, were transduced into *Il2rb*$^{-/-}$ bone marrow cells and engrafted into recipient mice[61]. Likewise, crossing *Il2rb*$^{-/-}$ mice to transgenic mice expressing a constitutively active form of STAT5b (*Stat5b-CA* mice) restored Treg development in the thymus[61]. Additional support for the role of STAT5 in Treg development came from two studies that demonstrated that conditional deletion of STAT5 in DP thymocytes (i.e., *Cd4-Cre x Stat5a/b*$^{FL/FL}$ mice) had minimal effects on CD4SP thymocytes with the exception of CD4^{+}FOXP3^{+} thymic Tregs[61, 85]. Together, these findings indicate that STAT5 activation downstream of IL2R is required for thymic Treg development.

Two groups have demonstrated that $CD4^+CD25^+FOXP3^-$ thymocytes are direct precursors of $FOXP3^+$ Tregs, which require only an additional IL2R/STAT5-dependent signal to express FOXP3. First, Hsieh and colleagues showed that adoptive transfer of $CD4^+CD25^+FOXP3^-$ thymocytes, but not $CD4^+CD25^-FOXP3^-$ thymocytes, into the thymii of wild type hosts resulted in the development of $CD4^+FOXP3^+$ Tregs[74]. Similar results were observed upon adoptive transfer into MHCII-deficient mice demonstrating that the conversion process did not require additional signals via the TCR[74]. In addition, stimulation of sorted Treg progenitors with IL2 *in vitro* led to induction of *Foxp3* mRNA within a few hours followed by the development of $CD4^+FOXP3^+$ Tregs 24 hours later. These findings were subsequently confirmed by Burchill and colleagues[75].

Interestingly, IL2R/STAT5 signaling also influences selection of the thymic Treg TCR repertoire. Several studies indicate that the Treg TCR repertoire is biased toward self-reactivity, although there is some overlap with the conventional $CD4^+FOXP3^-$ TCR repertoire[66, 67, 68]. Initial studies by Burchill et al found that augmented STAT5 signaling clearly altered the Treg TCR repertoire. Specifically, this study evaluated the effect of forced STAT5 activation on the Treg TCR repertoire by tracking the frequency of $CD4^+FOXP3^+$ cells specific for a peptide called 2W1S bound to $I\text{-}A^b$ MHC class II molecules using peptide:MHCII tetramers (in this study the 2W1S:$I\text{-}A^b$ tetramer) in littermate control and *Stat5b-CA* mice[75]. A simple comparison of the ratio of $CD4^+FOXP3^-$

to $CD4^+FOXP3^+$ cells amongst total and 2W1S-specific T cells revealed that this TCR is dramatically underrepresented in the Treg pool of wild type mice. The frequency of $CD4^+FOXP3^+$ T cells specific for 2W1S:I-A^b in WT mice (~3-6%) was much lower than that observed for the average of all other Treg TCR specificities (~15%); in contrast, in *Stat5b-CA* mice the frequency of 2W1S:I-A^b specific Tregs was identical to the average of all other Treg TCR specificities. This finding demonstrated that for at least one TCR specificity regulation of STAT5 signaling had a profound effect on its distribution within the Treg repertoire. Thus, ectopic STAT5 activation removed the TCR selection bias that typically results in underrepresentation of 2W1S:I-A^b specific T cells in the Treg TCR repertoire[75].

To extend the observations on the role of STAT5 and the Treg TCR repertoire beyond a single TCR, a fixed TCRβ transgenic system was employed to partially restrict the repertoire, and sequencing was performed on over 1000 productive Vα2 rearrangements from thymocytes isolated from *Stat5b-CA* mice or their WT littermate controls. Tonic STAT5 signaling in *Stat5b*-CA mice led to a dramatic expansion in the diversity of productive rearrangements amongst $CD4^+FOXP3^+$ Tregs including substantial numbers of TCRs that were typically not found in the Treg TCR repertoire[75]. These initial studies have been confirmed more recently by Moran and Hogquist using an independent approach[65]. This latter study used BAC transgenic mice in which a GFP reporter had been knocked into the *Nur77*

gene locus. These mice accurately measure TCR signal strength as assessed by overall GFP expression. Importantly, this study confirmed that signal strength for TCRs expressed by Tregs is higher than that for conventional $CD4^+$ T cells. When the Nur77-GFP mice were crossed to *Stat5b-CA* mice, it was observed that the Treg TCR repertoire was much broader and included substantial numbers of T cells with TCRs that signaled with significantly lower signal strength as assessed using the NUR77-GFP reporter[65]. Together, these observations indicate that limiting IL2R/STAT5 signaling helps to focus the thymic Treg TCR repertoire on TCRs with higher intrinsic signal strength[75]. This suggests that IL2R/STAT5 signaling plays an important role in ensuring the preferential development of Tregs with higher reactivity to self-antigens, which may be important in preventing autoimmunity.

Several questions remain unanswered pertaining to the role of IL2 and STAT5 in promoting thymic Treg differentiation. First, what cell subsets in the thymus actually synthesize the IL2 required for completing the second step of Treg development? IL2 was originally described as a cytokine made by activated T cells to drive proliferation and survival of T cells, thus amplifying effector responses[86]. In this regard, a reasonable assumption might be that developing thymocytes produce the IL2 needed for thymic Treg development. However, thymocytes make exceedingly low levels of IL2 (if any) relative to splenocytes upon stimulation (S.A.M. and M.A.F. unpublished observation). The observation

that dendritic cells and B cells can also make IL2 suggest that these cell subsets might play an important role in producing the IL2 needed for Treg development in the thymus[87, 88]. Further work in which IL2 can be conditionally deleted in T cells, dendritic cells and B cells will be needed to definitively address this question. A second question has to do with what signals actually induce or regulate IL2 production in the thymus; such signals remain undefined. Finally, a defining feature of Treg progenitors is their high expression of GITR. However, the functional significance of this high level upregulation of GITR remains unclear, and a thorough line of investigation of this question forms the second chapter of this dissertation.

The molecular mechanism by which STAT5 affects *Foxp3* transcription is also unclear. STAT5 binding sites have been found in the *Foxp3* promoter region as well as within the CNS2 region of intron 1 in the *Foxp3* gene and several studies have shown STAT5 binding to those sites[61, 85]. The effect of STAT5 binding to these sites is not yet clear. Deletion of the entire CNS2 region including the STAT5 binding sites did not prevent Treg development although it did have an effect on stability of FOXP3 expression[83]. However, the CNS2 region is highly methylated in non-Tregs and typically completely demethylated in natural $FOXP3^+$ Tregs. If methylation of this region normally represses *Foxp3* transcription, then deletion of the entire region would remove the need for any factors that typically reverse this methylation state. Thus, whether STAT5

binding to CNS2 plays a role in Treg development is difficult to determine based on studies deleting the entire CNS2 region.

The critical co-factors that interact with STAT5 to promote Treg development are also poorly characterized. STAT5 is known to interact with a variety of both co-activators, such as CBP and p300, and co-repressors such as NCOR2[89, 90]. How these molecules function in Treg differentiation remains untested. Intriguingly, treatment of Treg progenitors with two distinct histone deactylase (HDAC) inhibitors prevented the IL2/STAT5-dependent conversion of Treg progenitors into Tregs[75]. Although this result at first appears counterintuitive, it is consistent with several reports demonstrating that STAT-dependent gene transcription frequently requires HDAC activity[91, 92]. Whether NCOR2 and associated HDACs are recruited to the *Foxp3* locus by STAT5 during thymic Treg differentiation, and if so, how this complex regulates *Foxp3* transcription, remains to be elucidated. Thus, the molecular mechanisms by which STAT5 alters transcription of genes involved in Treg differentiation remains to be established.

IL2, STAT5 and induced Tregs

An important feature of peripheral tolerance is the conversion of naïve $CD4^+$ T cells into induced regulatory T cells (iTregs) in peripheral lymphoid organs. iTregs have important roles in protecting against chronic inflammatory conditions, and likely play a key role in regulating immune responses to commensal

microorganisms[93, 94]. The differentiation of iTregs, like nTregs in the thymus, requires both TCR- and IL2-dependent signals. However, unlike nTregs, iTregs require transforming growth factor-β (TGFβ) for their differentiation[95]. Moreover, while the CARMA1/NFkB pathway is required for the development of nTregs it actually antagonizes iTreg differentiation[96]. Finally, the stability of iTregs is lower than that of nTregs[97], a feature which correlates with the greater degree of DNA methylation of the CNS2 region of the *Foxp3* gene in iTregs versus nTregs[98, 99]. Thus, iTregs differ in several ways from nTregs.

A role for IL2 in iTreg development was established many years ago in studies documenting the role of both TGFβ and IL2 in iTreg differentiation[100, 101]. Likewise, STAT5 also plays an important role in iTreg differentiation *in vitro*[85]. More recent studies have demonstrated that IL2 and STAT5 also play critical roles in maintaining stability of the iTreg lineage[97]. Specifically, these studies demonstrated that transfer of iTregs into congenic hosts resulted in loss of FOXP3 expression in the transferred iTregs. This result could be blocked by co-administration of agonist IL2: anti-IL2 complexes indicating that IL2 was required to maintain FOXP3 expression in iTregs *in vivo*. These studies further documented that the loss of *Foxp3* expression correlated with re-methylation of the CNS2 region (also referred to as regulatory T cell specific demethylated region or TSDR) of the *Foxp3* gene, and that IL2 stimulation prevented this re-methylation process. The mechanism by which this occurs remains unclear.

However, STAT5 binding sites are found in the CNS2 region, which may be important for maintaining *Foxp3* expression. It is also possible that STAT5 directly initiates demethylation of this region in naïve $CD4^+FOXP3^-$ T cells as they are being converted into $CD4^+FOXP3^+$ iTregs. Arguing against this possibility is evidence that STAT5 binds poorly to its cognate DNA binding site when it is methylated[102, 103]. However, only one of the three potential STAT5 binding sites found in the CNS2 region contains a CpG motif that could be methylated[61]. Thus, whether IL2 and STAT5 promote demethylation of CNS2 requires additional study.

STAT5 also governs Treg function. For example, Blazar and colleagues demonstrated that Tregs expressing a constitutively active form of STAT5b (*Stat5b-CA* mice) are superior to WT Tregs in protecting mice from Graft-versu-host disease[104]. This was due to a number of factors including (i) improved homeostasis of transferred Tregs, (ii) augmented Treg suppressor function, and (iii) reduced ability of *Stat5b-CA* effector T cells to differentiate into T_H1 and T_H17 cells. Supporting these observations, Malek and colleagues demonstrated that IL2 and STAT5 signaling are required for development of a population of $KLRG1^+$ Treg cells that appear to express elevated levels of many factors required for Treg function, such as IL10 (ref [105]). Interestingly, this report documented that quite modest levels of IL2/STAT5 signals are required for thymic Treg development and peripheral survival. In contrast, the development of $KLRG1^+$

Tregs, which represent a terminally differentiated form of Treg with augmented suppressor function, require much stronger IL2/STAT5 dependent signaling. In many ways this latter population resembles KLRG1^{+} effector CD8^{+} T cells suggesting that these two populations may develop via similar mechanisms.

STAT family influences on Treg differentiation

As mentioned above, STAT5 has been shown to inhibit the production of IL17 both *in vivo* and *in vitro*. The mechanism underlying this has been attributed to the ability of STAT5 to directly compete with IL6-dependent STAT3 binding to enhancers within the *Il17* gene locus[106]. STAT5 binding within the *Il17* gene locus correlated with recruitment of the co-repressor NCOR2, which is known to interact with STAT5. Furthermore, IL2 downregulates IL6 receptor expression on iTregs which further acts to prevent iTregs from differentiating into T_H17 cells[107]. Thus the balance between STAT5 and STAT3 signaling plays an important role in directing iTreg development.

Other cytokines including IL4 and IL12 have also been shown to antagonize Treg development[108, 109]. These cytokines activate STAT6 and STAT4 respectively, which are required for inhibiting Treg differentiation[108]. While the mechanism by which STAT6 and STAT4 inhibit Treg development remains to be precisely defined, both of these factors have been shown to reduce STAT5 binding to the

promoter and/or CNS2 region of the *Foxp3* gene[108]. Thus, cytokine crosstalk plays an important role in directing the differentiation of iTregs.

As mentioned above with regard to thymic Treg development, STAT5 interacts with a number of potential binding partners. The role of these binding partners in iTreg development remains to be defined. Interestingly, recent work from the O'Shea lab has shown that micro RNAs activated by TGFβ and retinoic acid receptor alpha (RARα) suppress the expression of one of these binding partners, the co-repressor NCOR2 (ref [110]). Specifically, this study demonstrated that conversion of iTregs into T follicular helper cells (T_{FH}) is limited when *mir10a* is expressed at high levels, such as is the case following TGFβ and RARα signaling in the periphery. It appears that *mir10a* may have a role in fixing the iTreg cell lineage by suppressing conversion of iTregs into either T_H17 or T_{FH} cells. Additional studies are needed to more precisely define the role of NCOR2 and other STAT5 interacting partners on the development and maintenance of regulatory T cells.

Conclusions

Work over the last twenty years has clearly documented an important role for IL2 and STAT5 in shaping the development of both natural and induced Tregs. More recent studies point to a critical role for IL2/STAT5 signals in modifying the functional activity of regulatory T cells. However, the molecular mechanisms by

which STAT5 and its many potential binding partners influence Treg biology are just beginning to be explored. A better understanding of how the IL2/STAT5 pathway governs Treg biology may allow for more targeted approaches for augmenting or inhibiting Treg function. Such information could be particularly useful in developing strategies to inhibit Treg responses to tumors or to augment Treg responses in autoimmunity.

Chapter 2

Costimulation via the tumor necrosis factor receptor superfamily couples T cell receptor signal strength to thymic differentiation of regulatory T cells

Disclaimer: the following chapter is reproduced from the author's 2014 publication in *Nature Immunology*[111].

Regulatory T (T_{reg}) cells express tumor necrosis factor receptor superfamily (TNFRSF) members, but their role in thymic T_{reg} development is undefined. We demonstrate that T_{reg} progenitors highly express the TNFRSF members GITR, OX40, and TNFR2. Expression of these receptors correlates directly with T cell receptor (TCR) signal strength, and requires CD28 and the kinase TAK1. Neutralizing TNFSF ligands markedly reduced T_{reg} development. Conversely, TNFRSF agonists enhanced T_{reg} differentiation by augmenting IL-2R/STAT5 responsiveness. GITR-ligand costimulation elicited a dose-dependent enrichment of lower-affinity cells within the T_{reg} repertoire. *In vivo*, combined inhibition of GITR, OX40 and TNFR2 abrogated T_{reg} development. Thus TNFRSF expression on T_{reg} progenitors translates strong TCR signals into molecular parameters that specifically promote T_{reg} differentiation and shape the T_{reg} repertoire.

Introduction

The development of T lymphocytes with randomly generated T cell receptors (TCRs) allows for diverse and productive immune responses, but also raises the risk for autoimmunity. The thymic medulla contains antigen presenting cells (APCs) that display self-peptide:major histocompatibility (MHC) complexes to immature CD4 and CD8 single positive (SP) thymocytes, and plays a critical role in limiting autoimmunity[32]. This occurs in part by deletion of a substantial fraction of autoreactive thymocytes via the process of negative selection. However, some autoreactive T cells escape deletion in the thymus, and thus alternative mechanisms to control autoreactivity have evolved including the production of thymic-derived regulatory T cells (T_{reg})[35]. The transcription factor Foxp3 plays a critical role in this process as inactivating mutations in *Foxp3* abrogate T_{reg} development and cause lethal multi-organ lymphoproliferative disease[42, 44]. More subtle perturbations in T_{reg} development and/or function, such as reduced responsiveness to interleukin-2 (IL-2), are also associated with autoimmune diseases such as Type 1 Diabetes[46, 48, 112]. T_{reg} development occurs via a multistep differentiation process that preferentially drives high affinity autoreactive T cells to express *Foxp3*. A key question is how thymocytes translate relative differences in TCR signal strength into distinct developmental programs that give rise to naïve $CD4^+$ T cells or regulatory T cells, respectively.

Thymic T_{reg} development occurs via a two-step process[74, 75]. An initial TCR-CD28-driven signaling event leads to expression of CD25 and CD122 (the α and β subunits of the IL-2 receptor) and c-REL-dependent chromatin remodeling at the *Foxp3* locus[72, 76, 77, 78, 79, 83]. A second, TCR-independent but cytokine-dependent step occurs when $CD25^{+}Foxp3^{-}$ T_{reg} progenitors receive an IL-2 signal, which via a STAT5-dependent process drives *Foxp3* expression to complete the Treg differentiation process[61, 74, 75].

How high affinity TCR stimuli drive a molecular program that results in T_{reg} differentiation remains poorly defined. A hallmark of T_{reg} progenitor cells that might play a role in this process is their high expression of the TNFRSF member TNFRSF18, (called GITR hereafter). As GITR expression precedes the induction of Foxp3 in developing T_{reg} cells, we hypothesized that costimulation via GITR may support conversion of T_{reg} progenitors into mature $Foxp3^{+}$ T_{reg} cells in the thymus.

In addition to GITR, several other TNFRSF members are expressed on T_{reg} cells[113]. We screened thymocytes by flow cytometry and found that GITR, TNFRSF4 (called OX40 herafter), and TNFRSF1B (called TNFR2 hereafter) are uniquely overexpressed on T_{reg} progenitors when compared to conventional CD4SP thymocytes. Multiple APCs including dendritic cell subsets and medullary thymic epithelial cells express the corresponding ligands GITR-L,

OX40-L, and TNF. We found that TNFRSF expression by T_{reg} progenitors strongly correlates with TCR signal strength. Thus, CD4SP thymocytes encountering the highest affinity TCR signals most strongly upregulate GITR, OX40 and TNFR2. This process occurred via a TAK1-and CD28-dependent pathway as TAK1- and CD28-deficient T_{reg} progenitors fail to express GITR, OX40, and TNFR2 and do not convert into mature Foxp3$^+$ T_{reg} cells. Stimulation of wild type T_{reg} progenitors with either GITR-L or OX40-L promoted their conversion into mature Foxp3$^+$ T_{reg} cells at much lower doses of IL-2. In contrast, the addition of neutralizing antibodies to TNFSF members in neonatal thymic organ cultures markedly inhibited T_{reg} development. Likewise, blocking signaling collectively through OX40, TNFR2 and GITR completely abrogated T_{reg} development *in vivo* indicating that GITR, OX40 and TNFR2 function in a cell-intrinsic manner to program T_{reg} differentiation. Finally, competition for TNFRSF costimulation skews the T_{reg} repertoire toward higher affinity TCRs, as increasing GITR-L availability dose-dependently broadens the T_{reg} repertoire by enriching for cells with reduced TCR signal strength. These findings support a model in which high-affinity TCR signals in CD4SP thymocytes are translated into a molecular program that entrains T_{reg} development via increased expression of specific TNFRSF members. Ligation of GITR, OX40 or TNFR2 on T_{reg} progenitors enhances the ability of T_{reg} progenitors to compete for limiting amounts of IL-2 and thereby helps define the developmental niche for T_{reg} cells in the thymus.

Results

T_{reg} progenitors highly express GITR, OX40 and TNFR2

A prominent feature of T_{reg} progenitors is their high-level expression of GITR, which is upregulated prior to Foxp3 during T_{reg} development (refs 8, 9 and **Fig. 2-1a**). Studies to elucidate a role for GITR in T_{reg} cells have primarily focused on the effects of costimulation on mature T_{reg} cells in peripheral lymphoid organs[114, 115, 116]. However, whether GITR costimulation plays any role during thymic T_{reg} development is unknown. Because of the large potential for redundancy within the TNFRSF, we sought to clarify which of these receptors are expressed during T_{reg} development. Staining Foxp3-GFP thymocytes with specific antibodies to evaluate expression by conventional (non-T_{reg}) CD4SP thymocytes, T_{reg} progenitors, and mature T_{reg} cells (gated as $CD4^+CD8^-CD25^-Foxp3^-$, $CD4^+CD8^-CD25^+Foxp3^-$, and $CD4^+CD8^-CD25^+Foxp3^+$, respectively) revealed that in addition to GITR, T_{reg} progenitors also highly express OX40 and TNFR2 (**Fig. 2-1a**). Mature $Foxp3^+$ T_{reg} cells continue to express high amounts of these TNFRSF members in the thymus (**Fig. 2-1a**) and in peripheral lymphoid organs (data not shown). T_{reg} progenitors do not globally overexpress TNFRSF members, as 4-1BB, CD30, and TNFR1 were not detectable above background in thymic T_{reg} progenitors or T_{reg} cells (**Fig. 2-1a** and data not shown). CD27, which is already expressed on DP thymocytes, is highly expressed by all CD4SP thymocytes but was not further upregulated by T_{reg} progenitors or mature $Foxp3^+$ T_{reg} cells (**Fig.**

2-1a, and data not shown). Thus, GITR, OX40, and TNFR2 are highly upregulated on T_{reg} progenitors during thymic T_{reg} development.

APCs in the thymic medulla express GITR-L, OX40-L and TNF

For signaling, homotrimeric TNFRSF members require ligation by specific TNFSF ligands[117]. Various APC subsets have been described to express TNFSF ligands upon activation in secondary lymphoid organs, but the constitutive expression of GITR-L, OX40-L, and TNF within the thymic APC subsets that actually mediate T_{reg} differentiation has not been defined. Medullary thymic epithelial cells (mTEC; $CD45^{-}EpCAM^{+}UEA1^{+}BP\text{-}1^{lo}$) constitutively express GITR-L, OX40-L, and TNF (**Fig. 2-1b**). Both GITR-L and TNF are expressed by $CD8\alpha^{+}$ and $SIRP\alpha^{+}$ conventional DC subsets ($CD11C^{+}B220^{-}$), and by plasmacytoid DCs ($CD11C^{+}B220^{+}$), whereas OX40-L expression is restricted to mTEC. Since TNFR2 can bind both membrane-bound and soluble TNF [118, 119], we evaluated TNF expression by surface staining and by intracellular staining after fixation and permeabilization. We were able to detect membrane bound TNF on DCs (data not shown) albeit at much lower levels than total TNF (**Fig. 2-1b**).

We used immunofluorescence microscopy to define the pattern of TNFSF ligand expression within the thymic microenvironment. Co-staining slides with anti-Keratin 5, which marks the thymic medulla (the location of thymic T_{reg} differentiation), showed a high degree of overlap with OX40-L expression (**Fig. 2-**

1c). Similar results were obtained when staining for GITR-L and TNF (data not shown). Thus, the specific ligands that bind the TNFRSF members present on developing T_{reg} cells are expressed by APCs at the site of thymic T_{reg} maturation.

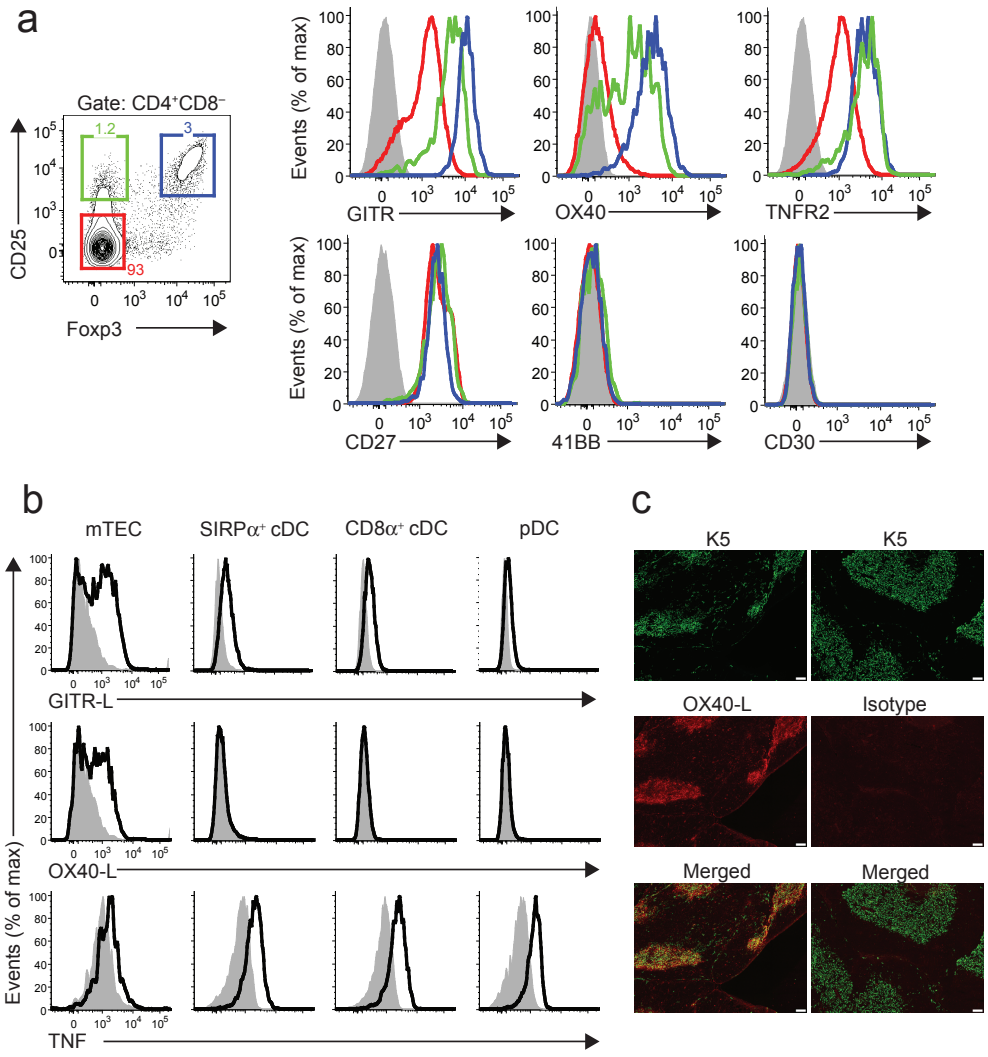


Figure 2-1. Expression of GITR, OX40, and TNFR2 on thymic T_{reg} progenitors and GITR-L, OX40-L, and TNF on APCs in the thymic medulla.

(**a**) Thymocytes from adult (6-10 week old) female or male Foxp3-GFP mice were stained with antibodies against the indicated TNFRSF members in conjunction with antibodies against CD4, CD8, and CD25. In the left panel, conventional (non-T_{reg}) CD4SP thymocytes were gated as $CD4^+CD8^-CD25^-Foxp3^-$ (red lines), T_{reg} progenitors as $CD4^+CD8^-CD25^+Foxp3^-$ (green lines), and mature thymic T_{reg}

cells were $CD4^+CD8^-CD25^+Foxp3^+$ (blue lines). The histograms to the right display the expression of TNFRSF within the indicated populations as compared to isotype-stained controls (gray shaded histograms). Data are representative of at least three separate experiments (n ≥ 3). (**b**) Expression analysis of the specific TNFSF ligands GITR-L, OX40-L, and TNF was conducted by flow cytometry on collagenase- and DNase I-digested thymii harvested from 6-8 week old male or female mice, which were magnetically depleted with anti-CD45 for enrichment of mTEC or enriched with anti-CD11C for DCs. Histograms show the expression of GITR-L (top), OX40-L (middle), and total TNF (membrane + intracellular, bottom) within the indicated populations gated as follows: mTEC ($CD45^-EpCAM^+UEA1^+$), $SIRP\alpha^+$ cDC ($CD11c^+B220^-SIRP\alpha^+CD8\alpha^-$), $CD8\alpha^+$ cDC ($CD11c^+B220^-SIRP\alpha^-CD8\alpha^+$), and pDC ($CD11c^+B220^+$). Data are representative of thymic DC or mTEC isolations pooled from 3-4 mice in at least three separate experiments (n ≥ 3). (**c**) Frozen thymic sections from 6-12 week old female or male mice were stained with anti-Keratin 5 (K5-FITC, green), anti-OX40-L or isotype control (PE, red), and corresponding images were merged (yellow) to evaluate the spatial distribution of TNFSF ligands within the thymus. (10X magnification, white bars represent 100 um; n=3, one of three independent experiments).

GITR, OX40 and TNFR2 correlate with TCR signal strength

When we gated on T_{reg} progenitors and plotted the expression of GITR versus OX40 (**Fig. 2-2a**) or GITR versus TNFR2 (data not shown), we found that cells expressing the most GITR also express the most OX40 and TNFR2, and vice versa. This finding prompted us to ask the question if TNFRSF expression is related to TCR signal strength in T_{reg} progenitors. For these experiments, we used Nur77-GFP BAC reporter mice, which provide a reliable readout of TCR signal strength via GFP expression[65]. We found a significant positive correlation between Nur77-GFP fluorescence and GITR, OX40 and TNFR2 expression in T_{reg} progenitors **(Fig. 2-2a,b**). In contrast, expression of CD27, a TNFRSF member recently reported to drive thymic Treg development via the inhibition of apoptosis[120], did not correlate significantly with Nur77-GFP (**Fig. 2-2b**). Therefore, thymic T_{reg} progenitors receiving the strongest TCR signals during development are engendered with the highest expression of GITR, OX40, and TNFR2. Moreover, mature T_{reg} cells express substantially more OX40 and GITR than T_{reg} progenitors (**Fig. 2-1a**) suggesting that T_{reg} progenitors expressing relatively more of these TNFRSF members have a selective advantage in thymic T_{reg} differentiation.

TAK1 and CD28 govern GITR, OX40 and TNFR2 expression

To identify the pathways downstream of the TCR that entrain expression of specific TNFRSF members we used mice that lack the MAPK kinase kinase

TAK1 (also known as MAP3K7) specifically in T cells (*Cd4*^{Cre} *x Tak1*$^{FL/FL}$ mice; Tak1 KO). TAK1 links TCR signaling to NF-κB activation[121] and thus is a key signaling intermediate downstream of the TCR. Previous studies have documented that TAK1-deficiency profoundly blocks thymic T_{reg} differentiation but did not examine effects on T_{reg} progenitors as these studies preceded the identification of this cell population[121, 122]. We found that T_{reg} progenitors could be detected in *Cd4*^{Cre} x *Tak1*$^{FL/FL}$ mice although their numbers are reduced (data not shown). These T_{reg} progenitors fail to express GITR, OX40, and TNFR2 on their surface and do not convert into mature Foxp3$^+$ T_{reg} cells (**Fig. 2-2c,d** and data not shown). In contrast, CD27 expression was not affected by TAK1-deficiency. Thus, TAK1 is required for TCR-induced expression of GITR, OX40, and TNFR2 on T_{reg} progenitors.

We also examined the surface expression of TNFRSF in T_{reg} progenitors from CD28-deficient mice, as CD28 plays an important role in T_{reg} development[70, 71, 73]. *Cd28*$^{-/-}$ mice showed a significant reduction in GITR and OX40 expression on T_{reg} progenitors relative to BALB/c controls; a more modest trend toward reduced TNFR2 expression was also observed (**Fig. 2-2c,d**, bottom panels). In contrast, CD27 surface expression on T_{reg} progenitors was unaffected by the absence of CD28. Similar results were obtained with *Cd28*^{AYAA} knockin mice (data not shown), which lack the distal proline-rich motif that was previously shown to be required for T_{reg} development[72]. This was not a strain-specific effect as we found

a similar reduction in the expression of GITR and OX40 on T_{reg} progenitors from *Cd28*$^{-/-}$ mice on the C57Bl/6 background (data not shown). Thus, CD28 signals are also required for upregulation of GITR and OX40 expression (and likely TNFR2) on T_{reg} progenitors.

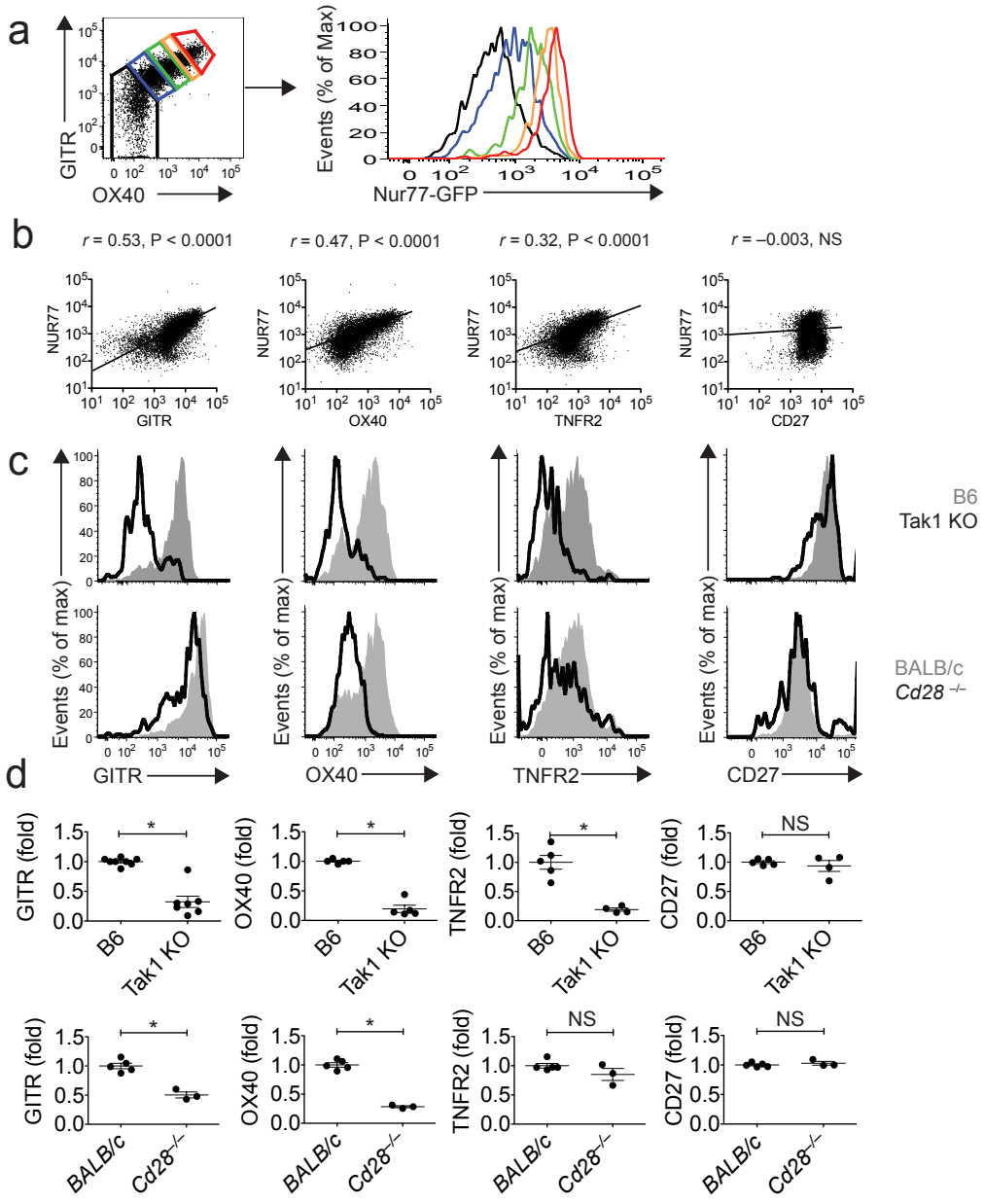


Figure 2-2. T_{reg} progenitors express select TNFRSF members in direct proportion to TCR signal strength. (**a**) Six-to-twelve week old female or male Nur77-GFP reporter mice were used to evaluate the role of TCR signal strength on the expression of TNFRSF members. T_{reg} progenitors were plotted on the basis of their expression of GITR and OX40 (left), and quartile gates representing

the spectrum of expression of these receptors were evaluated for Nur77-GFP expression in the corresponding histograms (right). Data are representative of four separate experiments (n = 4). (**b**) Raw values for GITR, OX40, TNFR2, and CD27 on T_{reg} progenitors were plotted versus Nur77-GFP expression. Lines represent the correlation between x and y variables and were used to calculate Pearson correlation coefficients (*r*). Correlation data for GITR are representative of 8 separate experiments (n = 8), for OX40 from 4 experiments (n = 4), for TNFR2 from 3 experiments (n = 3), and for CD27 from 3 experiments (n =3). (**c**) Histograms derived by gating on T_{reg} progenitors from *Cd4*^{Cre} x *Tak1*$^{FL/FL}$ mice (abbreviated as Tak1 KO*,* black histograms) and their wild type littermates (B6 background; gray shaded histograms; top panel) or *Cd28*$^{-/-}$ mice (black histograms) and their wild type littermates (BALB/c; gray shaded histograms; lower panel) are plotted demonstrating the expression of GITR, OX40, TNFR2, and CD27. (**d**) Cumulative data show the relative expression of GITR, OX40, TNFR2, and CD27 on T_{reg} progenitors from TAK1-deficient and CD28-deficient mice in comparison to wild type littermates. Raw MFI values obtained from each mouse after gating on CD25^{+}Foxp3^{-} T_{reg} progenitors were divided by the average MFI obtained from all wild type mice for normalization amongst separate experiments (mean ± SEM, for Tak1 KO experiments n ≥ 4 from at least two separate experiments, and for *Cd28*$^{-/-}$ n ≥ 3 from one experiment on the BALB/c background and n ≥ 3 from one experiment on the B6 background, * $P < 0.001$).

GITR, OX40 or TNFR2 costimulation drives T_{reg} maturation

To determine if signaling through GITR, OX40 or TNFR2 directly augments T_{reg} development by promoting maturation of T_{reg} progenitors, we utilized an assay that models step 2 of T_{reg} development *in vitro* (i.e., the IL-2-dependent conversion of T_{reg} progenitors into mature Foxp3+ T_{reg} cells). Purified T_{reg} progenitors sorted from Foxp3-GFP reporter mice were incubated in culture with low dose IL-2 with or without agonist TNFSF ligands. Conversion was assessed as the frequency of cells that acquired Foxp3-GFP expression during the course of the incubation. The addition of GITR-L–Fc, OX40-L–Fc, or TNF plus IL-2 enhanced conversion to Foxp3$^+$ T_{reg} cells by ~2-3 fold when compared to treatment with IL-2 alone (**Fig. 2-3a,b**). In contrast, three independent CD27 agonists (CD70–Fc, soluble CD70, and an agonist monoclonal antibody to CD27) did not enhance T_{reg} development (**Fig. 2-3a,b** and data not shown). T_{reg} progenitors do not express 41BB or CD30, and as expected, stimulation with 41BB-L or CD30-L did not affect T_{reg} development (data not shown).

One potential mechanism by which TNFRSF agonists promote T_{reg} development is to enhance sensitivity to IL-2. To directly test this, we examined CD25 expression on both Foxp3-GFP$^-$ T_{reg} progenitors and Foxp3-GFP$^+$ mature T_{reg} cells that had been stimulated with IL-2 ± GITR-L–Fc. CD25 expression was significantly enhanced on both T_{reg} progenitors and mature T_{reg} cells (**Fig. 2-3c,d**). To examine the relative effect of TNFRSF co-stimulation on IL-2-

dependent T_{reg} differentiation we carried out a dose-response experiment. Stimulation of T_{reg} progenitors with 1 U/mL IL-2 plus GITR-L or OX40-L resulted in 50% maximal conversion to mature T_{reg} cells; in the absence of GITR-L or OX40-L co-stimulation, almost 10-fold more IL-2 was required to achieve the same effect (**Fig. 2-3e**). Agonist GITR-L–Fc, OX40-L–Fc, or TNF did not promote T_{reg} maturation in the absence of IL-2 (data not shown). Finally, we observed that GITR-L–Fc costimulation enhanced IL-2-dependent STAT5 phosphorylation in T_{reg} progenitors, suggesting that GITR-L acts directly on the IL-2R signaling pathway (**Fig. 2-3f**). These data demonstrate that GITR, OX40, or TNFR2 costimulation augments IL-2 responsiveness in T_{reg} progenitors and allows them to effectively respond to low and likely physiologically relevant levels of IL-2.

An alternative mechanism by which TNFRSF costimulation enhances T_{reg} development is through the induction of anti-apoptotic proteins. To address this, we determined whether blocking apoptosis via the addition of the pan-caspase inhibitor, z-VAD-fmk, had a similar effect in promoting T_{reg} development to GITR-L–Fc. The addition of z-VAD-fmk significantly increased total cell recovery ($p<0.02$, data not shown); however, z-VAD-fmk did not enhance IL-2-dependent conversion of T_{reg} progenitors into mature T_{reg} cells (**Fig. 2-3g**). Thus, costimulation via GITR, OX40, and TNFR2 provide more than just pro-survival signals to developing T_{reg} cells.

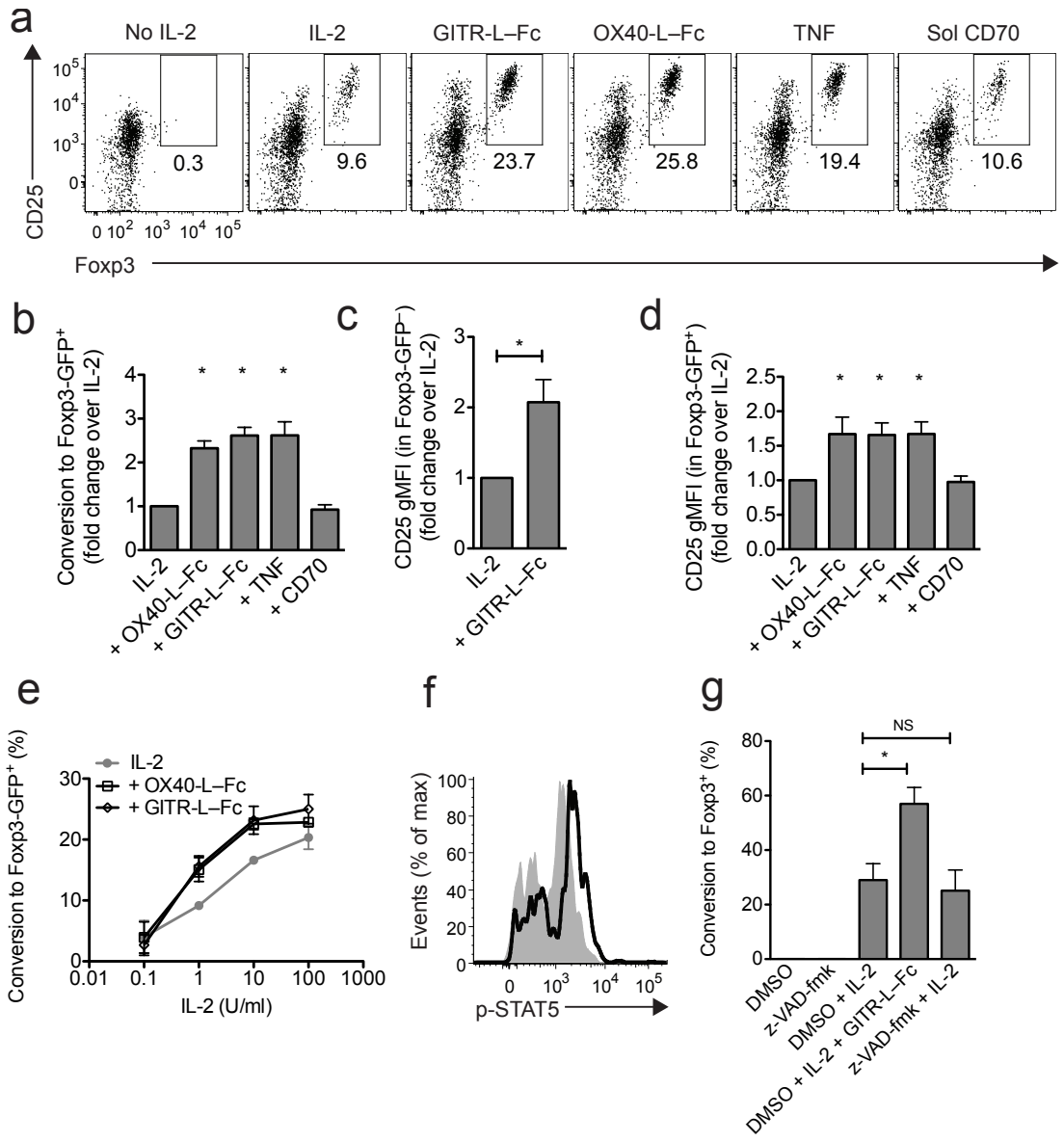


Figure 2-3. TNFRSF agonists enhance conversion of T_{reg} progenitors to Foxp3$^+$ T_{reg} cells. (**a,b**) Thymii from six- to eight-week old female or male Foxp3-GFP mice were harvested, pooled, and depleted of CD8SP, DP, and non-T cells prior to sorting purified CD4$^+$CD25$^+$Foxp3-GFP$^-$ T_{reg} progenitors. Sorted cells

were incubated in culture with or without IL-2 (1 U/mL) and 100 nM of the indicated TNFRSF agonists for 72 hours prior to flow cytometric analysis to determine the fraction of cells that acquired Foxp3-GFP during the course of the incubation (mean ± SEM from three independent experiments, n = 3, * P < 0.05). (**c**) The expression of CD25 amongst Foxp3-GFP^{-} T_{reg} progenitors after stimulation with IL-2 and GITR-L–Fc is plotted as fold change in geometric mean fluorescence intensity (gMFI) over IL-2 treatment alone (mean ± SEM derived from seven independent experiments, n = 7, * p < 0.05). (**d**) CD25 expression was measured as gMFI in the Foxp3-GFP^{+} gate after a 72h stimulation with IL-2 and GITR-L–Fc (mean ± SEM from three independent experiments, n = 3, * P < 0.05). (**e**) The efficiency of T_{reg} progenitor maturation with or without TNFRSF costimulation in response to a range of IL-2 concentrations is shown (mean ± SEM from three independent experiments, n = 3). (**f**) Intracellular staining for p-STAT5 was performed after stimulating sorted T_{reg} progenitors with IL-2 and/or TNFRSF agonists for 24 hours. Data are representative of one of two independent experiments (n = 2). (**g**) Shown are the percentages of sorted T_{reg} progenitors that converted to Foxp3-GFP^{+} T_{reg} cells during a 72h incubation with IL-2 and GITR-L–Fc or IL-2 and the pan-caspase inhibitor, z-VAD-FMK, using DMSO (v/v) as a vehicle control (mean ± SEM derived from three independent experiments, n = 3, * P < 0.05).

GITR- or OX40-deficiency modestly blocks T_{reg} development

We next sought to determine if TNFRSF members drive T_{reg} differentiation *in vivo* in a cell-intrinsic manner. To examine this we created bone marrow chimeras in which a 50-50% mixture of CD45.1/SJL wild type congenic marrow was mixed with CD45.2 *Gitr* $^{-/-}$ (also known as *Tnfrsf18)* bone marrow, depleted of mature lymphocytes, and engrafted into sublethally irradiated *Rag2* $^{-/-}$ recipients. After 10-18 weeks of reconstitution, chimeric recipients were euthanized and we assayed T_{reg} development by flow cytometry. As shown in **Fig. 2-4a**, *Gitr* $^{-/-}$ cells gave rise to conventional $CD4^+CD25^-Foxp3^-$ thymocytes equally as well as their wild type counterparts, but were outcompeted by wild type cells by approximately 30% in generating mature $CD25^+Foxp3^+$ thymic T_{reg} cells. T_{reg} progenitor populations were also slightly reduced in GITR-deficient cells, although the reduction was significantly less than observed in mature Foxp3+ T_{reg} cells. Similar results were observed in splenic T_{reg} cells (data not shown). We carried out similar studies using *Ox40* $^{-/-}$ (also known as *Tnfrsf4*) mice; we observed a 50% reduction in the ability of *Ox40* $^{-/-}$ -derived cells to give rise to $Foxp3^+$ T_{reg} cells (**Fig. 2-4b**). The effect of TNFR2 on thymic T_{reg} development has not been examined as closely but as TNFR2-deficient mice are viable it is unlikely that they are devoid of T_{reg} cells. Thus, deletion of any one TNFRSF member alone imposes only a modest cell-intrinsic T_{reg} developmental block.

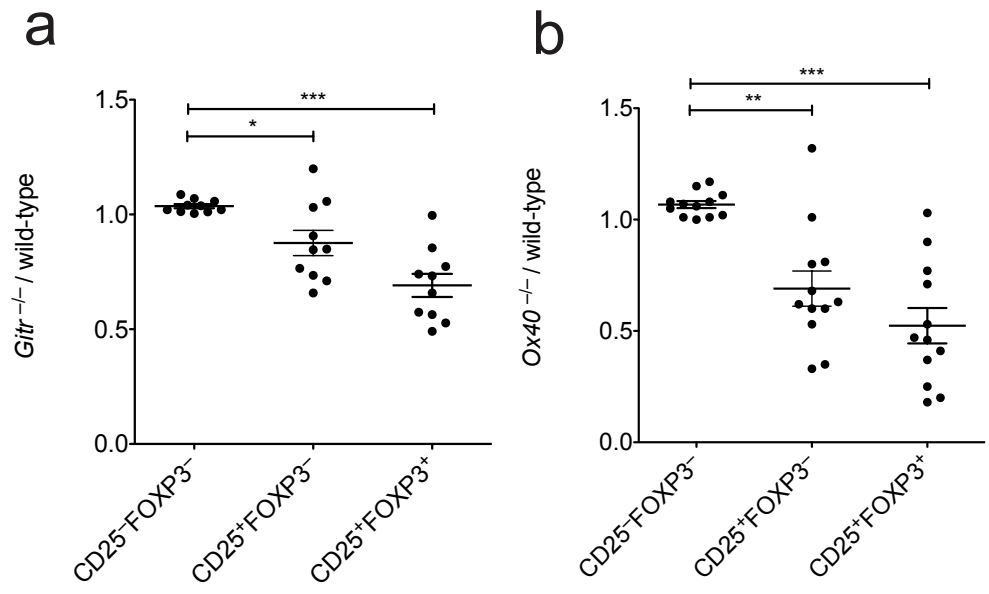


Figure 2-4. GITR- or OX40-deficiency imposes a modest cell-intrinsic thymic T_{reg} developmental block. Bone marrow from 8-12 week old female or male (**a**) *Gitr* $^{-/-}$ or (**b**) *Ox40* $^{-/-}$ mice (CD45.2) and age- and sex-matched congenic recipients was depleted of mature lymphocytes and mixed prior to engraftment into sublethally irradiated adult *Rag2* $^{-/-}$ mice. Ten to 18 weeks later thymii and spleen were analyzed for T_{reg} development and homeostasis by flow cytometry. (**a**,**b**) Shown are the percentages of $CD45.2^+$ vs. $CD45.1^+$ cells within conventional T cells ($CD25^-Foxp3^-$), T_{reg} progenitors ($CD25^+Foxp3^-$), and mature thymic T_{reg} cells ($CD25^+Foxp3^+$) normalized to the ratio of $CD45.2^+$ vs. $CD45.1^+$ within total CD4SP thymocytes. Results in panel **a** are from 10 individual chimeras from two independent experiments; results in panel **b** are from 12 individual chimeras from three separate experiments (mean ± SEM, $P < 0.05$ values calculated by ANOVA with Bonferroni's multiple comparison test).

TNFRSF blockade inhibits T_{reg} development and maturation

We next sought to determine if TNFRSF function redundantly to drive T_{reg} development by blocking multiple ligands simultaneously. Thymic organ cultures (TOCs) are a widely utilized technique to study thymocyte development in vitro with minimal manipulation[123]. We harvested thymic lobes from 1 day-old Foxp3-GFP reporter mice and subjected them to pair-wise comparison in TOCs for 14 days supplemented with either irrelevant isotype control antibodies or neutralizing anti-GITR-L, OX40-L, and CD70 or anti-GITR-L, OX40-L, CD70 and TNFR2. Neutralization of either three or four distinct TNFRSF members resulted in a dramatic reduction in the percentage of mature $CD25^{+}Foxp3^{+}$ T_{reg} cells within CD4SP thymocytes (**Fig. 2-5a,b**).

We also evaluated the acquisition of T_{reg} maturation markers by $Foxp3^{+}$ cells from TOCs with or without neutralizing antibodies to GITR-L, OX40-L and CD70 and found that inhibition of this costimulatory pathway markedly reduced not only the efficiency of T_{reg} generation, but also the expression of CD25 and Foxp3 (**Fig. 2-5c,d**). We also stained for the extracellular AMP nucleotidase, CD73, and the folate receptor, FR4, both of which mark highly functional T_{reg} cells[105, 124], and found significant reductions in the expression of both in $Foxp3^{+}$ cells when costimulation via GITR-L, OX40-L, and CD70 were blocked (**Fig. 5c,d**). Although the numbers of T_{reg} cells recovered were lower in cultures treated with neutralizing antibodies to GITR-L, OX40-L, CD70 and TNFR2 the same effects

on T_{reg} maturation markers were observed. Thus, neutralization of select TNFSF members inhibits thymic T_{reg} development and maturation.

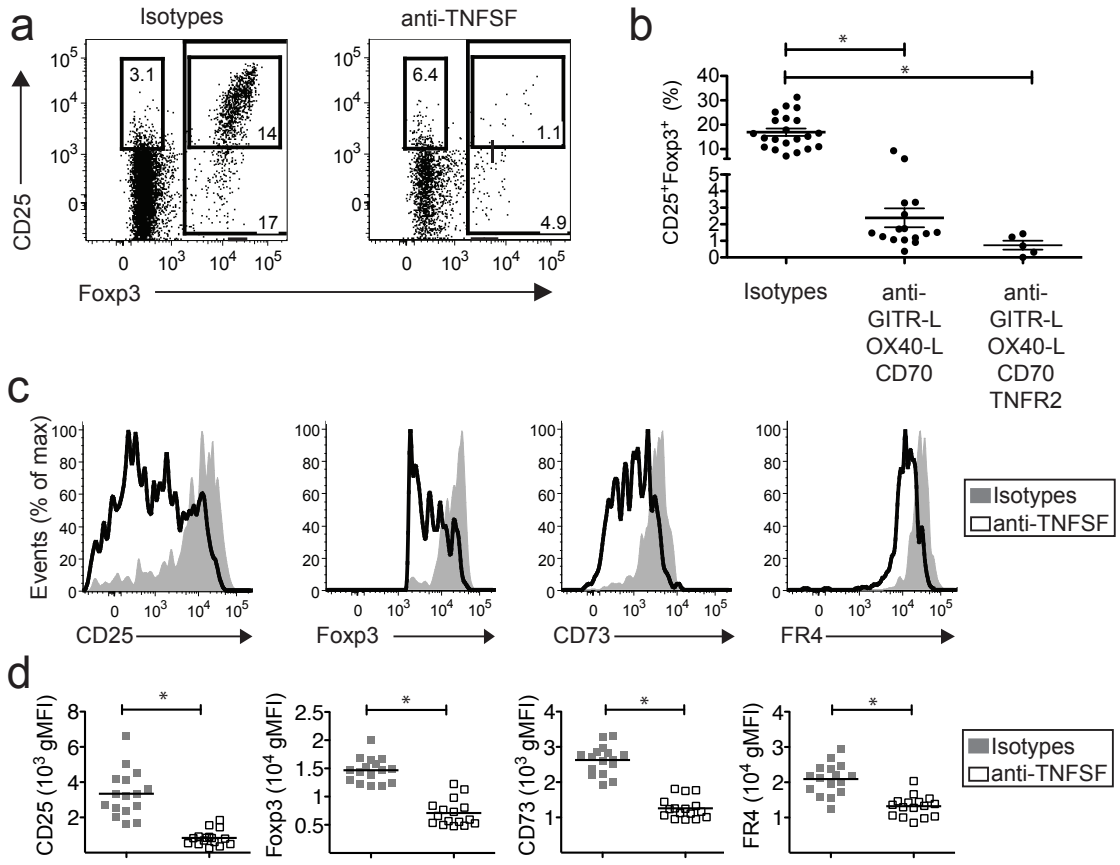


Figure 2-5. Antibody neutralization of TNFSF members in thymic organ cultures inhibits T_{reg} development and the acquisition of maturation markers. Neonatal Foxp3-GFP thymic lobes from one day-old pups were separated and plated in organ cultures with isotype controls or neutralizing antibodies to GITR-L, OX40-L, and CD70, or the combination of anti-GITR-L, OX40-L, CD70, and TNFR2. After 14 days, lobes were dissociated and T_{reg} development was analyzed by flow cytometry. (**a**) A representative comparison of CD4SP thymocytes from isotype- vs. antibody-treated thymic lobes is shown. (**b**) Shown are the percentages of $CD25^{+}Foxp3^{+}$ T_{reg} cells amongst total CD4SP

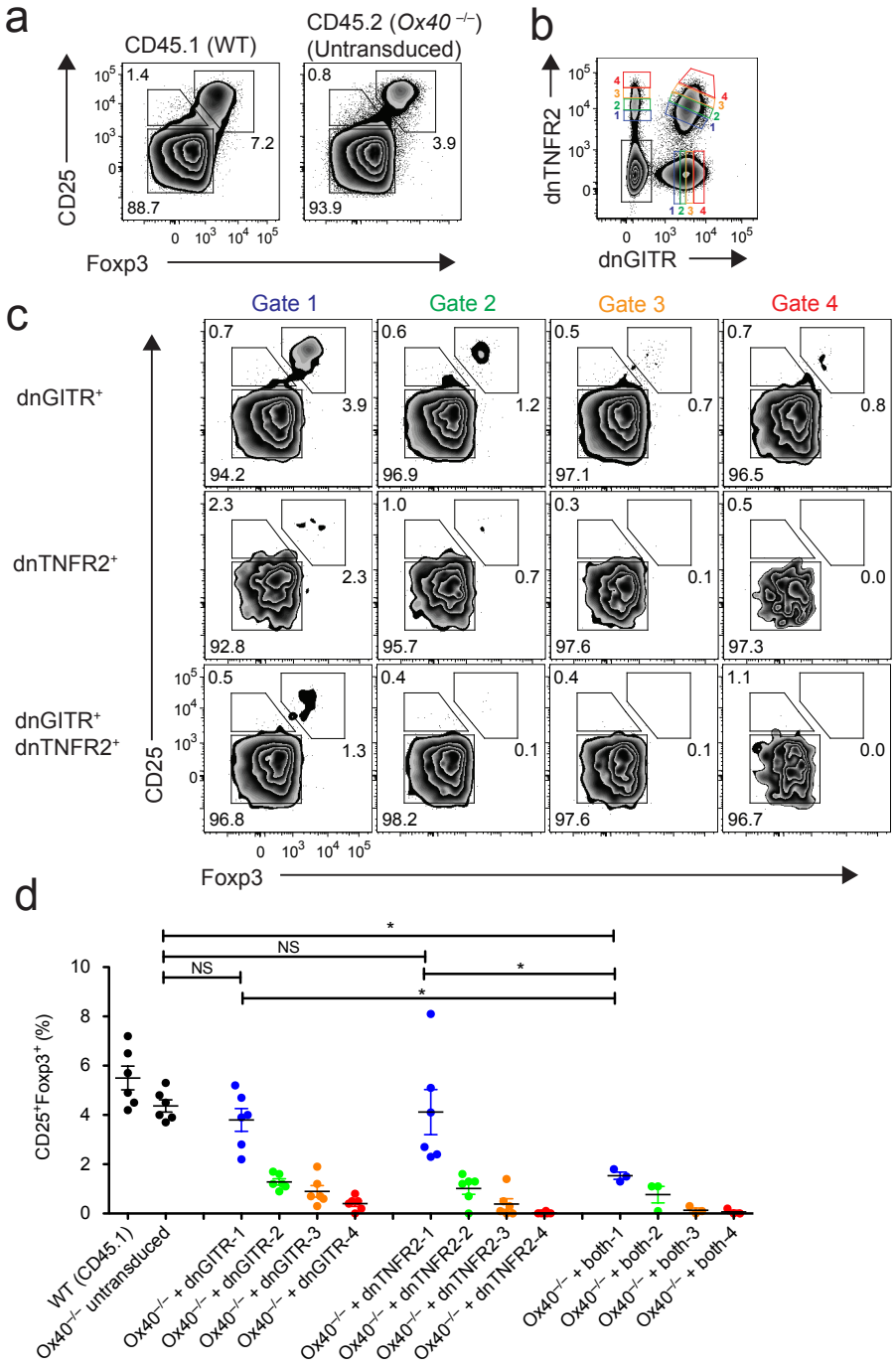
thymocytes (mean ± SEM, n=21 over four experiments for isotype controls, n=16 over three experiments for anti-GITR-L, OX40-L, and TNF; n=5 over two experiments for anti-GITR-L, OX40-L, CD70, and TNFR2; * $p < 0.05$ calculated by ANOVA with Bonferroni's multiple comparison test). (**c,d**) The expression of CD25, Foxp3, CD73, and FR4 expression in $CD4^{+}Foxp3^{+}$ cells from TOCs treated with isotype control antibodies- (solid gray) or TNFSF neutralizing Abs treated (open black) is displayed in the histograms and corresponding bar graphs below (n=16 from three independent experiments, mean ± SEM). P-values were generated using paired, two-tailed T tests (* $P < 0.0001$).

TNFRSF members collectively drive T_{reg} development

We next sought to determine whether inhibiting multiple TNFRSF members blocked T_{reg} development *in vivo* in a cell-intrinsic manner. For these studies we focused on OX40, GITR, and TNFR2 as their expression correlated with TCR signal strength while CD27 did not (**Fig. 2-2b**). One difficulty with these studies is that the genes encoding GITR and OX40 are spaced a mere ~10 kb apart on mouse Chromosome 4. *Tnfr2* is encoded nearby on Chromosome 4 as well. This overall pattern is the same in humans, except that *GITR, OX40, and TNFR2* are located on Chromosome 1. In mice, *Tnfsf18 (*encoding GITR-L) and *Tnfsf4 (*OX40-L) are also located next to each other on Chromosome 1. Because of this genomic organization, conventional double- or triple-knockout strategies to account for biologic redundancy in this pathway were not feasible. Instead, we exploited the fact that TNFRSF members must multimerize to function and generated tailless, dominant negative forms of GITR and TNFR2.

Dominant negative GITR (dnGITR) and dominant negative TNFR2 (dnTNFR2) were linked to IRES-GFP (pMIGR vector) and IRES-Thy1.1 (pMITR vector) reporter constructs and administered to *Ox40*$^{-/-}$ bone marrow (CD45.2) via retroviral transduction. Transduced cells were mixed with congenically marked wild type marrow (CD45.1 or CD45.1 x CD45.2), engrafted into sublethally irradiated *Rag2*$^{-/-}$ recipients, and reconstituted for 10-12 weeks. This strategy produced chimeric mice in which 5 populations of bone marrow cells could

compete to reconstitute the thymus: (1) wild type: CD45.1$^+$, (2) OX40-deficient: CD45.2$^+$, (3) OX40 plus GITR-deficient: CD45.2$^+$GFP$^+$, (4) OX40 plus TNFR2-deficient: CD45.2$^+$Thy1.1$^+$, and (5) OX40 plus GITR plus TNFR2-triple deficient (CD45.2$^+$GFP$^+$Thy1.1$^+$) (**Fig. 2-6a,b**). We then plotted the relative percentages of conventional CD4SP, T_{reg} progenitors, and T_{reg} cells within each of the gates shown in **Figure 2-6b** corresponding to increased expression of dnGITR, dnTNFR2, or both. T_{reg} progenitors were not significantly affected by reduced GITR, OX40, and TNFR2 signaling (data not shown). In contrast, superimposition of GITR- or TNFR2-deficiency in *Ox40*$^{-/-}$ cells led to a striking dose-dependent abrogation of T_{reg} development of >20 fold (**Fig 2-6c,d**). Importantly, we noted a further significant decrease in T_{reg} cells derived from cells deficient in all three TNF receptors. In fact, *Ox40*$^{-/-}$ cells expressing the highest levels of both dnGITR and dnTNFR2 completely lacked mature thymic T_{reg} cells (**Fig. 2-6c,d**, and for a summary of all statistical comparisons, see **Supplementary Table 1**). Thus, costimulation via GITR, OX40, and TNFR2 is collectively required for T_{reg} differentiation *in vivo*.


a
CD45.1 (WT)
CD45.2 (Ox40 −/−)
(Untransduced)
CD25
Foxp3
1.4
7.2
88.7
0.8
3.9
93.9
b
dnTNFR2
dnGITR
c
Gate 1
Gate 2
Gate 3
Gate 4
dnGITR+
dnTNFR2+
dnGITR+
dnTNFR2+
CD25
Foxp3
0.7
3.9
94.2
0.6
1.2
96.9
0.5
0.7
97.1
0.7
0.8
96.5
2.3
2.3
92.8
1.0
0.7
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0.1
97.6
0.5
0.0
97.3
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1.3
96.8
0.4
0.1
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0.4
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97.6
1.1
0.0
96.7
d
CD25+Foxp3+ (%)
NS
NS
*
*
*
WT (CD45.1)
Ox40−/− untransduced
Ox40−/− + dnGITR-1
Ox40−/− + dnGITR-2
Ox40−/− + dnGITR-3
Ox40−/− + dnGITR-4
Ox40−/− + dnTNFR2-1
Ox40−/− + dnTNFR2-2
Ox40−/− + dnTNFR2-3
Ox40−/− + dnTNFR2-4
Ox40−/− + both-1
Ox40−/− + both-2
Ox40−/− + both-3
Ox40−/− + both-4

Figure 2-6. GITR, OX40, and TNFR2 redundantly drive T_{reg} development in vivo. (a) Bone marrow from 8-12 week old male or female CD45.2^{+} *Ox40* $^{-/-}$ mice was harvested and prestimulated with IL-3, IL-6, and SCF prior to retroviral transduction with vectors encoding dominant negative GITR (dnGITR) and TNFR2 (dnTNFR2). Cells were mixed with congenically marked CD45.1^{+} control marrow and engrafted into adult sublethally irradiated *Rag*-deficient hosts for 10-12 weeks prior to analysis by flow cytometry. Representative flow cytometry plots derived by gating on CD45.1 WT or CD45.2 (*Ox40* $^{-/-}$) cells which were negative for GFP and Thy1.1 (untransduced) show the percentages of CD25^{-} Foxp3^{-} conventional CD4SP cells, CD25^{+}Foxp3^{-} T_{reg} progenitors, and CD25^{+}Foxp3^{+} T_{reg} cells amongst CD4SP thymocytes. (**b**) To evaluate dose-dependent effects of inhibiting multiple TNFRSF on T_{reg} development, *Ox40*$^{-/-}$ (CD45.2^{+}) thymocytes were plotted for their expression of dnGITR (GFP) and dnTNFR2 (Thy1.1) and gates were drawn on cells expressing increasing levels of GFP, Thy1.1, or both. Gates are labeled 1 through 4 and colored in blue, green, orange, and red, respectively. (**c**) Cells derived from gates in panel (**b**), representing increasing expression of dnGITR, dnTNFR2, or both, were evaluated for CD25 and Foxp3 expression to determine the frequencies of conventional CD4SP, T_{reg} progenitors, and mature T_{reg} cells within each gate (**d**) Cumulative data from one of three independent experiments are shown in the scatter plot below (mean ± SEM, n=6, * $P \leq 0.05$ as determined by 1-way ANOVA using Bonferroni's multiple comparison test).

TNFRSF costimulation shapes T_{reg} repertoire selection

The observation that TCR signal strength correlates with the expression of GITR, OX40, and TNFR2 in T_{reg} progenitors suggests that these receptors may play an important role in biasing the T_{reg} repertoire toward higher-affinity. To test this hypothesis, we generated Foxp3-RFP x Nur77-GFP reporter mice. We then sorted T_{reg} progenitors and stimulated them with IL-2 and increasing doses of agonist GITR-L–Fc. Increasing GITR-L concentration led to the development of a T_{reg} population with reduced expression of Nur77-GFP (**Fig. 2-7a**). This was not due to a loss of T_{reg} cells with high TCR signal strength, but rather inclusion of cells with lower Nur77-GFP expression. We found a clear negative correlation between GITR-L concentration and TCR signal strength within newly developed T_{reg} cells (r^2 = 0.81, **Fig. 2-7b**). These results demonstrate that altering the abundance of a TNFSF ligand directly affects selection of the T_{reg} repertoire.

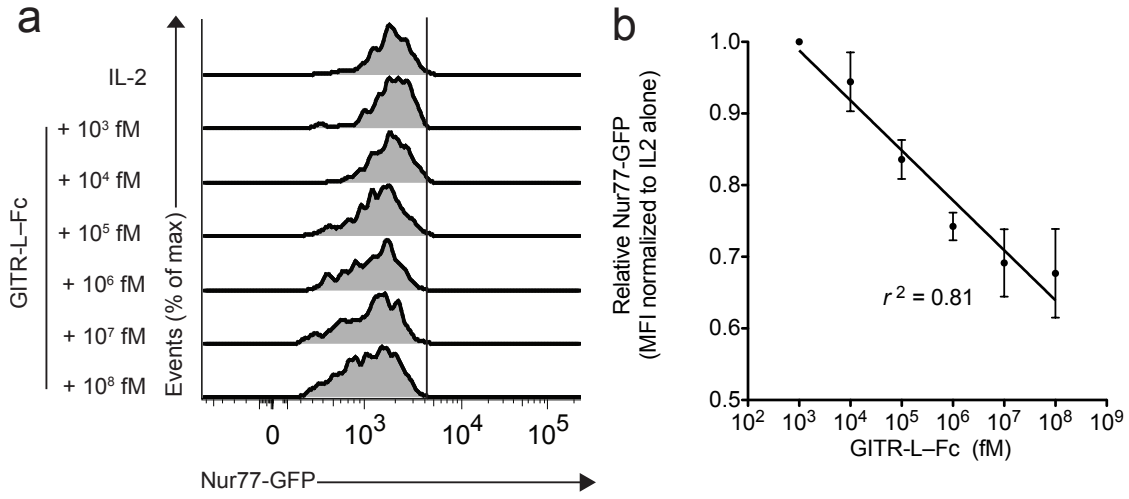

Figure 2-7. Excess TNFSF ligand broadens the T_{reg} repertoire to contain a greater fraction of cells with a lower affinity for self. (**a**) T_{reg} progenitors from 6-8 week old female or male Foxp3-RFP x Nur77-GFP dual reporter mice were isolated by cell sorting and stimulated in culture for 72h with 1 U/ml IL-2 and increasing concentrations of agonist GITR-L–Fc. Shown are stacked histograms displaying Nur77-GFP levels amongst converted Foxp3-RFP$^+$ T_{reg} cells. (**b**) Cumulative data from three independent experiments (n = 3) show a dose-dependent relationship between GITR-L–Fc concentration (x-axis) and the Nur77-GFP MFI (y-axis) within converted Foxp3-RFP$^+$ T_{reg} cells (as normalized to the value obtained with IL-2 treatment alone; mean ± SEM, n=3).

Discussion

T_{reg} development is a finely tuned process that is critical for preventing autoimmune disease. An intriguing feature of T_{reg} development is that this process discriminates between relative differences in TCR signal strength. T_{reg} cells with TCRs that recognize self-ligands with high-affinity predominate, although T_{reg} cells with lower-affinity TCRs can be found[67, 69]. The molecular program that accounts for this skewing process remains to be fully elucidated. One recent mechanism that has been proposed is TCR-induced expression of the NR4A family of transcription factors including NUR77, NOR1, and NURR1[125]. Expression of these transcription factors correlates with TCR signal strength[65] and these three transcription factors bind the *Foxp3* gene and are redundantly required for T_{reg} development[125]. This suggests that one potential mechanism to link TCR signal strength to T_{reg} development involves this transcription factor family. However, it is unlikely that this is the only mechanism that links TCR signal strength to T_{reg} development. For example, T_{reg} progenitors have clearly induced expression of NUR77 as documented by Nur77-GFP reporter mice yet they do not express Foxp3. In addition, in *Bim*$^{-/-}$ mice, many CD4SP thymocytes that have escaped deletion have high expression of NUR77 yet they do not differentiate into Foxp3^{+} T_{reg} cells[36]. TCR signals give rise to graded levels of NUR77 expression in thymocytes, yet Foxp3 is expressed in relative quantum increments with essentially no expression in T_{reg} progenitors and high levels in mature T_{reg} cells. If the effect of NUR77 family members on *Foxp3* transcription

was truly linear then one might expect a continuum of FOXP3 expression in T_{reg} progenitors culminating in that seen in mature T_{reg} cells; that is not observed experimentally suggesting that other mechanisms must also contribute to the process of linking TCR signal strength to T_{reg} differentiation.

We propose that the TNFRSF members GITR, OX40 and TNFR2 also play a critical role in converting strong TCR signals into molecular pathways that drive T_{reg} differentiation. In support of this hypothesis we observed that T_{reg} progenitors express much greater amounts of GITR, OX40, and TNFR2 relative to conventional $CD4^+CD25^-Foxp3^-$ thymocytes and that the level of expression of these receptors correlates directly with TCR signal strength as read out using Nur77-GFP reporter mice. This results in a competitive advantage as mature $Foxp3^+$ T_{reg} cells express even greater amounts of GITR and OX40. Blocking signaling via these receptors either by neutralizing antibodies or through combinations of gene knockouts and dominant negative receptors completely abrogates T_{reg} development. Thus, signaling through these receptors is collectively required for thymic T_{reg} development *in vivo*. Finally, the relative abundance of TNFSF ligands directly affects the TCR repertoire in developing T_{reg} cells. T_{reg} progenitors expressing the highest affinity TCRs, and hence the highest amounts of GITR, OX40, and TNFR2 can more effectively compete for their respective TNFSF ligands, and are thereby more likely to differentiate into mature T_{reg} cells.

The mechanism by which GITR, OX40 and TNFR2 promote T_{reg} development appears to primarily have effects on cell differentiation and not survival. GITR-L and OX40-L costimulation resulted in an ~10-fold increase in sensitivity to low doses of IL-2 that likely mimic what is available *in vivo*. This most likely reflects a direct effect on IL-2R signaling, as downstream STAT5 phosphorylation was also enhanced by costimulation with TNFSF agonists. Since GITR, OX40, and TNFR2 are known to signal via the NFκB pathway, we examined the expression of two NFκB-regulated genes that affect IL-2R signaling. We observed increased expression of CD25 on both T_{reg} progenitors and mature T_{reg} cells following costimulation with GITR-L, OX40-L, or TNF. In contrast, *Socs1* expression (which is induced by IL-2 signaling, and negatively regulated by NFκB via mIR-155[126]) was unaffected by GITR-L costimulation of T_{reg} progenitors (data not shown). When evaluating the effects of costimulation on mature T_{reg} cells, however, we observed that GITR-L reduced the *Socs1* expression induced by IL-2 stimulation by approximately 50% (data not shown). Thus, the predominant effect of GITR-L costimulation on enhancing T_{reg} progenitor sensitivity to IL-2 appears to be via CD25 induction.

An additional mechanism by which GITR, OX40, and TNFR2 could influence T_{reg} development is through the induction of anti-apoptotic genes[115, 117, 127, 128]. Such a mechanism has been proposed to account for the modest effect of CD27-CD70

on T_{reg} development[120]. We did observe that GITR, OX40, and TNFR2 costimulation weakly induced Bcl2 protein in T_{reg} progenitors (data not shown). However, blocking apoptosis with caspase inhibitors did not mimic the effect of GITR-L costimulation. Preliminary results with mice overexpressing Bcl2 indicate that ectopic anti-apoptotic gene expression does not prevent the defect in T_{reg} development imposed by TNFRSF blockade (data not shown). Thus, unlike CD27, which promotes cell survival, the induction of anti-apoptotic gene expression by GITR, OX40, or TNFR2 costimulation is not the dominant mechanism by which these TNFRSF drive T_{reg} differentiation.

It is unclear why three distinct TNFRSF members are required for T_{reg} differentiation. One possible explanation is that partial redundancy limits the chances of defects in T_{reg} development. A similar biological redundancy is observed for the NR4A transcription factor family in which neither NUR77, NOR1, or NURR1 is individually critical, but loss of all three stringently prevents T_{reg} development[125]. Alternatively, it is possible that selection for multiple TNFRSF members on mature T_{reg} cells is important for their later function. For example, ligation of OX40 or GITR on mature T_{reg} cells has been shown to limit their suppressor function[114, 116]. In this regard, enhanced selection of T_{reg} progenitors that express GITR and OX40 in the thymus could function as a possible safeguard to shut down their suppressive function during inflammatory responses to pathogens. Other reports have suggested that expression of TNFRSF

members is required to maintain survival of mature T_{reg} cells during inflammatory conditions[129, 130]. Finally, it is possible that higher expression of GITR and OX40 allows T_{reg} cells that most avidly recognize self-ligand to preferentially compete for IL-2 needed to sustain these cells in peripheral lymphoid organs.

In conclusion, our data support a model of T_{reg} differentiation in which developing thymocytes that bind MHC-II:self-peptide ligands with higher-affinity express greater amounts of GITR, OX40 and TNFR2. This elevated expression of select TNFRSF members on highly self-reactive T_{reg} progenitors results in a selective advantage that allows those cells to preferentially undergo maturation. Thus, upregulation of GITR, OX40, and TNFR2 by T_{reg} progenitors is a mechanism by which high-affinity TCR signals are translated into physical parameters that allow for more effective competition for IL-2 in a tightly regulated developmental niche. In agreement with previous reports[69], our model still allows for cells bearing TCRs with lower affinity for MHC-II:self-peptide complexes to emerge in the thymic T_{reg} population, but strongly favors T_{reg} differentiation in thymocytes receiving strong TCR signals since these cells most strongly upregulate TNFRSF receptors. This precisely matches the observed TCR affinity distribution in mature T_{reg} cells[65]. Thus, TNFSF ligand availability is an additional level of control by which thymic APCs define the T_{reg} developmental niche and couple TCR affinity to T_{reg} development.

Chapter 3

Comparing the developmental potential of two discrete populations of thymic Treg progenitors

Introduction

In 2008, back-to-back publications from the labs of Chyi Hsieh and Michael Farrar documented the existence of the $CD25^{+}Foxp3^{-}$ Treg progenitor population. It was clearly demonstrated that these progenitors required no additional T cell receptor stimulation, but only a signal from the IL-2 receptor to complete differentiation and develop into mature $CD25^{+}Foxp3^{+}$ Tregs[74, 75]. The high-level expression of CD25 by these cells presumably allows them to compete for limiting amounts of IL-2 available in the thymus. These observations fit well with former findings indicating that thymic Treg development requires *Il2rb* and subsequent signaling mediated by STAT5 activation[61].

In 2013, a publication arising from the laboratory of Al Singer proposed the existence of an alternative population of Treg progenitors. These cells do not express CD25, but express low levels of Foxp3 (reference [131]). Singer and colleagues sorted these putative Treg progenitors from the thymii of Foxp3 reporter mice and showed that by incubating them in culture with supra-physiologic doses of IL-2 (200 U/mL), that these cells converted into mature $CD25^{+}Foxp3^{+}$ Tregs. Subsequently, they attempted to compare these putative Treg progenitors to the formerly described population of $CD25^{+}Foxp3^{-}$ cells by sorting them from opposing congenic backgrounds, and co-injecting them intravenously into $Rag^{-/-}$ recipient mice along with effector T cells as a "source of IL-2." In this setting, the authors reported that $CD25^{-}Foxp3^{lo}$ cells matured into

$CD25^+Foxp3^+$ Tregs at an approximately 10-fold greater frequency than did the $CD25^+Foxp3^-$ Treg progenitors[131]. The experiments designed to gather these data were troubling in two ways. First, it has been demonstrated *in vitro* that $CD25^+Foxp3^-$ Treg progenitors convert into mature $CD25^+Foxp3^+$ Tregs at exceedingly low doses of IL-2, for example, 1 U/mL[111]. (We have found that Treg progenitor maturation into $CD25^+Foxp3^+$ Tregs becomes saturated at doses of IL-2 greater than 10 U/mL; MAF and SAM, unpublished data). Secondly, in regards to the *in vivo* competition experiments, thymic Treg progenitors mature in the thymus, and not the peripheral blood or peripheral lymphoid organs (which is where these progenitor cells would have likely seeded after an intravenous injection into recipient mice). A more thoughtful experimental design would be to compare these cells by co-injecting them into the thymii of recipient mice instead. Perhaps under more physiologic settings, different results would have been obtained.

Considering the well-accepted observation that thymic Treg development is an IL-2 and STAT5-dependent process[61], it becomes difficult to understand how cells lacking CD25 would be capable of outcompeting those bearing high levels of CD25 in a strictly IL-2 dependent process. We therefore set about conducting a series of experiments to carefully compare these two putative populations of Treg progenitors and to determine what their true developmental potential is both *in vitro* and *in vivo.*

Results

$CD25^{-}Foxp3^{lo}$ Treg progenitors convert into mature $CD25^{+}Foxp3^{+}$ Tregs with low-dose IL-2 *in vitro*

Singer and colleagues, in their 2013 manuscript, showed that $CD25^{-}Foxp3^{lo}$ Treg progenitors were able to mature into $CD25^{+}Foxp3^{+}$ Tregs when sorted and incubated in culture with a supra-physiologic, and likely saturating dose of IL-2 (200 U/ml). To determine if $CD25^{-}Foxp3^{lo}$ and $CD25^{+}Foxp3^{-}$ Treg progenitors can convert into mature $CD25^{+}Foxp3^{+}$ Tregs in vitro when provided low, and likely physiologically-relevant levels of IL-2, we sorted these populations from the thymii of Foxp3-RFP mice and stimulated them in culture for 72 hours with or without added IL-2 at a concentration of 1 U/ml. Not surprisingly, neither cell population converted into mature $CD25^{+}Foxp3^{+}$ Tregs without the addition of IL-2. However, in the presence of 1 U/ml of IL-2, both populations converted into mature $CD25^{+}Foxp3^{+}$ Tregs at a similar frequency. We observed no statistically significant differences at which this conversion process occurred (**Figure 3-1**). Thus, both $CD25^{-}Foxp3^{lo}$ and $CD25^{+}Foxp3^{-}$ Treg progenitors can convert into mature $CD25^{+}Foxp3^{+}$ Tregs *in vitro* when provided low-dose IL-2.

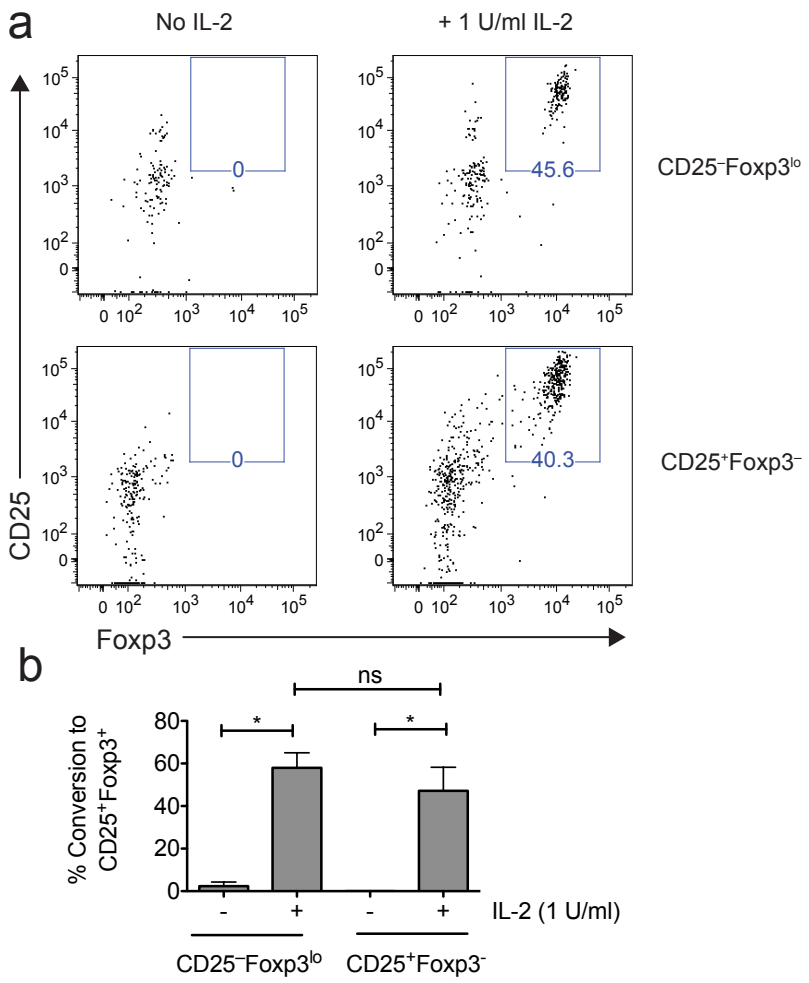

Figure 3-1. CD25^{-}Foxp3^{lo} and CD25^{+}Foxp3^{-} Treg progenitors convert into mature CD25^{+}Foxp3^{+} Tregs at similar rates when provided low-dose IL-2. (**a**) Thymii from Foxp3-RFP mice were harvested and depleted of non-CD4SP thymocytes prior to cell sorting of CD25^{-}Foxp3^{lo} and CD25^{+}Foxp3^{-} Treg progenitors. These populations were subsequently stimulated in culture for 72 hours with or without added IL-2 at a concentration of 1 U/ml prior to flow cytometric analysis to determine the frequency at which these cells matured into

the $CD25^{+}Foxp3^{+}$ mature Treg phenotype. (**b**) Cumulative data collected from three representative experiments as in panel (**a**) are presented. * $P<0.05$, ns = not significant. P-values were generated using ANOVA with Bonferroni comparison.

$CD25^{-}Foxp3^{lo}$ Treg progenitors have increased levels of CD122 compared to their $CD25^{+}Foxp3^{-}$ counterparts

After observing that $CD25^{-}Foxp3^{lo}$ Treg progenitors convert at a similar rate to $CD25^{+}Foxp3^{+}$ Tregs in response to low-dose IL-2 when compared to their $CD25^{+}Foxp3^{-}$ counterparts, one possibility we considered was that these cells express elevated levels of CD122, or the β chain component of the IL-2 receptor. This chain is the signaling component of the IL-2 receptor and upon ligand binding, three key tyrosine residues located in its intracellular domain become phosphorylated, leading to JAK1/3 autophosphorylation, and subsequent recruitment and activation of STAT5 (reference [84]). Thus, we screened these two populations of Treg progenitors by flow cytometry for the expression of CD122. Indeed, $CD25^{-}Foxp3^{lo}$ Treg progenitors had elevated levels of CD122 when compared to $CD25^{+}Foxp3^{-}$ cells (**Figure 3-2**). These results suggest that one mechanism by which $CD25^{-}Foxp3^{lo}$ Treg progenitors can effectively convert into mature $CD25^{+}Foxp3^{+}$ Tregs is by virtue of their elevated expression of the signaling component of the IL-2 receptor.

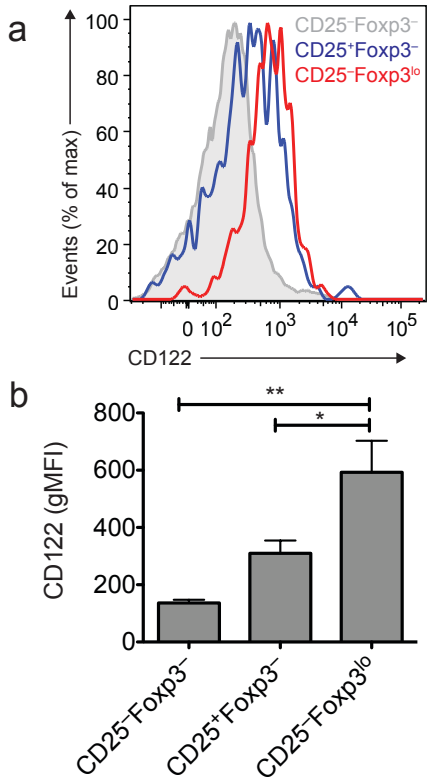

Figure 3-2. CD25^{-}Foxp3^{lo} Treg progenitors have increased levels of CD122 compared to CD25^{+}Foxp3^{-} cells. (**a**) Thymocytes were isolated and stained with a panel of antibodies to evaluate the expression of CD122 amongst CD25^{-} Foxp3^{-} conventional CD4SP cells (grey shaded histogram), CD25^{+}Foxp3^{-} Treg progenitors (blue histogram), and CD25^{-}Foxp3^{lo} Treg progenitors (red histogram). (**b**) Cumulative data are presented from two separate experiments analyzing a total of five mice and plotting the geometric mean fluorescence intensity (gMFI) of CD122 within the indicated populations of Treg progenitors. Statistical comparisons were made using ANOVA with Bonferonni's comparison. * $p < 0.05$, ** $p < 0.005$.

CD25^{-}Foxp3^{lo} Treg progenitors have resided in the thymus longer than their CD25^{+}Foxp3^{-} counterparts.

Comparison by HSA and Qa-2 staining allows for assessment of thymic maturation. As CD4SP thymocytes mature, their expression of Qa-2 increases, and that of HSA decreases[132]. In order to evaluate potential differences in maturation between these populations of Treg progenitors, we stained thymocytes for these markers and found that CD25^{-}Foxp3^{lo} Treg progenitors express higher levels of Qa-2 and lower levels of HSA when compared to their CD25^{+}Foxp3^{-} counterparts (**Figure 3-3**). Thus, this alternately described lineage of Treg progenitors have resided in the thymus longer than the CD25^{+}Foxp3^{-} Treg progenitor population. In fact, the expression of Qa-2 and HSA by CD25^{-} Foxp3^{lo} cells more closely resembles that of mature CD25^{+}Foxp3^{+} Tregs. The significance of this age difference is unclear, but may indicate that Treg progenitors not bearing CD25 develop via another mechanism that requires more time to complete; perhaps by residing longer in the thymus and by expressing elevated levels of CD122 (see **Figure 3-2**). A second possibility is that CD25^{-} Foxp3^{lo} cells result from intense competition for limiting IL-2 in the thymic medulla. Perhaps CD25^{+}Foxp3^{-} cells that failed to receive an IL-2 signal, but which initially received a TCR signal sufficient to induce NF-kappaB activation and remodeling of the *Foxp3* locus have internalized CD25 and may be ultimately destined for negative selection. Additional experiments would be required to test this hypothesis.

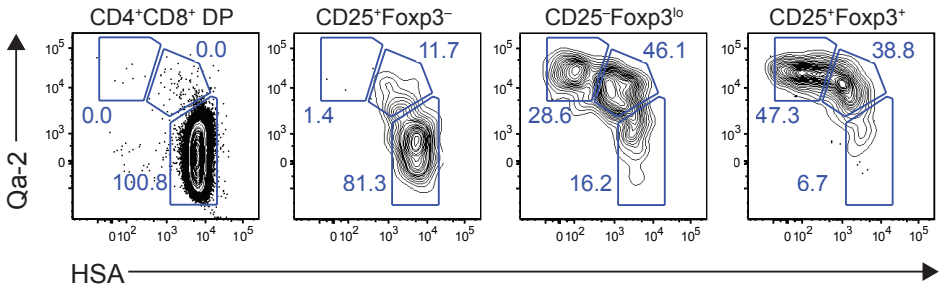

Figure 3-3. CD25^{-}Foxp3^{lo} Treg progenitors are more phenotypically mature than CD25^{+}Foxp3^{-} Treg progenitors. Thymocytes were isolated and stained with antibodies to CD4, CD8, CD25, Foxp3, Qa-2, and HSA (CD24). The left-most plot represents CD4^{+}CD8^{+}, double-positive (DP) thymocytes, and was used to initially place gates. These gates were subsequently applied to the right-hand three plots, which were pre-gated only on CD4-single positive thymocytes prior to selecting the indicated populations of Treg progenitors and mature CD25^{+}Foxp3^{+} Tregs. Data are representative from two separate experiments and a total of three mice.

CD25^{-}Foxp3^{lo} Treg progenitors also express TNFRSF and are responsive to costimulation

A key question is whether CD25^{-}Foxp3^{lo} cells are also affected by TNFRSF costimulation. We found that CD25^{-}Foxp3^{lo} T_{reg} progenitors also express GITR and OX40 in direct proportion to TCR signal strength (**Fig. 3-4a-d**). Stimulation of sorted CD25^{-}Foxp3^{lo} T_{reg} progenitors in the presence of low dose IL-2 plus GITR-L–Fc dose-dependently enhanced maturation to CD25^{+}Foxp3^{+} T_{reg} cells (**Fig. 3-4e**). Furthermore, as GITR-L concentration was increased, the average Nur77-GFP levels decreased in cells that differentiated into CD25^{+}Foxp3^{+} T_{reg} cells (**Fig. 3-4f**). Thus, TNFRSF costimulation plays a critical role in the differentiation of all potential T_{reg} progenitors into mature T_{reg} cells, and competition for TNFSF ligands regulates TCR repertoire selection during T_{reg} development.

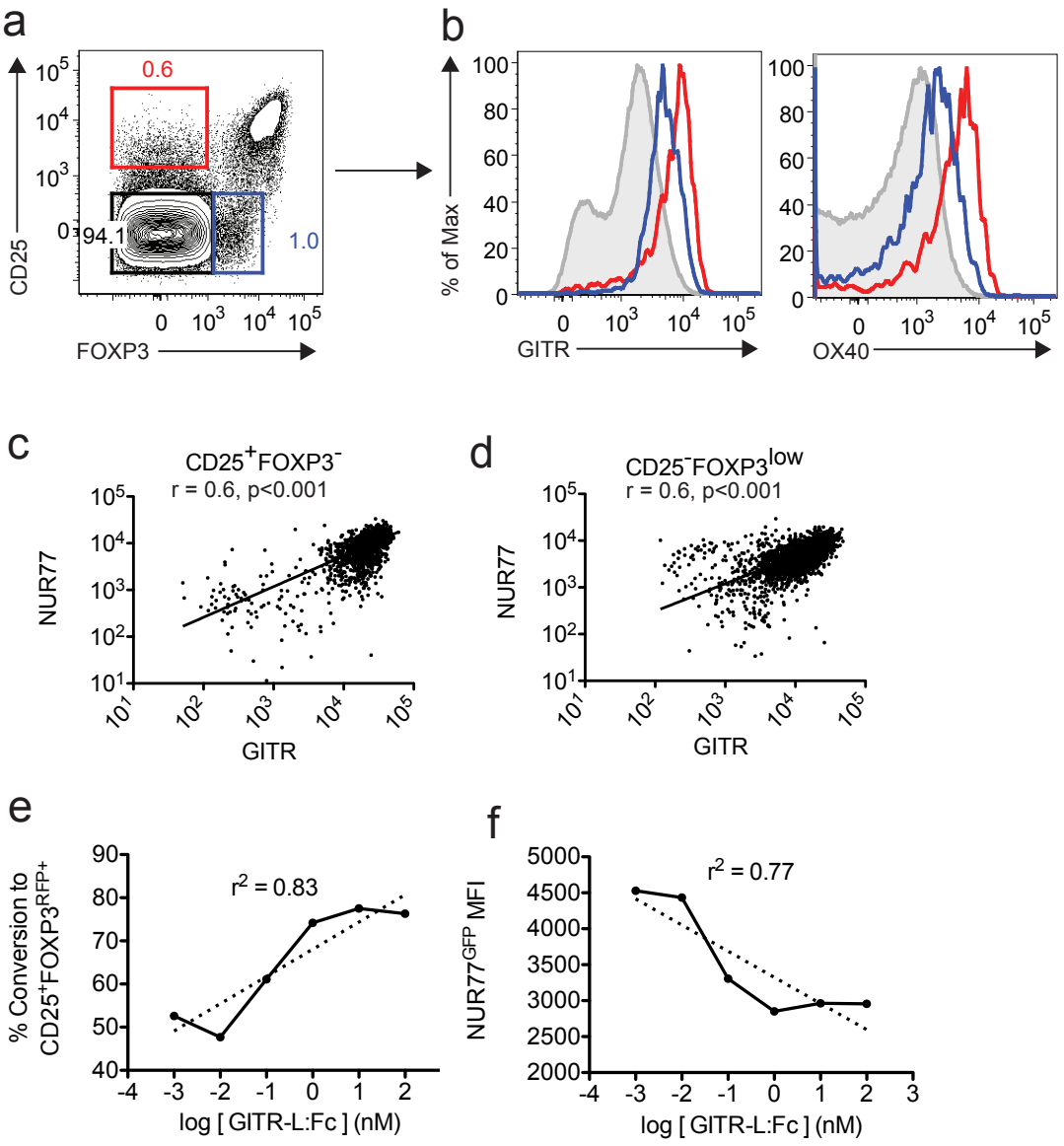

Figure 3-4. CD25^{-}Foxp3^{lo} Treg progenitors express TNFRSF in proportion to TCR signal strength and are responsive to TNFRSF costimulation. (**a**,**b**) CD25^{+}Foxp3^{-} Treg progenitors, and the alternately described population of Treg progenitors that are CD25^{-}Foxp3^{lo} are gated in red and blue, respectively, and are compared to conventional CD4SP (CD25^{-}Foxp3^{-}; gray shaded histogram) for

expression of GITR and OX40. Raw values for GITR and Nur77-GFP from (**c**) $CD25^{+}Foxp3^{-}$ and (**d**) $CD25^{-}Foxp3^{lo}$ Treg progenitors were plotted and used to calculate Pearson correlation coefficients. P-values assess whether the degree of correlation was statistically significant. (**e**) $CD25^{-}Foxp3^{lo}$ Treg progenitors were sorted from Foxp3-RFP x Nur77-GFP reporter mice and incubated with 1 U/mL IL-2 and increasing concentrations of GITR-L:Fc. The percentage of cells which upregulated CD25 and converted into mature $CD25^{+}Foxp3^{+}$ Tregs after 72h are shown in the scatter plot with a regression line applied. (**f**) The MFI of Nur77-GFP in newly formed $CD25^{+}Foxp3^{+}$ Tregs is shown after sorting $CD25^{-}Foxp3^{lo}$ Treg progenitors from Foxp3-RFP x Nur77-GFP reporter mice and stimulating for 72h with 1 U/mL IL-2 and increasing concentrations of GITR-L:Fc.

Discussion

The work by Singer and colleagues was important in identifying a novel, previously unappreciated population of thymic Treg progenitor cells, which paradoxically matured into $CD25^{+}Foxp3^{+}$ Tregs in the presence of IL-2, despite lacking CD25 expression. How this population could compete for limiting amounts of IL-2 without the high affinity alpha chain component of the receptor was initially difficult to understand. Upon careful review of this manuscript, we concluded that the experiments used to generate these data were flawed in two ways. First, saturating, and likely supra-physiologic doses of IL-2 were provided in the *in vitro* maturation assays. Secondly, the *in vivo* competition experiments were done by injecting these Treg progenitor populations intravenously, rather than intra-thymically. It is important to realize that Treg progenitors mature in the thymus, and not in the periphery.

We conducted similar *in vitro* assays using sorted populations of $CD25^{+}Foxp3^{-}$ and $CD25^{-}Foxp3^{lo}$ Treg progenitors and stimulated them in culture with low-dose IL-2 (1 U/ml), and were initially surprised to find that the $CD25^{-}Foxp3^{lo}$ cells converted into $CD25^{+}Foxp3^{+}$ Tregs at a similar frequency compared to their $CD25^{+}Foxp3^{-}$ counterparts. One hypothesis we considered was that the $CD25^{-}Foxp3^{lo}$ Treg progenitors might be able to mature without CD25 expression if they had increased levels of CD122, which is the signaling component of the IL-2 receptor. Indeed, careful flow cytometric analysis of these Treg progenitor

populations revealed that $CD25^{-}Foxp3^{lo}$ cells expressed roughly 2-fold more CD122 than did $CD25^{+}Foxp3^{-}$ cells. Furthermore, we revealed that $CD25^{-}Foxp3^{lo}$ cells are phenotypically more mature than are $CD25^{+}Foxp3^{-}$ Treg progenitors on the basis of their expression of Qa-2 and HSA. Thus, perhaps $CD25^{-}Foxp3^{lo}$ Treg progenitors are able to mature into $CD25^{+}Foxp3^{+}$ Tregs without initially expressing CD25 simply because they express more IL-2Rβ and because they reside in the thymus longer than the $CD25^{+}Foxp3^{-}$ cells.

We previously found that $CD25^{+}Foxp3^{-}$ Treg progenitors express the TNF receptor superfamily members GITR, OX40, and TNFR2 in direct proportion to TCR signal strength, and that these cells depend upon costimulation via these receptors in order to mature into $CD25^{+}Foxp3^{+}$ thymic Tregs. Thus, we evaluated this novel population of $CD25^{-}Foxp3^{lo}$ Treg progenitors to determine if they met similar criteria. We found that $CD25^{-}Foxp3^{lo}$ Treg progenitors express GITR and OX40, although they bear slightly lower levels than $CD25^{+}Foxp3^{-}$ cells. The expression of these TNFRSF was also directly proportional to TCR signal strength as measured using Nur77-GFP reporter mice. As is the case for $CD25^{+}Foxp3^{-}$ Treg progenitors, $CD25^{-}Foxp3^{lo}$ cells mature into $CD25^{+}Foxp3^{+}$ Tregs at a greater rate when costimulated with agonists to TNFRSF in the presence of IL-2. Moreover, when GITR-L concentration was increased, the average Nur77-GFP levels in cells that differentiated into $CD25^{+}Foxp3^{+}$ Treg cells decreased. Thus, TNFRSF costimulation plays a role in the differentiation of

all potential Treg progenitors into mature Treg cells, and competition for TNFSF ligands regulates TCR repertoire selection during Treg development.

Future Directions

The most robust methodology for directly determining if one or both of these populations of Treg progenitors are direct precursors of mature Foxp3^{+} thymic Tregs is to conduct TCR sequencing analysis and look at the degree of similarity in the TCRs used by these populations. We have generated Foxp3-RFP reporter mice bred onto a background with a partially fixed TCR repertoire to conduct these experiments. Hsieh and Rudensky were the first to demonstrate faithful analysis of TCR usage was possible by sequencing Vα2 (TRAV14) TCRα chains associated with a fixed (transgenic) TCRβ chain[66]. This strategy partially restricts the TCR repertoire by enforcing allelic exclusion at the TCR alpha chain locus using mice that are heterozygous for the Cα null mutation. We have recently generated *Foxp3-RFP* x *Nur77-GFP* x *Tcliβ* transgenic x Tcr Cα$^{+/-}$ in our lab and used flow cytometry to sort CD25^{-}Foxp3^{-}, CD25^{+}Foxp3^{-}, CD25^{-}Foxp3^{lo}, and CD25^{+}Foxp3^{+} cell subsets from a total of four animals. Cells were sorted into RNA extraction lysis buffer, RNA was isolated, and cDNA was synthesized using random primers. We have shipped these samples to our collaborator, Cal Williams, M.D., Ph.D, at the Medical College of Wisconsin, who is currently performing TCR sequencing analysis.

In order to compare $CD25^{+}Foxp3^{-}$ and $CD25^{-}Foxp3^{lo}$ Treg progenitors in a competitive *in vivo* experiment, we have bred Foxp3-GFP reporter mice onto the Thy1.1 congenic background. This will allow us to sort these two populations of Treg progenitors on opposing backgrounds and then co-inject them into the thymii of recipient CD45.1 mice using ultrasound guidance. We will then remove the thymii of recipient mice three days after injection, pull down donor cells using magnetic beads, and then determine which fraction of Foxp3-GFP^{+} cells has arisen from the two input populations based on Thy1.1 vs. Thy1.2 expression. We have begun setting up these experiments now and are eagerly awaiting data to determine the answers to several questions. First, we will be able to definitively determine the developmental potential of each of these populations in a competitive setting. Our hypothesis is that done correctly, when engrafted into the thymii of recipient mice (as opposed to the peripheral blood and lymphoid organs), that $CD25^{+}Foxp3^{-}$ and $CD25^{-}Foxp3^{lo}$ Treg progenitors will both mature into $Foxp3^{+}$ Tregs at a similar rate. Secondly, it may be possible that one of these cell populations is a precursor to another. For example, if $CD25^{+}Foxp3^{-}$ Treg progenitors are not provided with adequate IL-2, perhaps they down-regulate CD25 and evolve into the $CD25^{-}Foxp3^{lo}$ population. Indeed, this fits with unpublished data from S.A.M. and M.A.F. looking at Treg development in $Cd4^{Cre}$ x $Il2^{FL/FL}$ mice in which T cells cannot produce IL-2; in this setting we observed a 2-3 fold increase in the percentage of $CD25^{-}Foxp3^{lo}$ cells in the thymus. It would also be of value to perform these intrathymic engraftment

experiments using *Cd45.1* x *Foxp3-DTR* mice pre-treated with diphtheria toxin to remove endogenous Tregs. This scenario would provide competitive pressure to allow the two input populations of Treg progenitors to compete for development and then seed the peripheral lymphoid organs with mature $Foxp3^+$ Tregs. It is quite obvious when looking at $Foxp3^+$ cells in the spleen and lymph nodes that there is a very broad spectrum of expression of CD25 in peripheral Tregs. One might hypothesize that $CD25^+Foxp3^-$ Treg progenitors go on to form $CD25^+Foxp3^+$ Tregs in the periphery, while perhaps the $CD25^-Foxp3^{lo}$ cells form $CD25^{lo}Foxp3^+$ peripheral Tregs. Finally, by separately injecting these input populations of Treg progenitors into the thymii of *Foxp3-DTR* mice, it would also be possible to determine the suppressive function of the resultant Tregs that arise from the two populations of Treg progenitors, since *Foxp3-DTR* mice will die within 3 weeks of autoimmunity if not provided with donor Tregs.

Chapter 4

Conclusions and Models

Conclusion

$CD4^+Foxp3^+$ regulatory T cells are crucial for maintaining immune homeostasis and preventing autoimmune disease. While this subset of $CD4^+$ T cells is numerically small, the consequences of its absence are profound. This is exemplified in the rare but devastating condition called IPEX in which boys lacking functional *Foxp3* fail to form Tregs and die from multi-organ autoimmunity prior to the age of three if not given a bone marrow transplant. More subtle defects in Treg development and/or function are associated with numerous human autoimmune diseases and inflammatory states. The precise mechanisms that govern Treg development are therefore of interest to immunologists and are critically important in order to improve therapies for autoimmunity.

In Chapter 2 of this thesis, we demonstrated that thymic $CD25^+Foxp3^-$ Treg progenitors express three TNFRSF members: GITR, OX40, and TNFR2. We showed that the ligands that bind these receptors are present on the antigen presenting cell subsets in the thymus that actually mediate Treg differentiation. Using Nur77-GFP reporter mice initially created by Amy Moran during her graduate studies at the University of Minnesota, we demonstrated that the expression of GITR, OX40, and TNFR2 is directly proportional to TCR signal strength in developing Tregs. That is, the cells that experience the strongest signals via the TCR during development express the highest levels of GITR, OX40, and TNFR2. By purifying Treg progenitors from Foxp3-GFP reporter

mice, we demonstrated that costimulatory signals provided via GITR, OX40, or TNFR2 enhance differentiation into mature $CD25^{+}Foxp3^{+}$ Tregs by driving up the expression of CD25 and more effectively allowing these progenitor cells to compete for limiting amounts of IL-2. We found elevated levels of P-STAT5 in Treg progenitors costimulated with GITR-L to confirm these observations.

In vivo, genetic ablation of GITR or OX40 led to a modest, but statistically significant cell-intrinsic impairment of Treg development in the thymus. We hypothesized that this defect would be augmented if multiple TNFRSF could be eliminated concurrently and therefore adopted two strategies to test this hypothesis. First, we utilized neonatal thymic organ culture and multiple TNFRSF blocking antibodies and revealed a much more substantial Treg developmental defect. In the small number of cells that actually did mature into $Foxp3^{+}$ Tregs in thymic organ cultures treated with TNFRSF blocking antibodies, we found that four markers of Treg maturation were significantly lower: CD25, Foxp3, FR4 and CD73. To test the hypothesis that TNFRSF redundantly drive Treg development *in vivo*, we designed dominant negative forms of GITR and TNFR2, and introduced those constructs into OX40-deficient bone marrows via retroviral transduction. When set up in competitive mixed bone marrow chimeric recipients, this strategy revealed a near complete blockade of Treg development when at least two of three TNFRSF expressed by Treg progenitors were ablated.

Finally, we wished to test the hypothesis that TNFRSF costimulation would shape the Treg TCR repertoire. To do this, we created Foxp3-RFP x Nur77-GFP mice and used cell sorting to isolate Treg progenitors from those animals. Upon incubating Treg progenitors with IL-2 and increasing doses of TNFRSF agonist, we found that as the dose of TNFSF ligand was increased, the average Nur77-GFP levels were decreased in the cells that became Foxp3^{+} during the course of the incubation. Cells with lower TCR signal strength that would have otherwise failed to reach a level of IL-2 responsiveness sufficient for Treg development—if provided with TNFRSF costimulation—gained CD25, elevated P-STAT5, and ultimately entered into the Treg lineage.

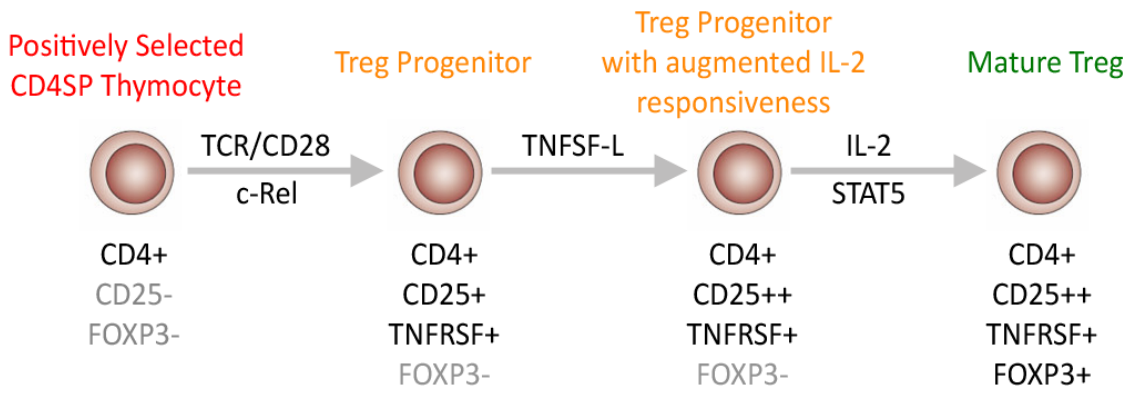

Figure 4-1. Revised model of thymic Treg development. A fraction of CD4SP thymocytes, by virtue of the random nature of TCR gene rearrangement, will experience high-affinity TCR:self-peptide:MHC class II interactions in the thymic medulla. This, in concert with adequate costimulation via CD28 leads to induction of the NF-kappaB pathway, and c-Rel translocates to the nucleus. This signaling event drives expression of CD25, as well as GITR, OX40, and TNFR2 (abbreviated here as 'TNFRSF+'). TNFRSF-positive Treg progenitors which subsequently receive costimulation via TNFSF-ligands (TNFSF-L) gain elevated levels of CD25, which allows them to more effectively compete for limiting amounts of IL-2. Transmission of an IL-2 signal via STAT5 ultimately drives Foxp3 expression and entrance into the thymic Treg lineage.

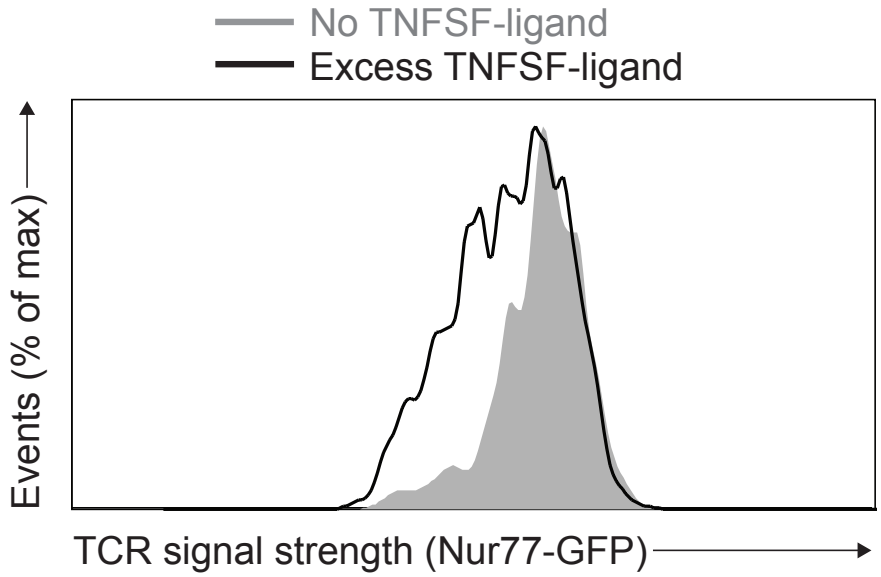

Figure 4-2. Model of the effects of TNFRSF costimulation on Treg TCR repertoire selection. Two theoretical distributions of TCR affinity amongst thymic Tregs are displayed. If no TNFSF-ligand is available for costimulation (grey shaded histogram), only cells bearing TCRs of the highest affinity gain enough IL-2 sensitivity to effectively compete for cytokine and ultimately gain entrance into the Treg lineage. In conditions of excess TNFSF-ligand (black open histogram), competition for costimulation via TNFRSF is eliminated. Under these circumstances, cells with lower-affinity TCRs, which normally would not have sufficient IL-2 responsiveness to compete for cytokine, now gain added CD25, P-STAT5, and ultimately entrance into the Treg lineage. This precisely matches the TCR distribution in $CD4^+Foxp3^+$ thymocytes seen by Moran *et al* upon crossing Nur77-GFP reporter mice onto animals expressing a constitutively activated form of STAT5 (reference [65]).

In Chapter 3 of this thesis, we aimed to compare the developmental potential of two populations of thymic Treg progenitors: the well-known population of progenitors which are $CD25^{+}Foxp3^{-}$, and the more recently described $CD25^{-}Foxp3^{lo}$ population. We showed that both of these cell types, despite their contrasting expression of the high-affinity alpha chain component of the IL-2 receptor, responded equivalently to low-dose IL-2 and could mature into $CD25^{+}Foxp3^{+}$ Tregs *in vitro*. One hypothesis we explored to explain this observation is that perhaps $CD25^{-}Foxp3^{lo}$ Treg progenitors might express higher levels of CD122, the beta chain of the IL-2 receptor that actually mediates activation of STAT5 and the signal transduction pathway downstream of receptor ligation. Indeed, we found that $CD25^{-}Foxp3^{lo}$ Treg progenitors express roughly 2-fold increased levels of CD122 when compared to $CD25^{+}Foxp3^{-}$ cells. In addition, we found that $CD25^{-}Foxp3^{lo}$ Treg progenitors have resided in the thymus longer than their $CD25^{+}Foxp3^{-}$ counterparts. Thus, one potential mechanism by which $CD25^{-}Foxp3^{lo}$ Treg progenitors are able to respond to IL-2 in the absence of CD25 is that they express increased levels of CD122 and remain in the thymus longer in order to differentiate. We also investigated the expression of members of the TNFRSF by $CD25^{-}Foxp3^{lo}$ Treg progenitors. We found they also express GITR and OX40, and do so in direct proportion to TCR signal strength. *In vitro*, $CD25^{-}Foxp3^{lo}$ Treg progenitors also dose-dependently responded to TNFRSF costimulation. As levels of GITR-L were increased, the efficiency at which $CD25^{-}Foxp3^{lo}$ Treg progenitors converted into $CD25^{+}Foxp3^{+}$

Tregs also increased. Lastly, we showed that as is the case for $CD25^{+}Foxp3^{-}$ Treg progenitors, the TCR repertoire of the $CD25^{-}Foxp3^{lo}$ Treg progenitor population is also biased by TNFRSF costimulation. Thus, TNFRSF costimulation is an important mediator of Treg maturation and repertoire selection for all potential subsets of thymic Treg progenitors. Ongoing studies involving TCR sequencing and *in vivo* competition assays after intra-thymic injection will allow us to fully characterize these two populations of cells and truly understand their developmental potential as progenitors of thymic Tregs.

Chapter 5

Methods

Mice.

All mice were housed in specific-pathogen free facilities at the University of Minnesota and experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee. *Gitr* $^{-/-}$ mice were previously described[115] and provided by Drs. Carlo Riccardi and Ethan Shevach. *Ox40* $^{-/-}$, Foxp3-GFP, Foxp3-RFP, *Rag2* $^{-/-}$, and *Cd4*^{Cre} mice were purchased from the Jackson Laboratory (Bar Harbor, ME, stock numbers 012839, 006772, 008374, 008449, and 013234 respectively). *CD45.1* (B6.SJL) mice were purchased from the NCI (Bethesda, MD). Nur77-GFP BAC reporter mice[65], *Tak1*$^{FL/FL}$ and *Bcl2*-Tg mice were previously described[133, 134]. *Cd28*$^{-/-}$ and *Cd28*^{AYAA} knock-in mice were previously described[135]. All mice used were on the C57Bl/6 background, except for *Cd28*$^{-/-}$ and *Cd28*^{AYAA} mice and their wild type littermates, which were on a BALB/c background. Mice were randomly selected for experiments from our mouse facility in age-matched cohorts. The investigators were not blinded to genotype during data acquisition.

Tissue preparation and cell isolation.

For analysis of thymocyte and Treg development thymii and peripheral lymphoid organs were mechanically dissociated into PBS with 2% FBS and 2 mM EDTA, pH 7.4, using frosted glass slides. Cell suspensions were passed through 70 μm filters and washed prior to staining. For analysis of thymic DC and epithelial cells (TEC), thymii were dissected into 5 mL complete RPMI supplemented with

Collagenase D (120 Mandl U/mL) and DNase I (3 U/ml, both from Roche Applied Sciences, Indianapolis, IN) and diced into ~20 pieces and placed in a 37˚C incubator for 30 minutes. Pieces were further homogenized by pipetting and the addition of EDTA (20 mM final concentration) which was immediately diluted 10-fold with buffer and the suspension filtered through 70 μm mesh. Thymic DC were magnetically enriched by labeling with anti-CD11C FITC (N418, eBioscience, San Diego, CA) and secondarily with anti-FITC microbeads (Miltenyi Biotec, Auburn, CA). Thymic epithelial cells were enriched by magnetic depletion of leukocytes using biotinylated anti-CD45 antibody (30F11, eBioscience) and streptavidin-conjugated microbeads (Miltenyi Biotec).

Flow cytometry and antibodies.

All analysis was conducted in the Center for Immunology Flow Cytometry Core Facility using BD LSR II and Fortessa cytometers (BD, San Jose, CA). For surface staining, cells were stained for 20 minutes with 1:100 dilutions of fluorochrome-conjugated antibodies prior to washing and analysis or intracellular staining. Anti-mouse CD4 (GK1.5 or RM4-4), CD8 (53-6.7), CD25 (PC61.5), CD122 (TMb1), Foxp3 (FJK-16s), GITR (DTA-1), OX40 (OX86), TNFR2 (TR75-32), CD27 (LG.7F9), 4-1BB (17B5), GITR-L (YGL386), OX40-L (RML134L), CD70 (FR70), CD45 (30-F11), MHC Class II (I-A/I-E, M5/114.15.2), BP1 (6C3),

Thy1.2 (30-H12 or 53.21), CD11C (N418), FR4 (eBio 12A5), CD73 (TY/11.8), CD103 (2E7), KLRG1 (2F1), and CD45R/B220 (RA3-6B2) were from eBioscience (San Diego, CA). Fluorescein labeled Ulex Europaeus Agglutinin I (UEA I) was purchased from Vector Laboratories (Burlingame, CA). FITC Anti-GFP was from Rockland Immunochemicals (Gilbertsville, PA). Intracellular detection of Foxp3 and P-STAT5 was performed as previously described[75].

Immunofluorescence microscopy.

Tissues were frozen in 2-methylbutane surrounded by dry ice. Frozen blocks were cut into 7 μm thick sections and fixed in acetone. Nonspecific binding was blocked for 1 hour with 5% bovine serum albumin in PBS. Sections were then stained with hamster anti-CD11C conjugated-FITC; rabbit A488 conjugated anti-K5; UEA-1 conjugated-FITC; goat anti-OX40-L; rat anti-TNF PE (eBioscience); or rat anti-GITR-L PE (eBioscience). Signal from PE-conjugated anti-TNF and anti-GITR-L or irrelevant isotype controls was amplified with rabbit anti-PE (NB120-7011; Novus Biologicals) followed by staining with donkey anti-Rabbit Cy3 (Jackson Immunoresearch). Jackson Immunoresearch secondary antibodies conjugated to various fluorochromes were used for staining of unconjugated antibodies including Cy3–conjugated donkey anti-rat, Alexa-488-conjugated donkey anti-rabbit, and Cy3-conjugated donkey anti-goat. Sections were incubated for 1 hour in the dark at room temperature, and then washed with PBS 3 times prior to the next stain. After the final wash, slides were stained with DAPI

(4,6-diamidino-2-phenylindole; Invitrogen) for 10 minutes before Fluoromount G was added with a cover slip. Tiled images were acquired with an automated Leica DM5500B microscope and analysis was done with Adobe Photoshop.

Treg progenitor conversion assays.

Treg progenitors were isolated as previously described[75]. Briefly, 6-8 week old Foxp3-GFP thymii were dissected, dissociated, and pooled (up to 8 mice per experiment). CD4SP cells were enriched by magnetically depleting with biotinylated anti-CD8, CD11B, CD11C, CD19, CD45R/B220, MHC class II, NK1.1, and Ter119 antibodies (eBioscience) followed by secondary labeling with streptavidin-conjugated microbeads (Miltenyi Biotec). Enriched CD4SP cells were stained with fluorchrome-conjugated anti-CD4, CD25, and streptavidin prior to sorting $CD4^+CD25^+GFP^-$ cells using a BD Facs Aria sorter (BD Biosciences). Purified T_{reg} progenitors were incubated in complete RPMI and supplemented with human IL-2 (1 Unit/mL, from the NIH repository at Frederick National Laboratory) with or without 100 nM recombinant GITR-L–Fc, OX40-L–Fc (both from Imgenex, San Diego, CA), recombinant murine TNF (Peprotech, Rocky Hill, NJ), recombinant CD70-Fc (Sino Biologicals, Beijing, China), soluble CD70 (Enzo Life Sciences, Farmingdale, NY), agonist CD27 mAb (RM27-3E5)[136], or recombinant mouse CD30-L and 4-1BBL (the latter two of which were crosslinked using 5 ug/ml anti-poly His antibody, both from R&D Systems, Minneapolis, MN). After 72 hours, cells were harvested, stained with anti-CD4

and CD25 antibodies and analyzed by flow cytometry for the percentage of cells that express GFP after incubation.

Thymic organ cultures.

Neonatal thymic organ cultures were performed as previously described[123]. Briefly, thymic lobes were harvested from 1-day-old Foxp3-GFP mice and set up in pairwise comparisons using isotype control or anti-TNFSF blocking antibodies. Neutralizing anti-OX40-L (RM134L) and anti-CD70 (FR70) were obtained from Bio X Cell (West Lebanon, NH). Neutralizing anti-GITR-L (Clone 337122) was from R&D Systems (Minneapolis, MN). Neutralizing anti-TNFR2 (Clone TR75-54) was previously described[137]. Thymic lobes were bisected and placed atop Millipore Cellulose Ester Gridded 0.45 μm filters (CAT HAWG01300) sitting on Gelfoam absorbable gelatin sponges (Pfizer, NY, NY). These filters were placed in 6 well plates with 2.1 mL of complete RPMI per well supplemented with 20 μg/mL of the indicated antibodies, and up to 6 bisected lobes placed on each filter. Medium and antibodies were replenished on day 7 and bisected lobes were collected and stained with primary antibodies for 20 minutes, washed, and analyzed by flow cytometry on day 14.

Mixed bone marrow chimeras.

Rag2$^{-/-}$ recipient mice were sublethally irradiated 24 hours prior to engraftment using an X-ray irradiator (Radsource RS2000 Biological Irradiator, Radsource,

Suwanee, GA) and administering two doses of 250 rads separated by a two-hour rest. Bone marrow was harvested from tibias and femurs of donor mice, dissociated into single cell suspensions, and magnetically depleted of contaminating mature T and B cells by labeling with biotinylated anti-CD3, CD4, CD8, CD19, and CD45R antibodies (eBioscience) and secondarily with streptavidin-conjugated microbeads (Miltenyi Biotec) prior to passage through magnetic columns. Cell suspensions were washed in PBS and injected intravenously via the tail vein. Chimeric recipients were engrafted for 10-18 weeks prior to analysis.

Preparation of dominant negative retroviral vectors.

Dominant negative retroviral vectors were cloned into pMIGR[138] or a modified version of this vector in which Thy1.1 replaces GFP, hereafter called pMITR. Sequences encoding truncated TNFRSF members were prepared by amplifying the extracellular and transmembrane domains of GITR and TNFR2 from a murine cDNA library and inserting stop codons immediately after the transmembrane domain. Specific primers utilized were as follows: for GITR dominant negative (Forward CTGCTCGAGCTGACCATGGGGGCATGGGCCAT, Reverse CTGGCGGCCGCCTGTCACAGGTCCTCCTCTGAGATCAGCTTCTGCATTGATGCCATTTGCCTCCTCAGCTGCCATATG), and for TNFR2 dominant negative (Forward CTGCTCGAGCTGACCATGGCGCCCGCCGC, Reverse

CTGGCGGCCGCCTGTCATTTGTCATCGTCATCCTTGTAGTCGGAGGGCTTC TTTTTCCTCTGCACC). Linear products were trimmed with *XhoI* and *NotI* and ligated into pMIGR or pMITR prior to transformation of chemically competent *E. coli* DH5α cells and plating on carbenicillin. LB starter cultures were prepared, digested, and picked for large culture. Mini- and maxipreps were performed using PureLink® kits according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Direct-Sanger sequencing was performed by the University of Minnesota Genomics Core facility and base calling was done by hand.

Bone marrow retroviral transduction

Subconfluent 293FT cells (ATCC, Manassas, VA) were transfected using Effectene reagent (Qiagen, Valencia, CA) with pMIGR-dominant negative GITR (dnGITR) or pMITR-dominant negative TNFRF2 (dnTNFR2) constructs in parallel with pHit60 and pHit123 packaging vectors for retrovirus production[139]. After 16 hours, sodium butyrate was added to the culture medium to a final concentration of 10 mM to enhance viral production. Eight hours later, medium was replaced to begin collecting retroviral particles. Supernatants were collected and medium replaced at 24 and 48 hours. After filtration through 0.45 μm filters, viral particles were centrifuged onto Retronectin-coated 6 well plates (Clontech Laboratories, Mountain View, CA) prior to overlaying *Ox40*$^{-/-}$ bone marrow cells at a density of 3 million cells per well. Bone marrows used for transduction had been isolated the previous day, depleted of T and B cells, and stimulated with 10 ng/mL IL-3

and IL-6, and 50 ng/ml SCF (Peprotech) to induce cell division. Medium and cytokines were replenished at the first and second retroviral transductions. Twenty-four hours after the second transduction, bone marrow was collected and *Ox40*$^{-/-}$ cells were mixed 80%/20% with congenically marked wild type bone marrow that had been stimulated identically in culture with cytokines. The mixture was washed in PBS and intravenously injected into sublethally irradiated *Rag1*$^{-/-}$ *or Rag2* $^{-/-}$ recipients and thymic T_{reg} was analyzed 10-12 weeks later. Bone marrow chimeric recipient mice that died due to a failure of engraftment were excluded from analysis.

Statistical analysis

P-values were generated using two-tailed T-tests; paired T-tests were used in the case of mixed bone marrow chimeras. Multiple sample comparisons were analyzed by one-way ANOVA. P-values in figure 2 were calculated based on Pearson correlation coefficients. All values were calculated using Prism (GraphPad Software, LaJolla, CA).

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