COSTIMULATION AND IL-2 IN NATURAL REGULATORY T CELL DEVELOPMENT

A DISSERTATION
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF THE UNIVERSITY OF MINNESOTA
BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

Dr. Michael A. Farrar, Advisor

September, 2010

Acknowledgements

In retrospect, many people were instrumental in preparing me for my success. One of them is my advisor, Michael Farrar. Without his mentorship, patience and understanding, I would not have become the Scientist I am today. However, much of what I have learned about regulatory T cells came from those who came before me, hence special thanks to both Matthew Burchill and Jianying Yang. I would also like to thank the members of my laboratory both past and present, more specifically, Lynn Heltemes Harris, Mark Willette, Shawn Mahmud, and Casey Katerndahl. However, I could not have learned how to do science without the combined knowledge and comradery from all my colleagues in the Center for Immunology. Last, I would to thank the Scientist in my life, Ruud, who has made me a better scientist, but foremost a better person.

Dedication

This thesis work is dedicated to my parents, Kong Xiong and Thai Vang, who are sowing poppy seeds in the fields of Laos and to my husband Ruud who, in this life, hold those seeds in the cup of his hands.

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Chapter 1

Introduction

More than 30 years ago, the idea of suppressor T cells was coined (1, 2). By its definition alone, the word "suppressor" posits that these cells would be capable of inhibiting the activity and function of other cells. It was proposed that loss of this suppressor function would result in autoimmunity. This idea of dominant tolerance was hard to resolve because little was known about these suppressor T cells and they could not be isolated. In addition, what was thought to be evidence of an antigen specific material derived from suppressor T cells was later found to be an apolipoprotein (3). Furthermore, the newly identified subregion (I-J) thought to reside within the major histocompatibility complex (MHC) region of suppressor T cells was not observed by genomic screening. Nor were transcripts ever found, in the original mice, of which these reports were first made (4, 5). Thus, the idea of suppressor T cells was largely discredited.

The earliest evidence that a loss of tolerance was due to an inherent cell defect arising from the thymus came from experiments involving thymectomy of mice that were less than three days old (6, 7). These mice developed autoimmunity proving that the source of dominant tolerance was due to cells of thymic origin. The second line of evidence came in 1995. Using CD25 as a surface marker for suppressor T cells, Sakaguchi and colleagues were able to show that $CD4^+CD25^+$ T cells were instrumental in controlling autoimmunity. In this landmark paper, the authors depleted the $CD4^+CD25^+$ fraction and transferred the remaining $CD4^+$ $CD25^-$ T cells into thymectomized mice. These mice developed autoimmunity, which was prevented when

$CD4^+$ $CD25^+$ suppressor T cells were added as well (8). Today, these suppressor T cells are more commonly referred to as regulatory T cells or "Tregs" henceforth.

Tregs can be indentified using the transcription factor Foxp3

The ability to use the surface marker CD25 to isolate Tregs was a major advance in the field; however, CD25 proved not to be a unique marker for Tregs as activated T cells also express it. At about the same time, several groups were interested in identifying the genetic defect in humans with Immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome and in *scurfy* mice. The authors were able to show that IPEX patients and *scurfy* mice had mutations in a gene called *Foxp3* (forkhead box p3) that mapped to the X-chromosome and that mutations in *Foxp3* led to a lymphoproliferative syndrome (9-12). Subsequently in 2003, the Ramsdell, Sakaguchi and Rudensky laboratories independently published data showing that the lineage specification factor for regulatory T cell development was Foxp3 (13-16). The identification of the transcription factor Foxp3 provided a specific marker for the identification of Tregs.

There are two types of $CD4^+$ $Foxp3^+$ regulatory T cells: natural Tregs and peripheral Tregs. The natural (innate) Tregs are those of thymic origin, while the peripheral (inducible) Tregs are those that are converted from $CD4^+$ $Foxp3^-$ non-Tregs into $CD4^+$ $Foxp3^+$ Tregs in the peripheral lymphoid organs. Both natural and inducible Tregs express high surface levels of CD25 (IL-2 receptor α, IL-2Rα), CD122 (a component of the IL-2R, IL-2Rβ), L-selectin (CD62L), glucocorticoid-indcued tumor

necrosis factor receptor (GITR), and cytotoxic T-lymphocyte antigen-4 (CTLA4). The role of CD62L, GITR and CTLA4, has been implicated in Treg homing to secondary lymphoid organs, activation and function respectively (17-19). Use of these markers, CD62L and GITR on Tregs can ameliorate graft versus host disease (GVHD) while the latter has been implicated in Treg homeostasis (17, 20-22). Although it is unclear whether there exists other undefined roles for these markers, what is clear is that $CD4^+$ $Foxp3^+$ Tregs, whether natural or inducible, express these markers.

Thymic architecture and summary of T cell development

One of the fundamental questions in the Treg field is how do these cells develop? In general, T cell development occurs in the thymus. The thymus is composed of two lobes and can be further divided into two regions: the cortex and medulla. The cortico-medullary junction (CMJ) serves as the boundary that divides the cortex from the medulla. The cortex and medulla contain cortical thymic epithelial cells (cTECs) and medullary thymic epithelial cells (mTECs), respectively, with a large concentration of thymic dendritic cells (tDCs) in the medulla. During T cell development, an early thymic precursor (ETP) from the bone marrow enters near the CMJ. As the cell matures, it moves from the CMJ into the cortex, from the cortex to the outer sub-capsular region and then from the sub-capsular region to the medulla. These discrete anatomical locations houses cells that will serve to direct the fate of developing T cells.

Thymic precursors originate in the bone marrow as hematopoietic progenitors that make their way to the thymus. Once in the thymus, an early ETP undergoes several

stages of development. Due to the lack of expression of CD4 and CD8, these stages are called double negative (DN). Using CD44 and CD25 as surface markers on ETP, the DN stages can be tracked (23). In the order of the most immature to the mature, the DN stages are designated as: DN1, DN2, DN3 and DN4 (23). It is at the DN2 stage ($CD44^+$ $CD25^+$) that recombinase activation genes (RAGs) are turned on and rearrangement of the T cell receptor (TCR) γ, δ and β loci begins. Once at the DN3 ($CD44^-$ $CD25^+$) stage, rearrangements of the TCRs are in full swing and commitment to either the γ/δ or α/β lineage is thought to occur through successful arrangement and pairing with the correct receptor. For example, for commitment into the γ/δ T cell lineage, this would require pairing of the γ/δ/CD3 complex (24). For α/β T cells, these cells enter into a process called β-selection. This process pairs the invariant pre TCRα chain with the TCRβ chain, thereby ensuring that an in-frame β chain is generated. Once β-selection is complete, the T cells rapidly expand, express both CD4 and CD8 (double positive (DP) cells), and then begin rearrangement of the TCRα locus (23). After successful rearrangement of the TCRα locus, DP cells are then paired with the previous TCRβ chain and a complete TCR is formed.

Following the generation of the newly formed TCR, T cells either die by neglect or undergo positive and negative selection. The basic principle of this process is to allow the production and maturation of T cells capable of recognizing foreign antigens while limiting the recognition of the host. During positive selection, the TCR on thymocytes recognizes self-peptide complexes presented in the context of MHC expressed on cTECs. It is thought that a low TCR affinity ligand is required for positive

selection (25-27). Once T cells are positively selected they up regulate the chemokine receptor, CCR7, which allows them to rapidly migrate from the cortex to the medulla (28). At the CMJ, the T cells that react strongly to high affinity peptide MHC signals undergo negative selection. However, it has been reported that negative selection can occur in the cortex as well (29). Unlike the low TCR affinity ligands required for positive selection, negative selection deletes T cells capable of recognizing high affinity ligands. The result of positive and negative selection gives rise to mature T cells with a diverse TCR repertoire that are able to recognize ligands of intermediate affinity with a low propensity to react to self.

The two-step model of Treg development

A two-step model has been proposed for Treg development (30, 31). In step 1 of this model, signals emanating from the TCR and CD28 initiate regulatory T cell development. In step 2, cytokine signaling completes the developmental pathway by turning on Foxp3 (Figure 1). It is interesting to speculate on the role of cytokines in regulatory T cell development. Two explanations have been proposed regarding the role of IL-2 in Treg development. First, that cytokine signaling via STAT5 can turn on Foxp3 and second, that strong IL-2 receptor (IL-2R) signals can rescue non-Tregs that are destined to undergo negative selection and divert them into the Treg lineage (31-33). In the latter explanation and not the former, recent published work have highlighted the importance of the IL-2 receptor in protecting cells from apoptosis. In this study, the authors wanted to investigate the idea that high affinity signals could induce the survival of antigen specific T cells. The authors purified OT-I transgenic T cells and

stimulated in vitro for 5 days with either high, intermediate or low affinity ligands. They found that stimulation with low affinity peptides resulted in lower amounts of IL-2Rα expression and this correlated with decreased Mcl-1 protein (an anti-apoptotic protein belonging to the Bcl-2 family of apoptotic molecules). In contrast, when they stimulated with a high affinity ligand, this was shown to have increased IL-2R expression on the T cells. Subsequently, Mcl-1 expression was also increased. The authors concluded that a high affinity signal directs T cell survival by an IL-2R -> Mcl-2 loop (34). Although, the authors do not look at the role of IL-2 in thymic Treg survival, their findings have interesting implications in the Treg field. Namely that perhaps high IL-2R signaling can inhibit apoptosis during negative selection and possibly direct these IL-2Rα hi expressing cells into the regulatory T cell lineage. It is possible that this might be the case since the IL-2Rα is expressed on 70-80% of $CD4^+$ $Foxp3^+$ Tregs. This would support explanation two as discussed (31). In addition, using the marker for CD25, both the Hsieh and Farrar laboratories have identified a Treg progenitor that is negative for Foxp3 and is characterized by the expression of CD25, IL-2Rβ (CD122), and GITR ($CD4^+$ $Foxp3^-$ $CD25^+$ $CD122^+$ $GITR^+$). Purification of these $CD4^+$ $Foxp3^-$ $CD25^+$ $CD122^+$ $GITR^+$ Treg progenitors and culturing in the presence of IL-2 was able to covert them into $Foxp3^+$ Tregs (30, 31). It is unknown whether IL-2 signaling can promote Treg survival during negative selection but it is intriguing to speculate perhaps that IL-2 signaling could be promoting both Foxp3 expression and thymic Treg survival after negative selection however, this needs to be further investigated. Combined with the role for the IL-2R signaling in directing regulatory T cell commitment, a two-step model is required for Treg development.

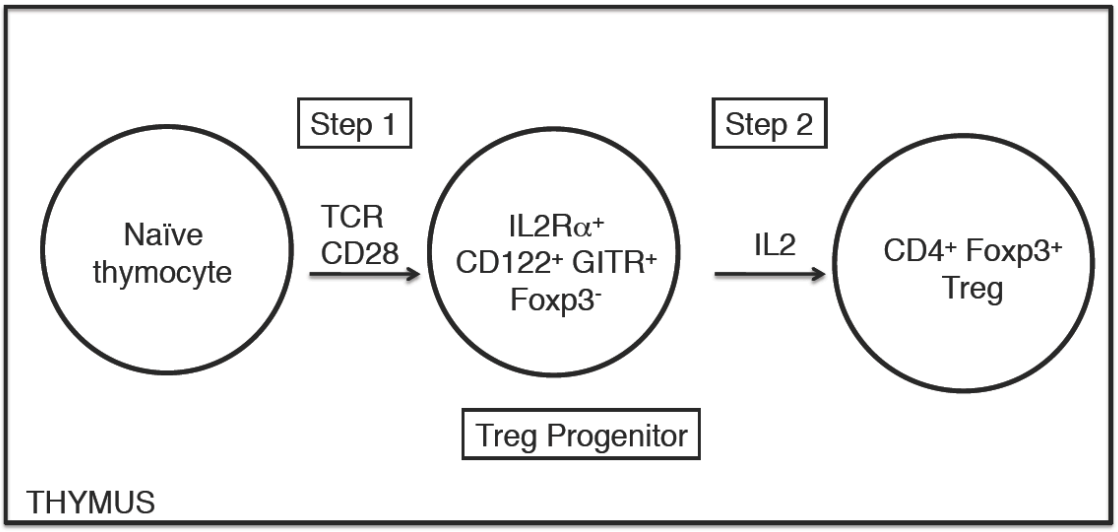
Signal 1: a T cell instructive model of Treg development

Unlike the conventional T cells, it has been proposed that Tregs arise through a process called agonist selection. This model dictates that Tregs are selected by higher affinity ligands and that this process is TCR dependent. It is thought that the selection threshold is slightly below that for negative selection but above those signals required for positive selection of conventional T cells. Evidence to support this model came from studies using a TCR transgenic system in which T cells express a TCR that recognizes the high affinity ligand, influenza-virus haemaglutinin (HA). Jordan and colleagues were able to show that TCR transgenic T cells specific for HA induced $CD4^+$ $Foxp3^+$ Tregs while the non-Tregs were selectively deleted (35). Similar results were reported elsewhere using other TCR transgenic mice (36-38). In addition, thymic $CD4^+$ $Foxp3^+$ Tregs have higher expression of CD25, CTLA4 and CD5 than developing conventional T cells. This is potentially because expression of these markers is a hallmark of activated T cells that have received an acute or chronic signal through their TCRs. Thus, expression of these markers could indicate that developing Tregs have seen a high affinity ligand through their TCR.

Fig. 1-1: A two-step model of Treg development.

Treg development requires two signals. In the first step, an early Treg precursor receives a TCR/CD28 signal. This gives rise to a putative Treg progenitor characterized by its expression of CD25, CD122 and GITR, but is largely Foxp3$^-$ ($CD4^+$ Foxp3$^-$ $CD25^+$ $CD122^+$ $GITR^+$). In step 2, cytokine signaling but more specifically, IL-2 signaling completes this developmental pathway.

Fig. 1


Step 1
Step 2
Naïve thymocyte
TCR CD28
IL2Rα+ CD122+ GITR+ Foxp3-
IL2
CD4+ Foxp3+ Treg
Treg Progenitor
THYMUS

A key question is, what is the nature of the selecting ligands? It has been postulated that the ligands required for Treg selection are high affinity self-ligands. Evidence to support this came from studies where the authors retrovirally transduced Treg or non-Treg TCRα libraries into *RAG*$^{-/-}$ TCR transgenic T cells. When they transferred in these transgenic T cells either bearing Treg or non-Treg TCRs, they found that the retrovirally transduced Treg TCRs were more self-reactive. This was based on the ability of the retrovirally transduced Treg TCRs to expand and mediate autoimmune pathology when compared to the non-Tregs (39). Furthermore, co-culture of the retrovirally transduced Treg TCRs with antigen presenting cells (APCs) did not mediate a robust response (39). These results support a model where the Treg selecting ligands resemble self-ligands and not those of foreign ones. Furthermore, by sequencing and comparing the TCRs of conventional T cells and Tregs, Hsieh and colleagues demonstrated that the TCR repertoire of Tregs is just as diverse as conventional T cells albeit with some minor degree of overlap (30, 40).

In contrast, the Ignatowicz Laboratory has proposed that non-self antigens select Tregs. This idea is supported by studies in which Tregs can recognize a wide variety of exogenous antigens such as those derived from parasites, bacteria, neo-antigens, alloantigens and viruses (41-48). To address the relative contribution of self-antigen versus non-self in the selection of the regulatory T cells in the thymus, Pacholczyk et al. sequenced and compared the TCRs between Tregs and non-Tregs. They used the TCR^{mini} mouse model, in which the TCRα chain is driven by a mini-locus that can rearrange one Vα2 segment and two Jα26 or Jα2 segments. By sequencing the TCRs

from Foxp3$^+$ Tregs and Foxp3$^-$ naive non-Tregs, they found that the TCR repertoire was largely overlapping (49). These findings contrast with those of the Hsieh Laboratory in which they found that the TCR repertoire between Tregs and non-Tregs were distinct with a minor overlap (39). To address the differences in these findings, it may be that depending on the mouse model, the TCR repertoire between Tregs and non-Tregs can vary between 10% - 42% (50). Although more work needs to be done, what is clear is that Tregs require a signal through the TCR.

To extend these studies further, Bautista et al. found that by using Tregs that only express the G113 TCR in limited dilutions can induce CD4$^+$ Foxp3$^+$ Tregs. In these studies, the authors identified and cloned individual Treg TCRs; one of which they called G113. Afterwards, TCR transgenic mice were made in which all the T cells expressed the Treg G113 TCR. Surprisingly, these mice had very few thymic CD4$^+$ Foxp3$^+$ Tregs (51). They thought that perhaps they were not inducing efficient Treg development due to intraclonal competition. To examine this in more detail, the authors made mixed bone marrow chimeras in which they mixed in varying ratios of G113$^+$ T cells to wild-type T cells that express a polyclonal TCR repertoire. They found that unlike high clonal concentrations of G113$^+$ T cells (1:1 or 1:5), at low clonal concentrations (1:50), they were able to induce 40% CD4$^+$ Foxp3$^+$ Tregs (51). This work demonstrates that the thymic niche for regulatory T cells is small and some clonal competition exists that suppresses the development of Tregs.

Can we postulate what these signals, or unknown factors might be that the Tregs are competing for? A reasonable hypothesis might be the competition for ligands, cytokines such as IL-2 or, an unknown factor from an accessory cell. Although we still have questions about the nature of the Treg selection environment, studies have shown that Tregs require peptide presented in the context of MHC.

The role of co-stimulation via CD28 in Treg development

Besides a TCR dependent requirement for Treg development, it has been proposed that signals through CD28 are also required for this process. CD28 is expressed on most $CD4^+$ T cells and it is thought that signals through CD28 provide an accessory signal to augment TCR signaling. CD28 is a 44-kDa member of the immunoglobulin superfamily whose ligands include B7.1 (CD80) and B7.2 (CD86), both of which are expressed on mTECS and cTECS. B7.1 is only expressed on DCs following Toll-like receptor (TLR) activation; in contrast, B7.2 is constitutively expressed on DCs and mTECs. CD28 is thought to regulate T cell proliferation, cytokine production, anergy and survival (52). The CD28 intracellular domain has two motifs that are required for the recruitment of effector molecules. The first consists of the Y^{170}MNM sequence and is required to recruit SH2 binding proteins such as Phosphoinositide 3-kinases (PI3K), Grb2 and Gads. The second motif consists of the proline rich sequence P^{187}YAPPand is thought to interact with Lck and other Tec kinases (53). On one hand, the N-terminal proline recruits SH3 binding proteins such as Tec and Itk, on the other hand, the C-terminal proline recruits Lck, Grb2 and Filamin A (54). Both Lck and Itk are kinases that phosphorylate the tyrosine (Tyr) residue in the

P^{187}YAPP motif, which then allows for the recruitment of SH2 binding proteins. The functional significance of the Y^{170}MNMmotif is the production of IL-2 via PKCθ and the induction of Bcl-XL (an anti-apoptotic protein) via mTOR. The importance of the PYAP motif is the involvement of CD28 in recruiting Lck into the immunological synapse (IS) by filamin-A resulting in the augmentation of TCR signaling. However, the P^{187}YAPP motif has been shown to recruit other signaling molecules such as Protein Kinase θ (PKCθ) to the IS (55). Due to the differential pathways that exists downstream of CD28, the question remains, how does CD28 signaling regulate Treg development?

Co-stimulation is important in lymphocyte biology. More specifically, it has been proposed that ligation of B7.1 and B7.2 are required for Treg development (56, 57). In Tregs it has been shown that a mutation in the Lck binding region of the CD28 PYAP motif abrogated Treg development (56). This was thought to be attributed to a defect in the ability of CD28 to regulate IL-2 production. To examine this issue, the authors mixed *CD28*$^{-/-}$ bone marrow (BM) with wild-type BM and reconstituted this into lethally irradiated C57BL/6J hosts. They were able to show that this effect was IL-2 independent as wild-type BM did not correct the IL-2 deficiency in *CD28*$^{-/-}$ BM (56). However, the results were based on using CD25 as a marker for Tregs and it is unknown at what stage does CD28 play a role in Treg development? For example, is CD28 required for the generation of Treg progenitors or for the conversion of Treg progenitors into mature Tregs? Furthermore, it is unknown what pathways downstream of CD28 can drive $CD4^+$ $Foxp3^+$ Treg development?

There are several pathways downstream of CD28. Notably, these pathways feed into NFκB activation. There are two NFκB pathways: canonical and the non-canonical. The NFκB/Rel family members consists of: NFκB1 (unprocessed form p105 & processed form p50), NFκB2 (unprocessed form p100 & processed form p52), RelA (p65), RelB, and c-Rel (Figure 2). In both the canonical and non-canonical pathway, NFκB/Rel family members associate with each other forming either homo or heterodimers. The dimers are sequestered in the cytoplasm by the inhibitor of κ B (IκB), of which there are seven members (see Figure 2). In order for the dimers of NFκB/Rel family members to be activated, IκB must be phosphorylated and targeted for proteasomal degradation via ubiquitination. In the canonical pathway, PKCθ is recruited into the immunological synapse (IS) by CD28. It has been shown in conventional T cells that PKCθ deficient cells cannot induce NFκB transcription (58). The role of PKCθ is to phosphorylate caspase recruitment domain (CARMA1, CARD11, or BIMP3) which recruits BCL10 and MALT1 forming the Carma1/Bc110/MALT1 (CBM) complex. Another molecule, Protein Kinase B (PKB or AKT) has also been shown to activate IKK in conventional T cells. However, PKB cannot do so by itself but requires its association with PKCθ (59, 60). This CBM complex ubiquitinates the Inhibitor of κ B kinase (IKK) complex composed of the adaptor protein, IKKγ (NEMO), and the two catalytic subunits IKKα and IKKβ. In the non-canonical pathway (also known as the alternative pathway, not shown), NEMO is not included in the IKK complex and consists of only IKKα and IKKβ. In the alternative pathway, both are activated and phosphorylated by the serine/threonine

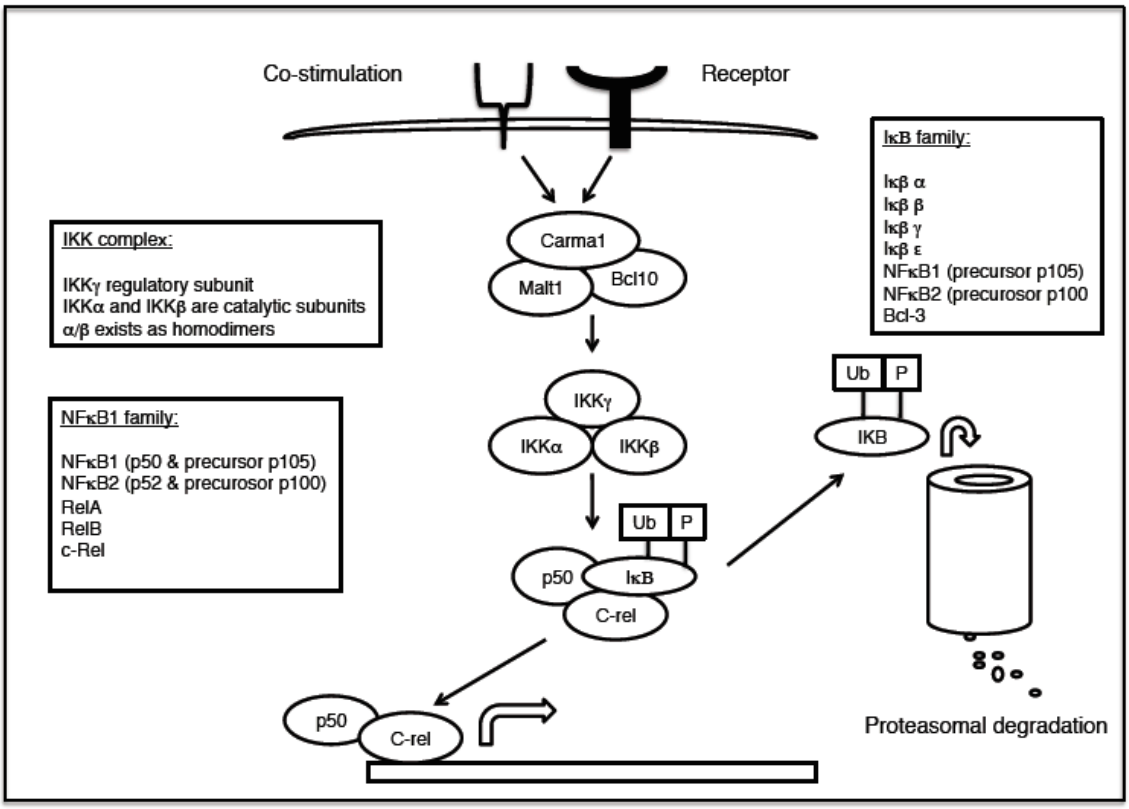
protein kinase, NFκB Inducing Kinase (NIK) In turn, the IKK complex phosphorylates the serine residues on the amino terminus of IκB, thereby leading to IκB degradation by the proteasome and the liberation of NFκB/Rel binding partners. The resultant NFκB/Rel dimers then translocate to the nucleus to mediate target gene transcription (Figure 2).

The NFκB pathway was shown to be key in Treg development as *Carma1*$^{-/-}$ mice had fewer CD4^{+} Foxp3^{+} Tregs (61). Furthermore, Molinero et al. were able to show that not only did *Carma1*$^{-/-}$ mice had fewer mature Tregs, they had a defect in Treg progenitors (61). Evidence to indicate that the NFκB pathway directed Treg development came from studies in which PKCθ, BCL-10 and IKKβ deficient mice had decreased numbers of Tregs (62-64). However, for these studies, it was unknown the relative contribution of these "players" in directing Treg progenitor development. Furthermore, these studies predated the discovery of Foxp3 and much of these studies used CD25 as marker to identify Tregs. What remains to be seen is the contribution of which NFκB family members and their involvement in Treg progenitor development?

Fig. 1-2: Co-stimulation and TCR signals lead to the activation of the NFκB pathway.

NFκB is complexed with the inhibitory protein IκB in an inactivated state in the cytosol. Due to extracellular stimuli and upstream signaling factors, the enzyme IκB kinase (IKK) phosphorylates IκB resulting in its ubiquitination, dissociation of IκB from NFκB dimers and eventual degradation of IκB by the proteasome. Afterwards, the activated NFκB dimers are translocated into the nucleus where it is able to bind to its DNA response elements (RE) thereby activating transcription.

Fig. 2


Co-stimulation
Receptor
IKK complex:
IKKγ regulatory subunit
IKKα and IKKβ are catalytic subunits
α/β exists as homodimers
NFκB1 family:
NFκB1 (p50 & precursor p105)
NFκB2 (p52 & precurosor p100)
RelA
RelB
c-Rel
Carma1
Malt1
Bcl10
IKKγ
IKKα
IKKβ
Ub
P
p50
IκB
C-rel
p50
C-rel
IκB family:
Iκβ α
Iκβ β
Iκβ γ
Iκβ ε
NFκB1 (precursor p105)
NFκB2 (precurosor p100
Bcl-3
Ub
P
IKB
Proteasomal degradation

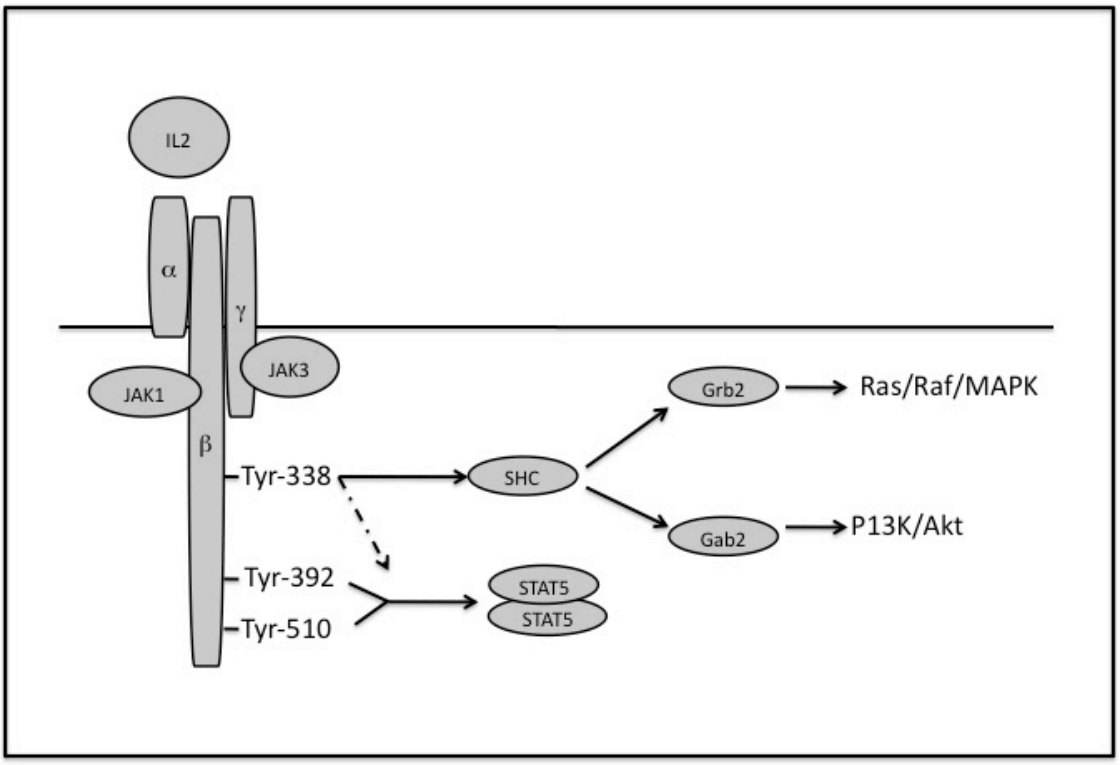
Signal 2: a role for cytokine signaling in Treg development

A second accessory signal for Treg development is provided by cytokines, most notably IL-2. IL-2 belongs to the family of common γ–chain (γc) receptor cytokines, which includes: IL-4, IL-7, IL-9, IL-15 and IL-21. The IL-2R signaling complex is composed of the IL-2Rα (CD25) , IL-2Rβ (CD122) and the common γ–chain (CD132) (Figure 3). The IL-2Rα chain has a short cytoplasmic domain and does not participate in signaling but helps in conferring high affinity binding of IL-2 to the IL-2R complex. Janus kinase 1 (JAK1) and Janus kinase 3 (JAK3) are associated with the IL-2Rβ chain and γc receptor respectively. Once IL-2 binds the tri-receptor complex, this brings the JAKs in close proximity to each other and allows them to phosphorylate multiple tyrosines in the IL-2Rβ chain leading to the recruitment of SH2 containing proteins. There are three main signaling pathways downstream of the IL-2R: PI3K, MAPK and signal transducer and activator of transcription 5 (STAT5) pathways (65). It is thought that phosphorylation of Tyr-338 allows the recruitment of SHC which then recruits Grb2 or Gab2 leading to the activation of MAPK or PI3K pathways respectively. Tyr-392, Tyr-510 and to a much lesser extent, Tyr-338, allows for the recruitment of STAT5. Once STAT5 is recruited to the IL-2Rβ chain, the JAKs phosphorylate Tyr-694 on STAT5a and Tyr-699 on STAT5b. This allows the STAT5a/b proteins to subsequently dimerize thereby allowing for their nuclear translocation and activation of gene transcription. The STAT5 dimers have been shown to bind to the sequence $TTCN_3GAA$ that was originally identified as a γ-interferon activated sequence (GAS) motif. The binding of STAT5 to the sequence leads to the transcription of genes

Fig. 1-3: IL-2R complex and its signaling moieties.

The IL-2R is composed of three subunits IL-2Rα, IL-2Rβ, and γc receptor. Signaling through the IL-2R results in three signaling pathways: MAPK, PI3K and STAT5. The phosphorylation of Tyr-338 recruits the adaptor protein SHC that binds Grb2 and Gab2, leading to activation of the Ras-Raf-MAPK and PI3K/Akt signaling pathways, respectively. Whereas the phosphorylation of Tyr-392 and Tyr-510 recruits STAT5 and leads to its activation; in the absence of these two tyrosine residues Tyr-338 can also induce STAT5 activation.

Fig. 3


IL2
α
γ
JAK3
JAK1
β
Tyr-338
SHC
Grb2
Ras/Raf/MAPK
Gab2
P13K/Akt
Tyr-392
Tyr-510
STAT5
STAT5

involved cell survival and cell cycle such as *Il2rα, Bcl2 and Cyclin d2* (66-68). The role of the IL-2/STAT5 axis has been implicated in lymphocyte biology, however, work by several groups has shown that signals through the IL-2R are required to drive Treg differentiation.

IL-2 signaling is required for efficient CD4$^+$ Foxp3$^+$ Treg development

STAT5 is a molecule downstream of the IL2 receptor that is required for Treg development. Work by Bensinger et al. has shown that STAT5 is turned on in CD4$^+$ CD25$^+$ Tregs in response to IL-2 stimulation (65). In support for the role of STAT5 in Treg development, using a constitutive active form of STAT5 (STAT5bca), Burchill et al. have shown that this led to an increase in Tregs in the thymus and peripheral organs (69). In contrast when mice were made that contained a STAT5 hypomorphic allele, there was a reduction in the Treg compartment (69-71). Subsequently, STAT5a/b floxed mice were generated in which STAT5 was deleted in all tissues resulting in perinatal lethality(72). To delete STAT5 from T cells specifically, the STAT5a/b floxed mice were crossed to CD4-cre mice demonstrating that in T cells that lack STAT5a/b, they were unable to differentiate into Tregs (33). Additionally, others and we have demonstrated that STAT5 can bind to the Foxp3 promoter using chromatin immunoprecipitation assays (33, 73). Furthermore, generation of mixed bone marrow chimeras in which either the STAT5 or Shc pathway was selectively activated supported a role for the former and not the latter in Treg development (33). More recently, we have also demonstrated that STAT5 can drive naïve a subset of non-Tregs

into the regulatory T cell compartment (31). Taken together, these studies support a role for STAT5 in directing Treg differentiation.

IL-2 signaling governs Treg homeostasis

IL-2/STAT5 signaling is important in the maintenance of the Treg lineage and promotes Treg expansion. In young *IL2*$^{-/-}$ and *IL2Rα*$^{-/-}$ mice, the percent of thymic CD4^{+} Foxp3^{+} Tregs are close to normal but they are largely CD25-. However, by 12-25 weeks of age, these mice develop multi-organ autoimmunity and a decline in CD4^{+} Foxp3^{+} Tregs is seen (74). Due to the absence of CD25 expression on the CD4^{+} Foxp3^{+} Tregs and the observation that these mice develop lethal autoimmunity, this suggested a role for IL-2 and IL-2 signaling in regulating the maintenance of the Treg lineage (32). To examine this further, work by Setoguchi et al. demonstrated that injection of IL-2 neutralizing antibodies to wild-type mice was able to reduce the number of CD4^{+} CD25^{+} (75). These results demonstrated that IL-2 signaling is required to maintain Treg homeostasis.

IL-2 signaling is required for Treg expansion

It was shown that IL-2 and IL-2 signaling can regulate Treg expansion. Tregs from wild-type mice were adoptively transferred into *IL2Rβ*$^{-/-}$ mice. In this system, the host can produce IL-2 and the donor cells have a functional IL-2R. In this model, host derived IL-2 was able to effectively expand the transferred CD4^{+} Foxp3^{+} Tregs (76). In a related study by Almeida et al., addition of an exogenous source of IL-2 to *IL2*$^{-/-}$ mice was able to clonally expand Tregs and ameliorate autoimmune pathology (77).

Furthermore, addition of IL-2, α-CD3 and α-CD28 to cell cultures can expand $CD4^+$ $Foxp3^+$ Tregs (78). In addition, STAT5 has been shown to regulate CD25 expression. *STAT5*$^{-/-}$ mice have fewer $CD4^+$ $Foxp3^+$ Tregs but we have found that by using a constitutively active STAT5, peripheral $CD4^+$ $Foxp3^+$ Tregs was enhanced (33, 69-71). Taken together, these results indicate that IL-2/STAT5 signals positively regulate Treg expansion.

IL-2 governs Treg function

Can IL-2 and STAT5 signals regulate $CD4^+$ $Foxp3^+$ Treg function? It has been reported that variations in the IL-2 gene can cause defects in Treg function (79). To examine the role of IL-2 and IL-2R signaling in Treg function, Lafaille and colleagues were able to reverse a mouse model of autoimmunity by transferring in *IL2*$^{-/-}$ $CD4^+$ T cells. Conversely, when they transferred in *IL2Rα*$^{-/-}$ $CD4^+$ T cells, they were not able to prevent autoimmunity. This highlights a role for IL-2 in promoting Treg function. To decipher the requirement for IL-2 in Treg suppressive function, the authors added neutralizing antibodies to a Treg suppression assay. They found that IL-2 neutralization was able to prevent suppression. In addition, Burchill et al. demonstrated that reconstitution with bone marrow from IL-2Rβ mutant mice that could only selectively activate the STAT5 pathway to *IL2Rβ*$^{-/-}$ mice was able to restore Treg function (33). These studies demonstrate that IL-2 dependent signals are required for Treg suppressive function.

The mechanisms of regulatory T cell function

It is clear that mutations in *Foxp3* result in multi-organ autoimmunity. How then, does a Treg exert its effects? Several mechanisms have been proposed but they generally fall into two categories: cell contact dependent and cell contact -independent. It is thought that the physical association between regulatory T cells and their target cells are required for suppression in vitro. This was shown in a transwell system where purified populations of regulatory T cells and effector cells were separated by a membrane. Without the direct contact of the Tregs with the effector cells, suppressor activity was blocked (80). Although it is still unclear what the exact mechanisms of contact-contact mediated suppression are, Gondek and colleagues showed that this could occur through a granzyme B-dependent, perforin-independent fashion (81). In addition, it has been proposed that Fas-Fas Ligand (FasL) interactions are thought to play a role in Treg mediated suppression (82). Contact-independent mechanisms also exist. For example, regulatory T cells can secrete interleukin 10 (IL-10) or Transforming growth factor β (TGFβ) to mediate suppression (83-85). However, the secretion and utilization of cytokines by Tregs to mediate suppression was variable depending on the disease and its affected organ (83-86).

In addition to the cell contact dependent and cell contact-independent mechanisms, inhibition of downstream signaling moieties in the T cell receptor (TCR) pathway has been shown to enhance Treg function. One such signaling molecule downstream of the TCR is PKCθ. In non-Tregs, PKCθ has been shown to be associated with the co-stimulatory molecule CD28. In this article, Zanin-Zhorov et al. purified

$CD4^+$ $CD25^+$ Tregs and treated these cells with the PKCθ inhibitor, compound 20 (C20) (87). They found that the treatment of Tregs with C20 improved Treg function. Using planar bilayers and antibodies to PKCθ, the authors were able to quantify PKCθ recruitment in the immunological synapse (IS) by imaging with total internal reflection fluorescence microscopy. The authors noticed that the PKCθ in $CD4^+$ $CD25^+$ Tregs was increased but was found to be located in the distal pole, that is, away from the Treg IS (87). However, in the non-Tregs, PKCθ was found to be located in the proximal portion of the non-Treg IS, or near the IS (87). In Tregs, this was associated with decreased PKCθ activity and attenuation of NF-κB activation at the Treg IS (87). The implication of this finding is that unlike the requirement of the TCR to instruct Treg development, Tregs seem to require *minor* instruction from the TCR to mediate their function. To examine this in vivo, the authors induced colitis using a colitis model of inflammation, *C57BL/10.PL TCR*$\alpha^{-/-}$ $\beta^{-/-}$ mice. They purified $CD4^+$ $CD25^+$ Tregs and treated them for 30 minutes with the PKCθ inhibitor, C20. Afterwards, they transferred these Tregs into *C57BL/10.PL TCR*$\alpha^{-/-}$ $\beta^{-/-}$ hosts. Unlike the non-treated Tregs, the C20 treated Tregs were able to prevent colitis (87). In this instance, inhibition of PKCθ specifically in $CD4^+$ $CD25^+$ Tregs was able to alleviate the symptoms associated with autoimmunity and rendered the Tregs functional. This would imply that the TCR has a minor role in directing mature Treg function. However, using an artificial Antigen Presentation system in which beads were coated with α-CD3/CD28, Hippen and colleagues was able to show that this expanded umbilical cord blood regulatory T cells (88). This showed that signaling moieties downstream of the TCR could be utilized to enhance Treg function and has important implications in the clinical setting.

The differences between the natural versus inducible Tregs

Although the topic of this thesis will deal mainly with natural Tregs, I will highlight some key differences between the natural versus inducible Tregs. First, natural Tregs require a high affinity TCR signal combined with co-stimulation through CD28, while the inducible Tregs require relatively optimal TCR stimulation without CD28 co-stimulation (89, 90). Second, while IL-2 seems to be the main cytokine that governs natural Treg development, both IL-2 and TGF-β have been shown to be involved in inducible Treg development. However the role for TGF-β in natural Treg development has been recently clarified. It was found that in 3-5 day old TGF-βRII deficient mice, these mice have fewer natural Tregs. This was attributed to a loss of protection from apoptosis of the natural Tregs by the TGF-β receptor (91). Last, unlike natural Tregs, which generally have stable Foxp3 expression, it has been proposed that inducible Tregs can lose expression of Foxp3. Inducible Tregs created by culturing in vitro with TGF-β have been shown to lose expression of Foxp3 (92, 93). Hence, it has been proposed that the role of Foxp3 may not be a Treg lineage specification factor. Instead, Foxp3 can promote and/or, maintain Treg function (94, 95). To summarize, these differences between the natural and the inducible Tregs can be classified into three categories, TCR and CD28 requirement, cytokine requirement and Foxp3 stability.

Objectives of this thesis:

Although the two-step model has proposed that a TCR/CD28 instructive signal first generates a Treg precursor that hinges on Foxp3 expression in which cytokines such as IL-2 completes this developmental process, three questions remain:

I) What are the molecular mechanisms downstream of CD28 that drive $CD4^+$ $Foxp3^+$ Treg development? Does these affect Treg progenitor or Treg development?

II) Can other cytokines such as IL-7 and IL-15 contribute to this process?

III) What accessory cells in the thymic microenvironment can produce the IL-2 required for $CD4^+$ $Foxp3^+$ Treg development?

Chapter II

CD28 and c-Rel-dependent Pathways Initiate Regulatory T Cell Development (96)

Abstract

Regulatory T cell (Treg) development proceeds via a two step process in which naïve $CD4^+$ thymocytes are first converted into $CD4^+CD25^+CD122^+GITR^+Foxp3^-$ Treg progenitors followed by a second step in which IL-2 converts these Treg progenitors into $CD4^+Foxp3^+$ Tregs. The co-stimulatory molecule CD28 is required for efficient Treg development. However, the stage at which CD28 affects Treg development remains undefined. Herein we demonstrate that *Cd28*$^{-/-}$ mice lack Treg progenitors. Furthermore, the P^{187}YAP motif in the cytoplasmic tail of CD28, which links CD28 to Lck activation, is required for this process. In contrast, the Y^{170}MNM motif, which links CD28 to PI3K activation, is not required for Treg progenitor development. Finally, the CD28/Lck pathway has been shown to activate the NFκB family of transcription factors. We demonstrate that c-Rel but not NFκB1 promotes the development of Treg progenitors. Thus, a CD28/c-rel-dependent pathway is involved in initiating Treg development.

Introduction

Regulatory T cells (Tregs) that develop in the thymus do so via a two step-process (30, 31). The first step involves the differentiation of $CD4^+$ thymocytes into $CD4^+CD25^+CD122^+GITR^+Foxp3^-$ Treg progenitors. In the second step these Treg progenitors are then converted into $CD4^+Foxp3^+$ Tregs by stimulation with IL-2. The first step of this differentiation process is thought to be governed by TCR-dependent signals (30, 51, 97). An important question is whether the co-stimulatory molecule CD28 also plays a role in this process.

Previous studies have demonstrated that *Cd28*$^{-/-}$ mice have a greatly reduced population of $CD4^+CD25^+$ T cells (98). The effect of CD28 on Tregs could be due to a role for CD28 in promoting IL-2 production by effector T cells, or due to a cell intrinsic effect. Work by Tai et al. using *Cd28* transgenic mice indicated that a PYAP motif within the cytoplasmic domain of CD28 is required for IL-2 production and the development of $CD4^+CD25^+$ T cells (56). However, by using WT versus *Cd28*$^{-/-}$ bone marrow chimeric mice they demonstrated that the effect of CD28 on Treg development was independent of IL-2. Thus, CD28 plays a cell intrinsic role in development of $CD4^+CD25^+$ T cells.

The above studies strongly suggested that CD28, acting via the PYAP cytoplasmic motif, was critical for regulatory T cell development. However, since these studies pre-dated the discovery of thymic Treg progenitors we do not know at what stage CD28 affects Treg development. In addition, these previous studies relied largely on CD25 to identify Tregs and thus may not accurately reflect the development of $CD4^+Foxp3^+$

Tregs. Moreover, the transgenic expression of CD28 used in the above studies is likely different from that expressed by the endogenous *Cd28* gene. This latter point is important, as even 2-fold differences in CD28 expression have been shown to have significant effects on CD28 function (99). Finally, the signaling pathways downstream of CD28 that entrain Treg development have also not been identified. To address this issue we made use of *Cd28*$^{-/-}$ mice, as well as two distinct *Cd28* knock-in mutants with defects in CD28-dependent PI3K (Y170F mutant) or Lck (AYAA mutant) activation, respectively (99, 100). We demonstrate that CD28 is involved at the first stage of Treg differentiation as *Cd28*$^{-/-}$ mice lack Treg progenitors. In addition, we demonstrate that CD28 drives Treg development via the PYAP motif, a motif that has been shown to activate the NFκB pathway (101-104). Finally, we demonstrate that the NFκB family member c-Rel, but not NFκB1, promotes the development of Treg progenitors. Thus a CD28/c-Rel dependent pathway is critical for initiating Treg differentiation in the thymus.

Materials and Methods

Intracellular Foxp3 flow cytometry

Thymii were harvested and single cell suspensions were prepared, stained and analyzed as previously described (31, 61). Antibodies used were obtained from eBioscience (San Diego, CA): CD3 (2C11), CD4 (RM4-5), CD8 (53-6.7), CD122 (5H4), CD25 (PC61.5), GITR (DTA-1), CD45.1 (A20) and Foxp3 (FJK-16s).

Mice

nfκb1$^{-/-}$, *Cd28*$^{-/-}$, *c-rel*$^{-/-}$, *nfκb1*$^{-/-}$ *x c-rel*$^{-/-}$, *Cd28 AYAA* and Cd28 *Y170F* KI mutant mice have been previously described (99, 100, 105-108). All mice were on the C57Bl/6 background.

Mixed bone marrow chimeras

Bone marrow cells from mutant (CD45.2) or WT (CD45.1) mice were harvested and mature hematopoietic cell subsets were depleted using biotinylated antibodies (CD3, CD4, CD8, CD25, B220 (RA3-6B2), CD19 (eBio1D3), Ter119 (TER-119), Gr-1 (RB6-8C5) and NK1.1 (PK136)) and Miltenyi magnetic beads. Cells from distinct mice were mixed at a 1:1 ratio and introduced into sublethally irradiated *rag2*$^{-/-}$ *x γc*$^{-/-}$ host mice by tail vein injection. Mice were analyzed 8 weeks after bone marrow reconstitution. To normalize for differences in the amount of stem cells injected from WT versus mutant bone marrow we first calculated the ratio of WT to mutant cells in DP thymocytes, which are the direct progenitors of both $CD4^+CD25^-Foxp3^-$ thymocytes and $CD4^+CD25^+Foxp3^-$ Treg progenitors. We then calculated the ratio of WT to mutant in

the non-Treg, Treg progenitor, and mature Treg compartments and divided those ratios by the ratio found in the corresponding DP compartment. This allows us to directly compare the relative contribution of mutant versus WT cells in these distinct cell populations.

Results and Discussion

CD28 is required for Treg progenitor development

To determine whether CD28 is required for development of Treg progenitors we used flow cytometry to identify this population in WT littermate control and *Cd28*$^{-/-}$ mice (Fig. 1A). *Cd28*$^{-/-}$ mice exhibit a clear reduction in CD4^{+}CD25^{+}CD122^{+}GITR^{+}Foxp3^{-} Treg progenitors as well as CD4^{+}Foxp3^{+} Tregs (Fig. 1B). We next examined Treg progenitors in two distinct CD28 knock-in (KI) mutant mice. The first CD28 KI involves mutation of a P^{187}YAP sequence within the cytoplasmic tail to A^{187}YAA; this mutation prevents CD28 interaction with Lck and results in loss of CD28-dependent IL-2 production (referred to as AYAA mutant). The second CD28 KI involves mutation of tyrosine-170 to phenylalanine and prevents CD28-induced activation of the PI3K pathway (referred to as Y170F mutant) (99, 100). As shown in figure 1B, the AYAA mutant mice exhibited defective development of Treg progenitors in the thymus; in contrast, Y170F mutant mice had no defect in Treg progenitor differentiation. Similar results were seen when examining the more mature CD4^{+}Foxp3^{+} Treg population in the thymus (Fig. 1B).

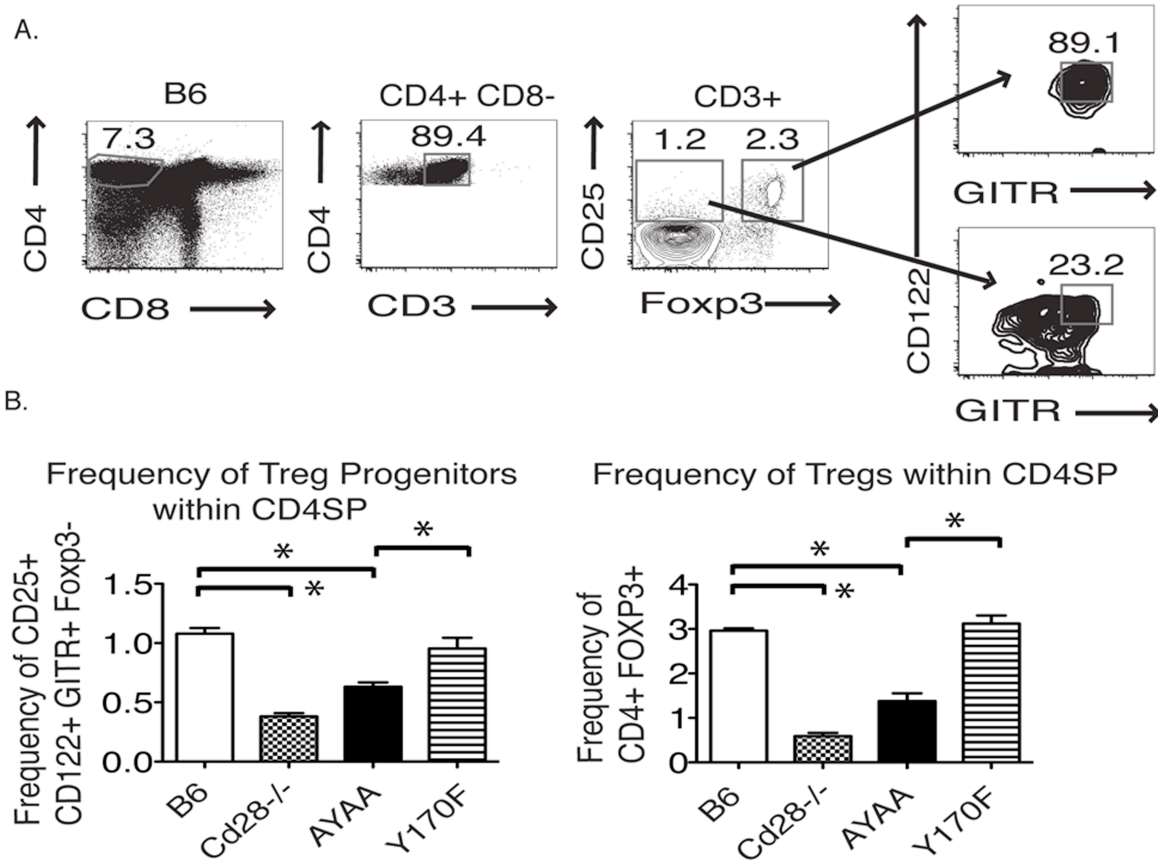
The CD28-Lck binding motif is required in a cell-intrinsic manner for Treg progenitor development

The results shown in figure 1 could reflect a requirement for CD28-dependent IL-2 production by effector T cells needed for survival of Treg progenitors. Alternatively, these results could be due to a cell intrinsic effect of the CD28/Lck pathway on the

Fig. 2-1. The CD28 Lck binding motif P^{187}YAP is required for Treg progenitor development.

(**A**) The gating strategy to identify Tregs and Treg progenitors is shown. The $CD122^{+}GITR^{+}$ gate used to identify Treg progenitors was based on staining observed for these markers on $CD4^{+}Foxp3^{+}$ Tregs. Shown are $CD4^{+}Foxp3^{+}CD122^{+}GITR^{+}$ Tregs (top right) and $CD4^{+}CD25^{+}CD122^{+}GITR^{+}Foxp3^{-}$ Treg progenitors (bottom right) from the thymus of a C57Bl/6 mouse. **(B)** The frequency of $CD4^{+}CD25^{+}CD122^{+}GITR^{+}Foxp3^{-}$ Treg progenitors (left) and $CD4^{+}Foxp3^{+}$ Tregs (right) within the $CD3^{+}CD4^{+}CD8^{-}$ thymocyte subset is shown. Error bars represent standard error of the mean. Data is representative of three independent experiments with 6 mice per group. * $p \leq 0.05$; determined by Anova using Bonferroni correction.

Fig. 1


A.
B6
7.3
CD4
CD8
CD4+ CD8-
89.4
CD4
CD3
CD3+
1.2
2.3
CD25
Foxp3
89.1
GITR
23.2
CD122
GITR
B.
Frequency of Treg Progenitors within CD4SP
Frequency of CD25+ CD122+ GITR+ Foxp3-
1.5
1.0
0.5
0.0
B6
Cd28-/-
AYAA
Y170F
Frequency of Tregs within CD4SP
Frequency of CD4+ FOXP3+
4
3
2
1
0
B6
Cd28-/-
AYAA
Y170F

conversion of naïve $CD4^+CD25^-Foxp3^-$ thymocytes into Treg progenitors. To examine this issue, we isolated bone marrow from *Cd28*$^{-/-}$, AYAA, or Y170F mutant mice, mixed them at a 1:1 ratio with CD45.1+ WT congenic mice and injected them into sublethally irradiated *rag2*$^{-/-}$ *x* $\gamma c^{-/-}$ host mice to generate mixed bone marrow chimeras. We then analyzed these mice by flow cytometry to assess the relative contribution of WT and mutant –derived cells to distinct thymocyte subsets. Both $CD4^+CD8^+$ double positive thymocytes and $CD4^+CD25^-Foxp3^-$ single positive thymocytes showed no obvious skewing of the ratio between WT and mutant progenitors from the initial input of ~1:1 (Fig. 2 and data not shown). In contrast, both *Cd28*$^{-/-}$ and *AYAA*-derived cells exhibited a significant decrease in their ability to contribute to Treg progenitor populations in these mixed bone marrow chimeras, although the magnitude of the defect for AYAA mutant cells was modestly but consistently less than that observed for *Cd28*$^{-/-}$ cells. A similar result was observed when examining the contribution of *Cd28*$^{-/-}$ and AYAA-derived cells to the $CD4^+Foxp3^+$ Treg subset. Finally, Y170F-derived cells were able to contribute to Treg progenitor and Treg subsets with equal efficiency as that seen for CD45.1+ WT–derived cells (Fig. 2). These results demonstrate that CD28 is required in a cell-intrinsic manner for development of Treg progenitors and that this is largely dependent on the CD28 P^{187}YAP, but not the Y^{170}MNM, motif.

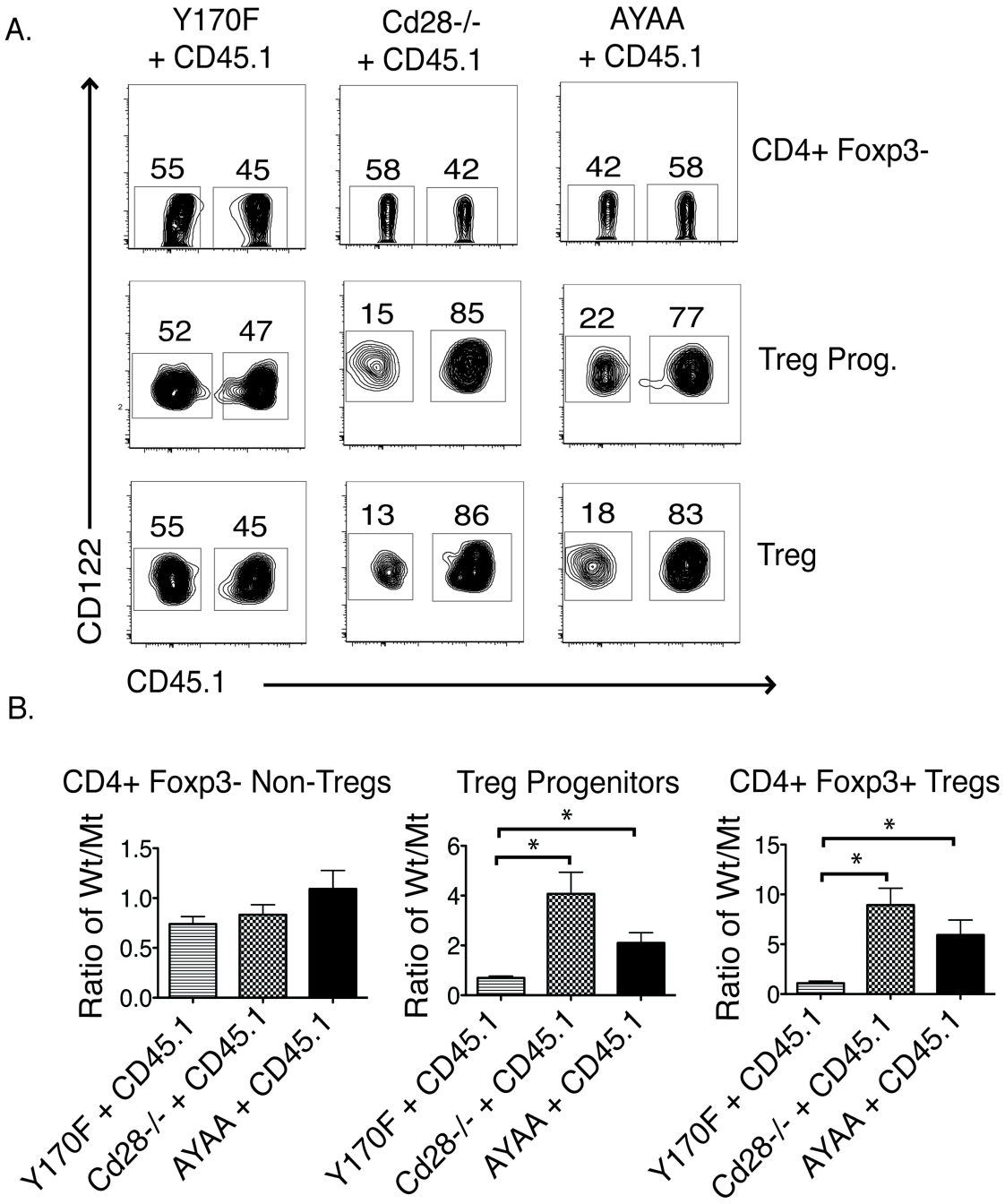
c-Rel but not NFκB1 is required for Treg progenitor development

The CD28-PYAP motif is known to govern a Lck/PKCθ pathway that subsequently regulates activation of the CARMA1/Bcl10/Malt1 complex (54). The CARMA1 complex in turn governs activation of both Jnk2 and the IKK complex leading

Fig. 2-2: The CD28 Lck binding motif is required for Treg progenitor development in a cell-intrinsic manner.

(A) Thymii were isolated and stained as described in the methods. Contour plots depict mutant ($CD45.1^-$) and WT ($CD45.1^+$) cells gated on $CD4^+CD25^-Foxp3^-$ thymocytes (top row), $CD4^+CD25^+CD122^+GITR^+Foxp3^-$ Treg progenitors (middle row) and $CD4^+Foxp3^+$ Tregs (bottom row). (**B)** Comparison of the ratio of WT to mutant cells between mixed chimeras in $CD4^+CD25^-Foxp3^-$ thymocytes (left), Treg progenitors (middle), and $CD4^+Foxp3^+$ Tregs (right). Ratios were normalized by comparison to the ratio of WT to mutant cells in double positive thymocytes to control for differences in the initial input ratio of WT to mutant cells. Error bars represent standard error of the mean. Data is representative of three independent experiments with 11-15 recipient mice per group. * $p \leq 0.002$ determined by unpaired t-test.

Fig. 2


A.
Y170F
+ CD45.1
Cd28-/-
+ CD45.1
AYAA
+ CD45.1
55
45
58
42
42
58
CD4+ Foxp3-
52
47
15
85
22
77
Treg Prog.
55
45
13
86
18
83
Treg
CD122
CD45.1
B.
CD4+ Foxp3- Non-Tregs
Treg Progenitors
CD4+ Foxp3+ Tregs
Ratio of Wt/Mt
1.5
1.0
0.5
0.0
6
4
2
0
15
10
5
0
*
Y170F + CD45.1
Cd28-/- + CD45.1
AYAA + CD45.1

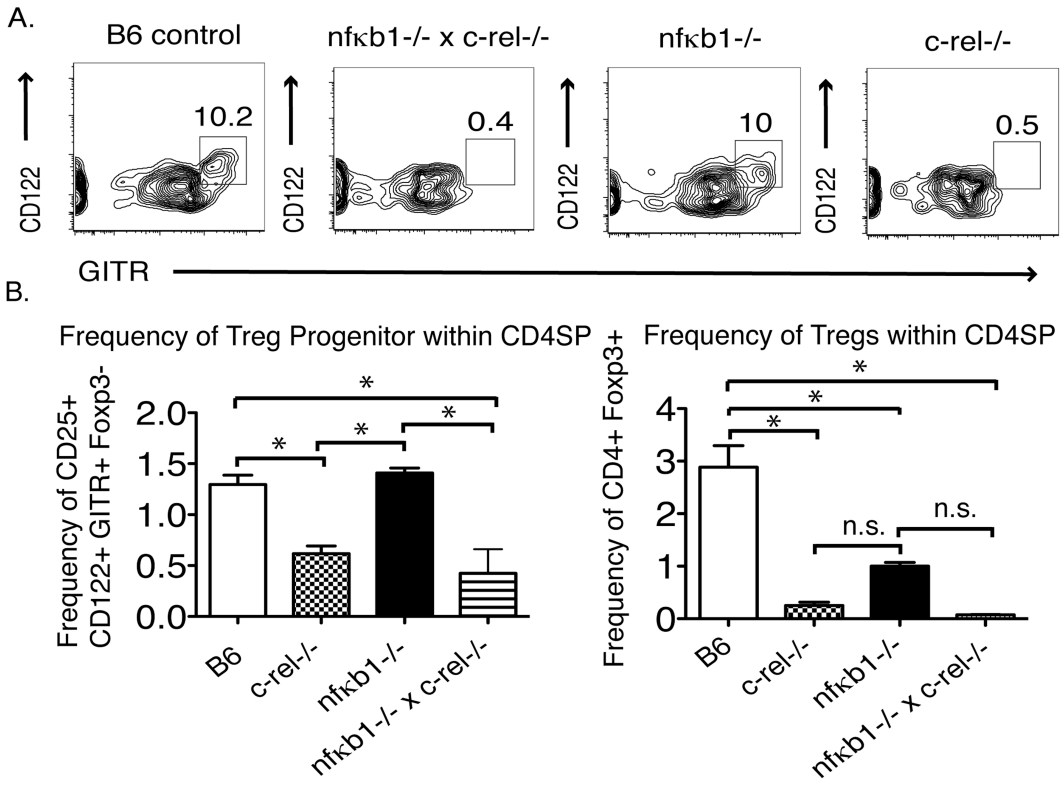
to activation of the classical NFκB pathway (54). Previous work has demonstrated that mice lacking both NFκB1 and c-Rel have defects in $CD4^+CD25^+$ T cells suggesting a role for this pathway in $CD4^+Foxp3^+$ Treg homeostasis and/or function (108). Likewise, mice expressing a $NF\kappa B1^{SSAA}$ knock-in mutation, which leads to impaired activation of NFκB1, RelA and c-Rel have also been shown to have reduced numbers of mature $Foxp3^+$ Tregs (109). To explore the role of the NFκB pathway in the development of Treg progenitors, and to more precisely identify which NFκB family members are required for this process, we examined these populations in *nfκb1*$^{-/-}$, *c-rel*$^{-/-}$ and *nfκb1*$^{-/-}$ *x c-rel*$^{-/-}$ mice. Compared to WT controls, *nfκb1*$^{-/-}$ *x c-rel*$^{-/-}$ mice were devoid of Treg progenitors and mature Tregs (Fig. 3). A similar result was seen in *c-rel*$^{-/-}$ mice. In contrast, *nfkb1*$^{-/-}$ mice showed no defect in Treg progenitors although they did exhibit a significantly smaller population of mature $CD4^+Foxp3^+$ Tregs in the thymus (Fig. 3).

To investigate whether c-Rel or NFκB1 is required in a cell intrinsic manner for Treg progenitor or mature Treg development, we made mixed bone marrow chimeras with CD45.1+ WT versus CD45.2+ *nfkb1*$^{-/-}$, *c-rel*$^{-/-}$, or *nfkb1*$^{-/-}$ *x c-rel*$^{-/-}$ bone marrow. Consistent with previous studies, *nfκb1*$^{-/-}$, *c-rel*$^{-/-}$ and *nfκb1*$^{-/-}$ *x c-rel*$^{-/-}$ derived cells showed no defect in the ability to generate $CD4^+CD25^-Foxp3^-$ thymocytes (Fig. 4A, top row). In contrast, both *c-rel*$^{-/-}$ and *nfκb1*$^{-/-}$ *x c-rel*$^{-/-}$ cells were unable to efficiently generate Treg progenitors and mature Tregs (Fig. 4A, middle and bottom rows). *Nfκb1*$^{-/-}$ derived cells did not have a defect in the ability to generate either Treg progenitors or mature Tregs in these mixed bone marrow chimeras (Fig. 4A-B). This latter result

Fig. 2-3: c-Rel but not NFκB1 is required for Treg progenitor development.

(A) Shown are contour plots gated on $CD4^+CD25^+CD122^+GITR^+Foxp3^-$ Treg progenitors from WT control (far left), *nfκb1*$^{-/-}$ *x c-rel*$^{-/-}$ (middle left), *nfκb1*$^{-/-}$ (middle right) and *c-rel*$^{-/-}$ (far right). (**B)** The frequency of Treg progenitors (left) and $CD4^+Foxp3^+$ Tregs (right) in $CD3^+CD4^+CD8^-$ thymocytes is shown. Data is representative of two independent experiments with three mice per group. * $p \leq 0.05$; determined by Anova using Bonferroni correction.

Fig. 3


A.
B6 control
nfκb1-/- x c-rel-/-
nfκb1-/-
c-rel-/-
10.2
0.4
10
0.5
CD122
GITR
B.
Frequency of Treg Progenitor within CD4SP
Frequency of CD25+ CD122+ GITR+ Foxp3-
2.0
1.5
1.0
0.5
0.0
B6
c-rel-/-
nfκb1-/-
nfκb1-/- x c-rel-/-
Frequency of Tregs within CD4SP
Frequency of CD4+ Foxp3+
4
3
2
1
0
n.s.
n.s.

suggests that the reduced number of mature Tregs in the thymus of *nfkb1*$^{-/-}$ mice reflects a role for NFκB1 in trans, most likely due to effects of NFκB1 in promoting IL-2 production by effector T cells.

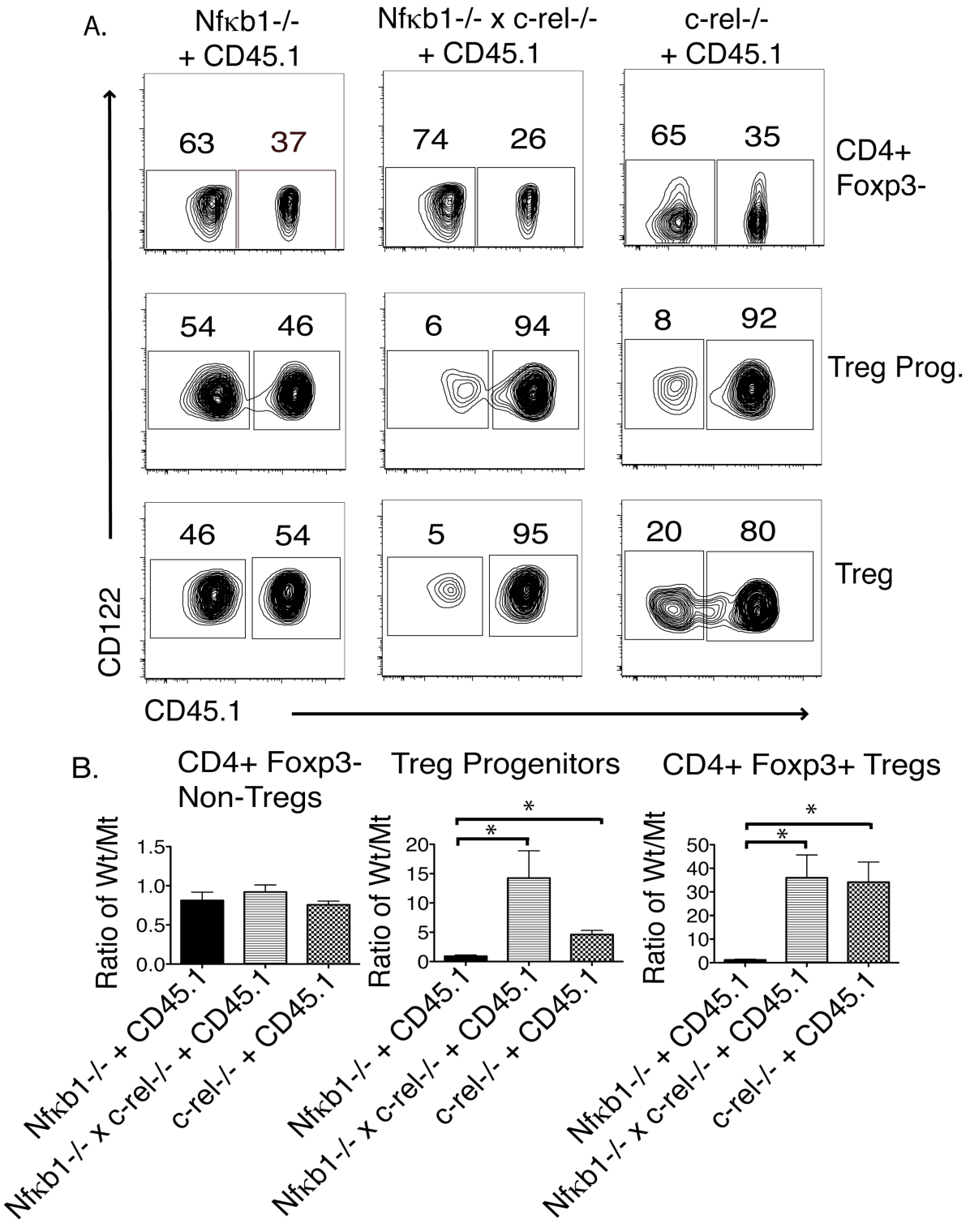
Our results demonstrate that CD28 and c-Rel-dependent pathway plays a critical role in initiating the development of Treg progenitors. This most likely reflects a role for c-Rel homodimers (or possibly c-Rel:RelA or c-Rel:NFκB2 heterodimers), as NFκB1 is not required for this process. Activation of the c-Rel pathway may involve signals initiated by the TCR although previous studies have shown that this is greatly enhanced by CD28 co-stimulation (102). Moreover, substantial evidence exists tying CD28 and the P^{187}YAP motif to c-Rel activation (54, 101-104). Taken together with the findings reported here, this suggests a critical role for a CD28/c-Rel dependent pathway in initiating the first step in Treg development.

c-Rel likely initiates Treg development via two distinct mechanisms. First, NFκB family members have been shown to play a role in regulating CD25 expression on T cells (110). Thus, c-Rel probably allows developing Tregs to respond to IL-2 by inducing high-level expression of the IL-2R complex on Treg progenitors. However, this cannot be the only mechanism by which c-Rel governs Treg development as crossing *Carma1*$^{-/-}$ mice, which are defective in c-Rel activation, to mice expressing a constitutively active form of STAT5 (*STAT5b-CA*) does not rescue Treg development (61). Since Treg development is restored in *IL2Rb*$^{-/-}$ *x STAT5b-CA*

Fig. 2-4: c-Rel but not NFκB1 is required in a cell intrinsic manner for Treg progenitor development.

(A) Contour plots depict the ratio of mutant (CD45.1-) versus wild-type cells (CD45.1+) gated on $CD4^+CD25^-Foxp3^-$ thymocytes (left), $CD4^+CD25^+CD122^+GITR^+Foxp3^-$ Treg progenitors (middle) and $CD4^+Foxp3^+$ Tregs (bottom). (**B)** Comparison of the ratio of wild-type to mutant cells between mixed chimeras in $CD4^+CD25^-Foxp3^-$ thymocytes (left), Treg progenitors (middle), and $CD4^+Foxp3^+$ Tregs (right). Ratios were normalized by comparison to the ratio of WT to mutant cells in double positive thymocytes to control for differences in the initial input ratio of WT to mutant cells. Error bars represent standard error of the mean. Data is representative of two independent experiments with 8 *nfκb1*$^{-/-}$ vs. WT, 9 *nfkb1*$^{-/-}$ *x c-rel*$^{-/-}$ vs. WT, and 4 *c-rel*$^{-/-}$ vs. WT mixed bone marrow chimeric mice. * $p < 0.02$ determined by unpaired t-test.

Fig. 4


A.
Nfκb1-/-
+ CD45.1
Nfκb1-/- x c-rel-/-
+ CD45.1
c-rel-/-
+ CD45.1
63
37
74
26
65
35
CD4+
Foxp3-
54
46
6
94
8
92
Treg Prog.
46
54
5
95
20
80
Treg
CD122
CD45.1
B.
CD4+ Foxp3-
Non-Tregs
Treg Progenitors
CD4+ Foxp3+ Tregs
Ratio of Wt/Mt
1.5
1.0
0.5
0.0
20
15
10
5
0
50
40
30
20
10
0
*
Nfκb1-/- + CD45.1
Nfκb1-/- x c-rel-/- + CD45.1
c-rel-/- + CD45.1

mice, the failure to rescue Tregs in *Carma1*$^{-/-}$ *x STAT5b-CA* mice suggests that c-Rel affects Treg differentiation via mechanisms other than just inducing IL-2R expression. An intriguing possibility is that c-Rel may bind to and prime the *foxp3* locus for subsequent cytokine-dependent transcription. We envision that c-Rel binding to the *foxp3* gene would result in epigenetic changes that would permit subsequent IL-2/STAT5-dependent signals to initiate *foxp3* transcription. Supporting this hypothesis, conserved NFkB binding sites exist in both the first and second introns of the *foxp3* gene (data not shown). Our results support a model in which CD28/c-Rel-dependent signals promote the development of Treg progenitors by inducing expression of the IL-2R complex on these cells and possibly by priming the *foxp3* gene, in an as yet undefined manner, for subsequent transcription (step I). These primed Treg progenitors would then be converted into mature Tregs by IL-2/STAT5-dependent signals that lead to actual *foxp3* transcription (step II), thereby completing Treg differentiation.

The role of NFκB signaling in CD4$^+$ Foxp3$^+$ Treg development

This thesis has discussed the role of CD28 and c-Rel in regulating Treg development. Evidence to indicate that the NFκB pathway was important in directing Treg lineage commitment was that mice deficient in PKCθ, Bcl-10 and IKK2 (IKKβ) have decreased numbers of Tregs in both the spleen and the thymus (62-64). At the time this paper (Chapter 2) was submitted, several papers subsequently appeared directly and indirectly linking the role of c-Rel to Treg development. Herein, I will highlight the main findings of these articles.

Although all the papers highlight the role of c-rel in Treg development, enhancement of a constitutively activated IKKβ kinase increased CD4$^+$ Foxp3$^+$ Tregs. In these studies, the Ghosh laboratory enhanced NFκB signaling by using a constitutively activated IKKβ kinase (known as IKKEE mice) under the control of the proximal Lck promoter. They found that in IKKEE mice, even though there was a 50% reduction in the total number of CD4SP cells, there was 20-25% increase in CD4$^+$ Foxp3$^+$ Tregs when compared to wild-type (111). We also confirmed these results using an independent system. We used a *loxP*-flanked STOP cassette that prevented transcription of a constitutively active form of IKKβ kinase (112). When we crossed these mice to CD4-cre, this allowed the deletion of the STOP cassette in CD4 T cells and activation of the constitutively active form of IKKβ kinase. Compared to the phenotype from Long et al., we saw an increase in CD4$^+$ Foxp3$^+$ Tregs but not to the extent as what they have published (unpublished work). To complement these findings, the authors used a non-degradable form of IκBα (IκBα-SR) in which the percentages

and the total numbers of $CD4^+$ $Foxp3^+$ Tregs were decreased compared to wild-type (111). Next, they wanted to address whether IKKEE signaling could bypass TCR signaling. To address this question, the authors crossed the IKKEE mice to *Rag1*$^{-/-}$ mice and found that they did not get $CD4^+$ $Foxp3^+$ Treg development. Hence, signals through IKKβ could not bypass the requirement for TCR signals in Treg development (111). This would imply that Foxp3 acquisition is dependent on the TCR possibly due to co-stimulation and requirements for TCR engagement.

Surprisingly the authors found that these $CD4^+$ $Foxp3^+$ Tregs from the IKKEE mice were not functional. Even though there was an increase in $CD4^+$ $Foxp3^+$ Tregs, these $CD4^+$ $Foxp3^+$ Tregs did not express CD25. CD25 is expressed on 70-80% of normal wild-type $CD4^+$ $Foxp3^+$ Tregs (32, 33). Since CD25 and IL-2 responsiveness has been shown to be involved in Treg suppression, the authors purified the $CD4^+$ $Foxp3^+$ Tregs from the IKKEE mice and performed a Treg suppression assay. Notably, these $CD4^+$ $Foxp3^+$ Tregs from the IKKEE mice did not suppress responder T cells (111). Interestingly enough, work by Dustin and colleagues have shown that inhibition of PKCθ in Tregs was able to enhance Treg suppressive function (87). This gives rise to a seemingly paradox in which the PKCθ signals are required for Treg development but block function of mature Tregs. In summary, even though there was increase in $CD4^+$ $Foxp3^+$ Tregs in the IKKEE mice and they expressed all the characteristics of normal wild-type Tregs with the exception of CD25, these were not functional Tregs.

How is it possible that we can get Foxp3 expression but absent CD25 expression? Perhaps one explanation is that the over expression of IKKβ can prematurely turn on Foxp3 but somehow bypass the requirement for CD25 expression. We favor a model where NFκB signaling can up regulate CD25 prior to Foxp3 expression during step one of the Two-step process of Treg development (30, 31). Indeed, work by the Chen Laboratory has shown that $CD4^+$ $Foxp3^+$ Tregs in *c-rel*$^{-/-}$ mice express less CD25 than wild-type controls (113). In addition, recent work by Deenick et al. support a role for c-Rel in the regulation of surface expression of CD25 (114). As discussed in this thesis, c-Rel may be regulating components of the IL-2R as *c-rel*$^{-/-}$ have fewer Treg progenitors. This would support a model where c-Rel can turn on CD25.

In addition for the role of c-rel in $CD4^+$ $Foxp3^+$ Treg development, what other NFκB family members are involved? Ruan et al. propose that both c-Rel and p65 (RelA) can activate the Foxp3 promoter (113). Using luciferase activity as a surrogate for Foxp3 expression, the authors co-expressed plasmids containing either c-Rel, p65, RelB, or, p50 into the Foxp3 promoter luciferase constructs. They found that c-Rel and to a lesser extent p65 could turn on luciferase activity (113). However, no luciferase activity was detected when co-expressing RelB or p50. In addition, RelB deficient and *p50*$^{-/-}$ mice have relatively normal $CD4^+$ $Foxp3^+$ Tregs (113). This provides strong evidence that c-Rel and to some extent, p65 are involved in Treg development.

How is *c-rel* exerting affects at the Foxp3 locus? To address this question, and by using different techniques, the Rudensky and Chen Laboratories mutated the c-Rel binding sites in the Foxp3 gene. In the Chen paper, they discovered that the Foxp3 promoter in mice contained two Rel-NFκB binding sites that were similar to that of humans: -382 to -376 and -327 to -321 (113). Using a Luciferase reporter construct in which they had mutated the c-Rel binding site -379 to -376 in the Foxp3 promoter and co-transfected c-Rel, they found that they had less luciferease activity as compared to vector controls (113). The Rudensky Laboratory took a slightly different approach in which they made green fluorescent protein (GFP) reporter mice that had each of three conserved non-coding (CNS) regions knocked out in the Foxp3 gene called CNS1-KO, CNS2-KO (Intron I), and CNS3-KO (Intron II). Upon closer examination of the CNS3 region, they found that it contained a c-Rel binding site similar to the CD28 response element found in the IL-2 locus. Using these knock out mice, they found that the CNS3 region was required for innate Treg development (115). CNS3-KO mice had a three-fold decrease in thymic $CD4^+$ $Foxp3^+$ Tregs but they did not see a difference in $CD4^+$ $Foxp3^+$ Tregs in the spleen (115). In contrast, CNS1 and CNS2 was found to be required for inducible Treg development and maintenance of Foxp3 expression respectively (115). They concluded that the c-Rel binding site in the CNS3 region of the Foxp3 promoter was required for thymic $CD4^+$ $Foxp3^+$ Treg development.

To examine whether the selective activation or inactivation could promote Treg development, the Ghosh Laboratory used a Luciferase reporter assay. In this system the luciferase reporter contained the Foxp3 promoter in which they either co-transfected

expression plasmids of IKKEE or IκBα-SR (non-degradable form of IκBα). They found that over expression of IKKβ could induce luciferase activity whereas IκBα-SR did not selectively enhance luciferase activity (111). These results support a role for IKKβ in regulating Foxp3$^+$ Treg development.

In summary, c-Rel is one of the upstream factors required for thymic CD4$^+$ Foxp3$^+$ Treg development. In contrast c-Rel does not drive inducible Treg development as in vitro culture of *c-rel*$^{-/-}$ CD4$^+$ T cells in the absence of IL-2 did not induce Foxp3$^+$ Tregs (116). However, the addition of IL-2 to *c-rel*$^{-/-}$ CD4$^+$ T cells was able to convert non-Tregs into CD4$^+$ Foxp3$^+$ Tregs. This was attributed to the requirement for c-Rel in driving IL-2 production and may not be due to a cell intrinsic defect for c-Rel in inducible Treg development. In light of recent publications, we can conclude that c-Rel is an important upstream factor promoting natural CD4$^+$ Foxp3$^+$ expression.

Chapter III

IL-2, -7, and -15, but Not Thymic Stromal Lymphopoietin, Redundantly Govern $CD4^{+}Foxp3^{+}$ Regulatory T Cell Development (117)

Abstract

Common γ chain *(γc)*-receptor dependent cytokines are required for regulatory T cell (Treg) development as $\gamma c^{-/-}$ mice lack Tregs. However, it is unclear which γc-dependent cytokines are involved in this process. Furthermore, TSLP has also been suggested to play a role in Treg development. Herein, we demonstrate that developing $CD4^{+}Foxp3^{+}$ Tregs in the thymus express the IL-2Rβ, IL-4Rα, IL-7Rα and IL-21Rα chains, but not the IL-9Rα or TSLPRα chains. Moreover, only IL-2 and IL-7 (and to a much lesser degree IL-15) were capable of transducing signals in $CD4^{+}Foxp3^{+}$ Tregs as determined by monitoring STAT5 phosphorylation. To examine this issue in more detail we generated *IL2Rβ*$^{-/-}$ *x IL7Rα*$^{-/-}$ and *IL2Rβ*$^{-/-}$ *x IL4Rα*$^{-/-}$ mice. We found that *IL2Rβ*$^{-/-}$ *x IL7Rα*$^{-/-}$ mice were devoid of Tregs thereby recapitulating the phenotype observed in $\gamma c^{-/-}$ mice; in contrast, the phenotype observed in *IL2Rβ*$^{-/-}$ *x IL4Rα*$^{-/-}$ mice was comparable to that seen in *IL2Rβ*$^{-/-}$ mice. Finally, we observed that Tregs from both *IL2*$^{-/-}$ and *IL2Rβ*$^{-/-}$ mice show elevated expression of IL-7Rα and IL-15Rα chains. Addition of IL-2 to Tregs from *IL2*$^{-/-}$ mice led to rapid down regulation of these receptors. Taken together, our results demonstrate that IL-2 plays the predominant role in Treg development, but that in its absence the IL-7Rα and IL-15Rα chains are upregulated and allow for IL-7 and IL-15 to partially compensate for loss of IL-2.

$CD4^+$ $Foxp3^+$ regulatory T cells (Tregs) that develop in the thymus play a key role in preventing autoimmune disease (86). Multiple signals are required to induce the development of Tregs. These include signals emanating from the TCR and the costimulatory molecule CD28 (56, 98, 118, 119). In addition, several studies have suggested that cytokines play a key role in this process (30, 31, 33, 76, 120, 121). However, which cytokines are involved has remained controversial. For example, it has been known for many years that mice lacking IL-2, or the IL-2Rα or IL-2Rβ chains, develop lethal autoimmune disease (122-124). This was initially attributed to defective Treg development as these mice lacked $CD4^+CD25^+$ T cells. More recent studies using the transcription factor Foxp3 as an identifier of Tregs found that young *IL2*$^{-/-}$ and *IL2Rα*$^{-/-}$ mice have relatively normal numbers of $CD4^+Foxp3^+$ Tregs (33, 125, 126). In contrast, both *IL2Rβ*$^{-/-}$ and *IL2*$^{-/-}$ *x IL15*$^{-/-}$ mice exhibited significant decreases in Treg numbers, suggesting that IL-2 and IL-15 play a redundant role in Treg development (33, 120).

Importantly, although Treg differentiation is inhibited in *IL2Rβ*$^{-/-}$ mice, Tregs are not completely absent (33) (120). This raises the possibility that other cytokines can also drive Treg differentiation. Along these lines, Watanabe et al. (127)have suggested a role for the cytokine thymic stromal lymphopoietin (TSLP). Specifically, they suggested that TSLP production by Hassall's corpuscles plays an important role in human Treg development (127). Likewise, the common γ-chain (*γc*), which is closely related to the TSLPR, has also been shown to be involved in Treg development. For example, others and we have demonstrated that mice lacking *γc* are devoid of Tregs (33,

126). The *γc* forms a component of multiple cytokine receptors including those for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 (128). Thus, two key questions in Treg development are 1) which *γc* -dependent cytokines can induce Treg development and 2) whether TSLP signals are also involved in this process. In this study, we demonstrate that developing Tregs express IL-2Rα, IL-7Rα and IL-15Rα and respond to IL-2, IL-7, and to a much lesser degree IL-15, by inducing STAT5 activation in $CD4^+$ $Foxp3^+$ thymocytes. Similarly, IL-2-induced conversion of $CD4^+$ $CD25^+$ Foxp3 thymic Treg progenitors into $CD4^+$ $Foxp3^+$ mature Tregs. IL-7 and IL-15 also induced conversion of thymic Treg progenitors into mature $CD4^+$ $Foxp3^+$ Tregs, albeit much less effectively; in contrast, TSLP showed no activity in this conversion assay. IL-4 signaling also does not appear to play a role in Treg development as $IL4R\alpha^{-/-}$ *x* $IL2R\alpha^{-/-}$ mice show comparable numbers of Tregs as that seen in $IL2R\beta^{-/-}$ mice. In contrast, $IL2R\alpha^{-/-}$ *x* $IL7R\alpha^{-/-}$ mice exhibit a developmental block, which mimics that seen in $\gamma c^{-/-}$ mice. Finally, the expression of IL-7Rα and IL-15Rα chains is suppressed in mature Tregs; this suppression does not occur in $IL2^{-/-}$ or $IL2R\beta^{-/-}$ mice, demonstrating that IL-7Rα and IL-15Rα down-regulation occurs via an IL-2/IL-2Rβ -dependent signaling pathway. Our findings demonstrate that IL-2, IL-7, and IL-15, are the critical *γc*-dependent cytokines that are responsible for promoting Treg development. In contrast, developing Tregs do not express the TSLPRα -chain, nor respond to TSLP, by inducing phospho-STAT5 (p-STAT5). Thus, at least in the mouse, TSLP does not appear to play a direct role in Treg development.

Materials and Methods

Mice

IL2$^{-/-}$, *IL2Rβ*$^{-/-}$, *IL7Rα*$^{-/-}$, and *IL4Rα*$^{-/-}$ mice were obtained from The Jackson Laboratories. *IL2Rβ*$^{-/-}$, *IL7Rα*$^{-/-}$, and *IL4Rα*$^{-/-}$ mice were crossed in our laboratory to obtain *IL-2Rβ*$^{-/-}$ *x IL7Rα*$^{-/-}$, and *IL4Rα*$^{-/-}$ *x IL2Rβ*$^{-/-}$ mice. Mice used were on a C57BL/6 background with the exception of the *IL4Rα*$^{-/-}$ and *IL4Rα*$^{-/-}$ *x IL2Rβ*$^{-/-}$ mice, which were on a mixed BALB/c x C57BL/6 background, and Foxp3-GFP reporter mice, which were on a mixed C57BL/6 x 129 background. Foxp3-GFP reporter mice were provided by Dr. Sasha Rudensky (University of Washington School of Medicine, Department of Immunology, Seattle, WA).

Flow cytometry and FACS analysis

Mice were sacrificed and lymph node, spleen, and thymus were isolated. Five million cells were used per staining condition. Cells were first pretreated with an Ab that blocks Fc receptor binding (Clone 24G2). Cells were subsequently stained with the following Abs from eBioscience: CD4-Alexa 700, CD8-allophycocyanin-Alexa Fluor 750 or CD8-FITC, CD3 -FITC, CD25-PE-Cy7 (PE-Cy 7), or CD25-allophycocyanin. In addition, biotinylated Abs for CD122 (IL-2Rα), CD124 (IL-4Rα), CD127 (IL-7Rα), and IL-21Rα, were obtained from eBioscience. Abs for IL-9Rα, TSLPRα, and IL-15Rα were obtained from R&D Systems. Isotype control Abs for TSLPRα and IL-15Rα were obtained from R&D Systems, while isotype controls for IL-9Rα were

obtained from eBioscience. Both the IL-9Rα and TSLPRα were biotinylated according to the manufacturer's instructions (Sigma-Aldrich, Cat. no. BTAG-1KT), while the IL-15Rα Ab was purchased in a biotinylated form. Streptavidin allophycocyanin from eBioscience was used as a secondary reagent to reveal staining with biotinylated Abs. Intracellular Foxp3 staining was done after fixation, permeabilization, and overnight incubation at 4°C as described previously (8).

To examine whether IL-2 alters IL-7Rα and IL-15Rα expression on Tregs in *IL2*$^{-/-}$ mice, we purified CD4 splenocytes from *IL2*$^{-/-}$ mice by MACS beads enrichment (Miltenyi Biotec). Purified cells were then stimulated with IL-2 (100 U/ml) for 4, 8, 12, and 24 h. Cells were then harvested and stained for IL-7Rα, IL-15Rα, and Foxp3 expression and analyzed on an LSR II flow cytometer (BD Biosciences).

Flow cytometry for p-STAT5

Single-cell suspensions were generated from isolated spleens and thymii from Foxp3-GFP reporter mice. These cell suspensions were pretreated with an Ab that blocks Fc receptor binding. Cells were then stained for the surface markers CD4, CD8, and CD25. Five million cells were then serum starved in 500 l of 1 DMEM for 30 min at 37°C, then stimulated with either 100 U/ml of IL-2 (PeproTech), or 50 ng/ml IL-4 (PeproTech), IL-7 (R&D Systems), IL-9 (R&D Systems), TSLP (R&D Systems), IL-15 (R&D Systems), or IL-21 (PeproTech) for 20 min. After stimulation, the cells were washed with 1 DMEM to remove all traces of the supernatant; they were then resuspended in 100 l of fixation medium from the Caltag Fix and Perm kit and

incubated at 37°C for 15 min. Afterward, 1 ml of 4°C 100% methanol was added and the cells were incubated overnight at 4°C in the dark. Intracellular p-STAT5 staining was done using the Caltag Fix and Perm kit and PE-conjugated anti-p-STAT5 (BD Biosciences). Nonstimulated cells were used as negative controls.

Treg conversion assay

The conversion assay of Treg progenitors into $CD4^+$ $Foxp3^+$ Tregs was conducted as previously described (10). In brief, $CD4^+$ $CD25^+$ $Foxp3^-$ Treg progenitors were sorted from Foxp3-GFP reporter mice (19) and placed in culture in the presence of the indicated amounts of cytokine. Twenty-four hours later cells were stained for CD4 and CD25 and analyzed for expression of these markers plus Foxp3-GFP using an LSR II flow cytometer.

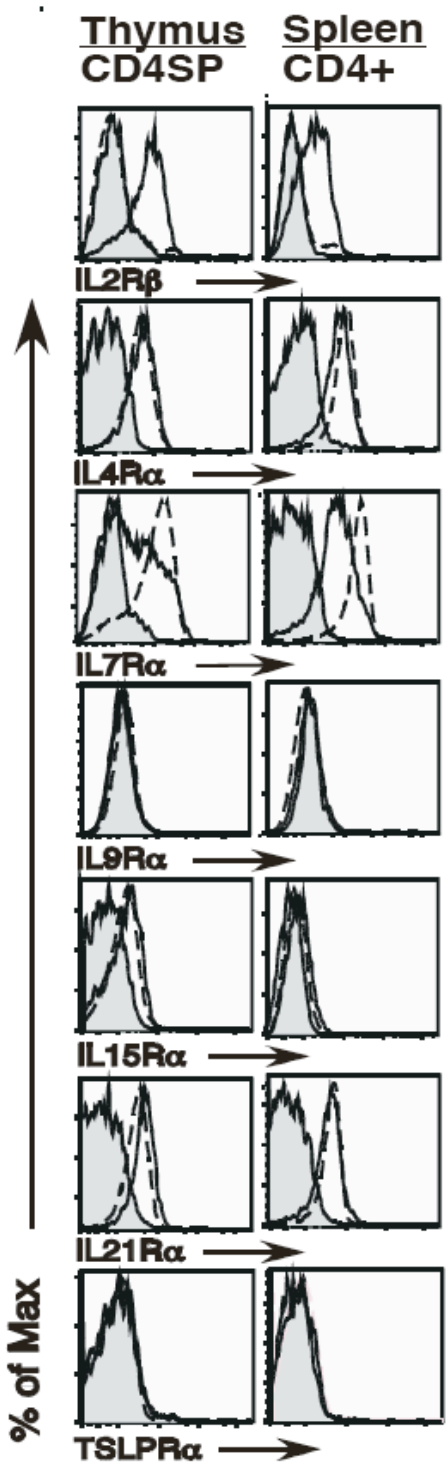
Results

To examine the role that different cytokines play in Treg development in the thymus, we first analyzed the expression of IL-2Rβ, IL-4Rα, IL-7Rα, IL-9Rα, IL-15Rα, IL-21Rα, and TSLPRα on $CD4^+Foxp3^+$ thymocytes and $CD4^+Foxp3^+$ splenocytes. We found four basic patterns of cytokine receptor expression. First, the IL-2Rβ -chain was selectively expressed on $CD4^+Foxp3^+$ thymocytes (Fig. 1). This pattern of expression was maintained in splenic $Foxp3^+$ vs $Foxp3^-$ T cells. Second, IL-9Rα and TSLPRα were not observed on either $Foxp3^+$ or $Foxp3^-$ thymocytes. To confirm that our staining for these receptors was working, we also stained peritoneal B1 B cells and $CD19^+$ pre-B cells, which have previously been reported to express the IL-9Rα and TSLPRα chains, respectively (17, 18). We detected IL-9Rα expression on peritoneal B1 B cells; as expected, we also observed TSLPRα expression on pre-B cells in the bone marrow, thereby indicating that our Abs to IL9Rα and TSLPRα are capable of detecting expression of these receptors (data not shown). Third, IL-4Rα and IL-21Rα were expressed equally on $Foxp3^+$ vs $Foxp3^-$ thymocytes and splenic T cells (Fig. 1). Last, we observed a dynamic expression pattern for the IL-7Rα and IL-15Rα chains. Expression of both of these receptors was observed in $CD4^+$ $Foxp3^+$ thymocytes, but was significantly reduced in splenic Tregs (Fig. 1). Thus multiple γc-dependent cytokine receptors, but not the TSLPRα -chain, are expressed on developing Tregs in the thymus. To assess whether receptor expression on Tregs correlated with function, we stimulated cells with IL-2, IL-4, IL-7, IL-9, IL-15, IL-21, and TSLP and examined STAT5 activation by intracellular staining for p-STAT5. We focused on STAT5 as

Fig. 3-1. *γc* receptor expression on $CD4^+Foxp3^+$ SP thymocytes and $CD4^+Foxp3^+$ splenocytes.

Thymus and spleen cells from 5-9 week old C57Bl/6 mice were harvested and stained with antibodies to CD4, CD8 and Foxp3, to identify distinct thymocyte and splenic T cell subsets, as well as antibodies to IL-2Rβ, IL-4Rα, IL-7Rα, IL-9Rα, IL-15Rα, IL-21Rα and TSLPRα. Shown are flow cytometry histograms of CD4 SP thymocytes (left column) and $CD4^+$ splenocytes (right column). Gray filled in histograms represent staining of the corresponding $Foxp3^+$ population with isotype control antibody. Solid lines and broken lines represent staining of $Foxp3^+$ and $Foxp3^-$ cells, respectively. A representative example of 4 independent experiments is depicted (n=17).

Fig. 1

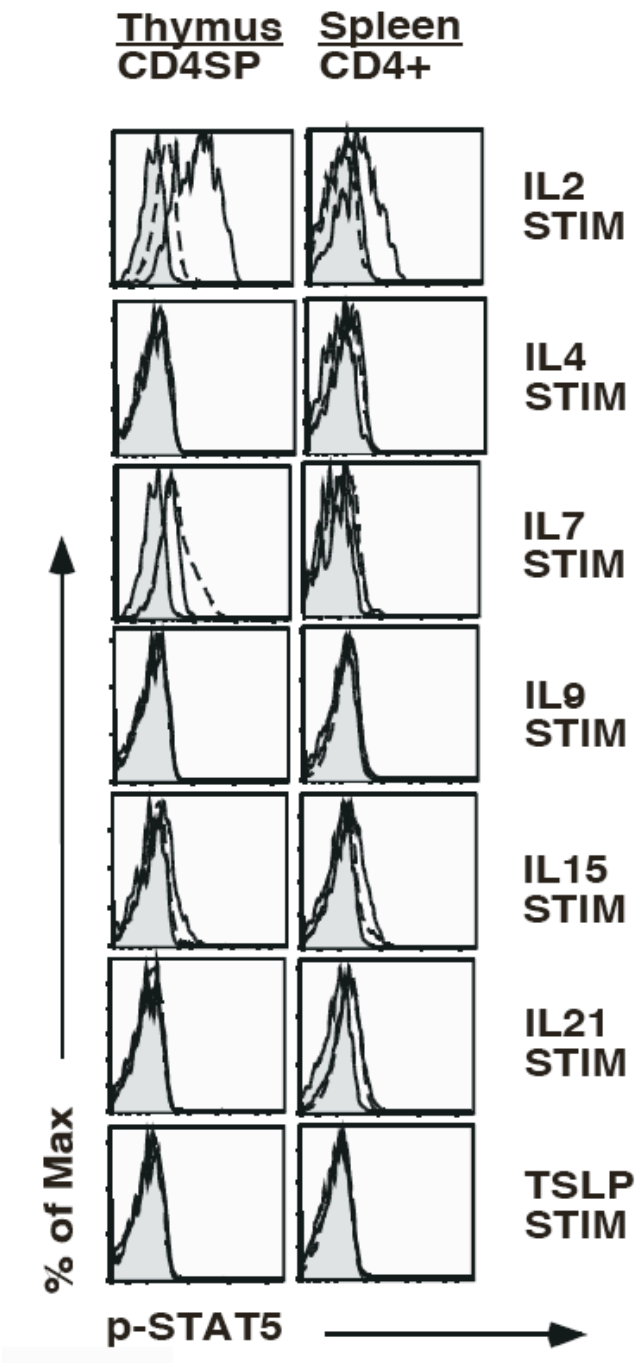

Thymus
CD4SP
Spleen
CD4+
IL2Rβ
IL4Rα
IL7Rα
IL9Rα
IL15Rα
IL21Rα
TSLPRα
% of Max

previous reports have indicated that STAT5 plays a critical role in Treg development (8, 10, 20). For these studies, we identified Tregs using Foxp3-GFP reporter mice; similar studies were also conducted using CD25 as a marker of Tregs. We identified three distinct response patterns. First, IL-2 induced robust STAT5 activation in $CD4^+$ $Foxp3^+$ thymocytes; splenic Tregs remained highly responsive to IL-2 stimulation (Fig. 2). Second, IL-7 and IL-15 induced modest STAT5 activation in $CD4^+$ $Foxp3^+$ thymocytes. However, these responses were almost completely eliminated in $CD4^+$ $Foxp3^+$ splenocytes (Fig. 2). Third, IL-4, IL-9, IL-21, and TSLP did not induce detectable STAT5 activation on $CD4^+$ $Foxp3^+$ thymocytes (Fig. 2). Similar results were obtained when gating on $CD4^+$ $CD25^+$ T cells, with the exception that under those staining conditions IL-4 led to very weak STAT5 phosphorylation in $CD4^+$ $Foxp3^+$ thymocytes and splenocytes (data not shown). A potential caveat with the IL-15 studies is that IL-15 could be presented by the IL-15Rα -chain via trans presentation in vivo (129). It is possible, therefore, that our ex vivo stimulation studies may not have allowed for optimal transpresentation of IL-15 to $CD4^+$ $Foxp3^+$ Tregs. Thus, developing thymic Tregs respond to IL-2 and IL-7, and to a lesser degree IL-15. Previous reports have suggested that TSLP plays a role in Treg development (130). However, *TSLPRα*$^{-/-}$ mice have no reported defects in Treg development or function (131). This latter observation is consistent with our failure to observe TSLPRα expression on developing Tregs. A potential caveat with our studies is that TSLPRα expression levels may be below the limits of detection by flow cytometery. Likewise, the amount of STAT5 phosphorylation induced by TSLP might be below the level that we can detect by flow cytometry. To explore this in more detail, we examined whether TSLPR mRNA was

Fig. 3-2. Cytokine stimulation and P-STAT5 expression on $CD4^+Foxp3^+$ thymocytes and splenocytes.

Single cell suspensions of thymocytes or splenocytes from Foxp3-GFP mice were serum starved for 30 minutes and then stimulated with IL-2, IL-4, IL-7, IL-9, IL-15, IL-21 or TSLP for 20 minutes. Cells were then stained with antibodies to CD4, CD8, CD25 and phospho-STAT5 as described in methods. Shown are histograms of phospho-STAT5 expression in CD4SP thymocytes (left column) and $CD4^+$ splenocytes (right column). Gray filled in histograms represent staining of unstimulated Foxp3-GFP^+ cells. Solid lines and broken lines represent staining of stimulated Foxp3-GFP^+ and Foxp3-GFP^- cells, respectively. A representative example of 2 independent experiments is depicted (n=3 mice). Similar results were obtained when using CD25 to identify Tregs (n=13 mice, data not shown).

Fig. 2


Thymus
CD4SP
Spleen
CD4+
IL2
STIM
IL4
STIM
IL7
STIM
IL9
STIM
IL15
STIM
IL21
STIM
TSLP
STIM
% of Max
p-STAT5

detectable by RT-PCR in CD4 Foxp3-GFP thymcoytes. These studies indicated that TSLPR mRNA could be detected at some level in developing Tregs (data not shown). A key question then is whether this results in expression of a receptor capable of inducing biological responses.

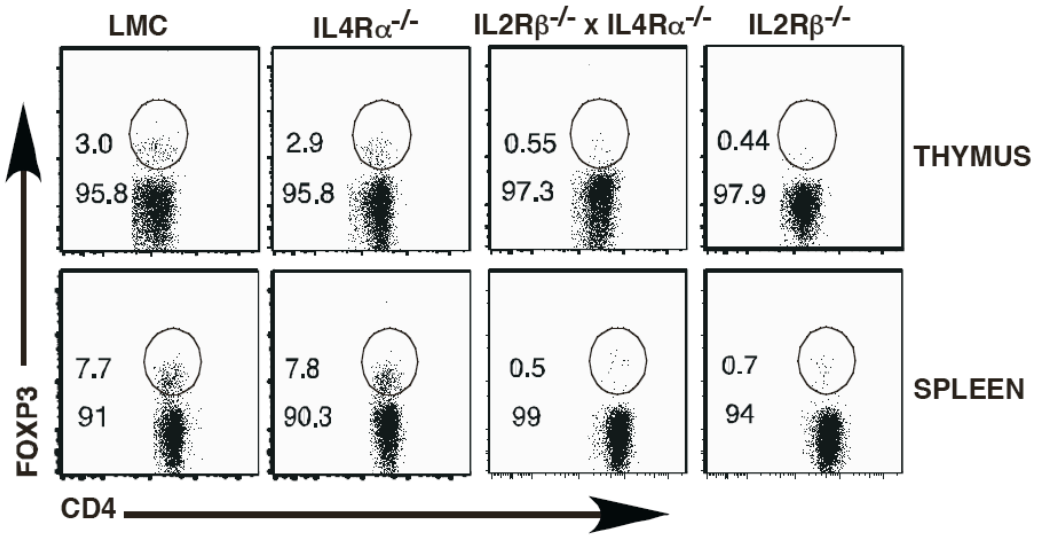
To address this question, we made use of the recent identification of $CD4^+$ $CD25^+$ $Foxp3^-$ thymocytes as penultimate Treg progenitors that can be converted into CD4 Foxp3 mature Tregs following stimulation with IL-2 (30, 31). For these studies, we isolated $CD4^+$ $CD25^+$ Foxp3-GFP^- Treg progenitors and stimulated them overnight with either IL-2, IL-7, IL-15, or TSLP. The cultured cells were then examined for Foxp3-GFP expression. As shown in Fig. 3, IL-2 induced clear conversion of Treg progenitors into $CD4^+$ Foxp3-GFP^+ Tregs. IL-7 and IL-15 were also capable of inducing the conversion of Treg progenitors into $Foxp3^+$ Tregs, although they were much less effective than IL-2. In contrast, TSLP stimulation failed to induce conversion of any Treg progenitors into $Foxp3^+$ Tregs. Thus, even if the TSLPR is expressed at very low levels on developing Tregs, it is incapable of inducing Treg differentiation following stimulation with TSLP.

Our observation that IL-2Rβ and IL-7Rα were the predominant receptors expressed on developing thymocytes suggested that IL-2, IL-7, and IL-15 were most likely the key *γc*-dependent cytokines that drive Treg development. However, given the expression of the IL-4Rα -chain on developing Tregs, we examined whether IL4-dependent signals also played a role in this process. *IL-4Rα*$^{-/-}$ mice show no decrease in

Fig. 3-3. Treg development in *IL4Rα*$^{-/-}$ and *IL2Rβ*$^{-/-}$ *x IL4Rα*$^{-/-}$ *mice.*

Thymus and spleen were harvested from 4-5 week old LMC, *IL4Rα*$^{-/-}$, *IL2Rβ*$^{-/-}$ and *IL2Rβ*$^{-/-}$ *x IL4Rα*$^{-/-}$ mice. Cells were stained with antibodies to CD4, CD8 and Foxp3 to identify Tregs. Shown are flow cytometry plots of thymus (top panel) or spleen cells (bottom panel) gated on CD4^{+} T Cells. A representative example of 6 independent experiments is depicted.

Fig. 3

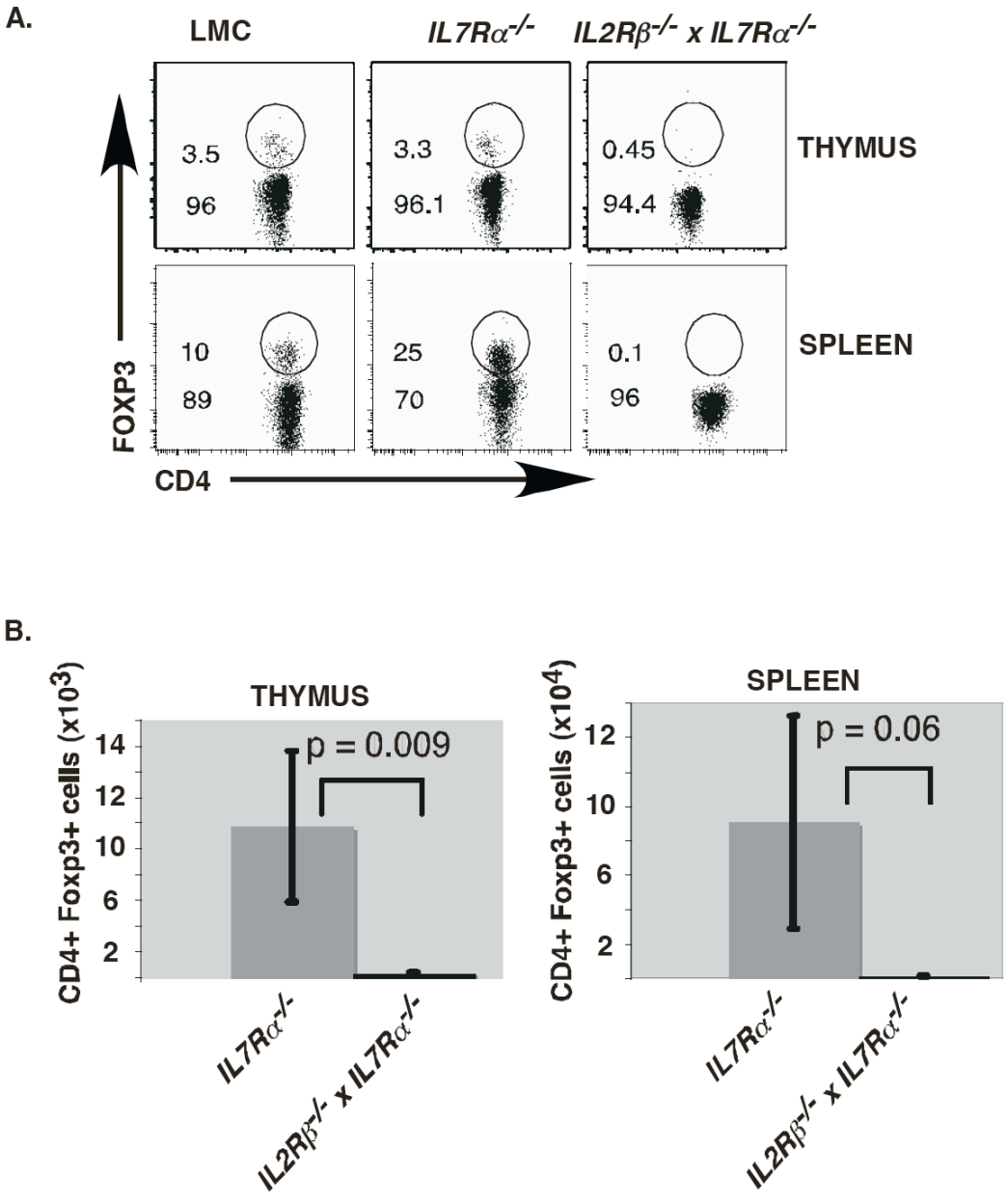

LMC
IL4Rα-/-
IL2Rβ-/- x IL4Rα-/-
IL2Rβ-/-
3.0
95.8
2.9
95.8
0.55
97.3
0.44
97.9
THYMUS
7.7
91
7.8
90.3
0.5
99
0.7
94
SPLEEN
FOXP3
CD4

the percentage of Tregs in the thymus relative to littermate control (LMC) mice (Fig. 4). Furthermore, splenic Tregs were also not reduced in *IL4Rα*$^{-/-}$ mice (Fig. 4). To examine whether IL-2Rβ and IL-4Rα -dependent signals played a redundant role in Treg development, we generated *IL4Rα*$^{-/-}$ *x IL2Rβ*$^{-/-}$ mice. As previously reported, *IL2Rβ*$^{-/-}$ mice exhibited reduced numbers of Tregs in both the thymus and spleen; a further reduction was not observed in *IL4Rα*$^{-/-}$ *x IL2Rβ*$^{-/-}$ mice (Fig. 4). These findings strongly suggest that IL-4Rα-dependent signals are not required for Treg development. It is important to note here that unlike our previous studies, which used *IL2Rβ*$^{-/-}$ mice on the C57BL/6 background, the *IL4Rα*$^{-/-}$ and *IL4Rα*$^{-/-}$ *x IL2Rβ*$^{-/-}$ mice in these experiments are on a mixed C57BL/6 x BALB/c background. We have consistently noticed that the *IL2Rβ*$^{-/-}$ mice on the C57BL/6 x BALB/c background mice have a more severe phenotype (i.e., fewer Tregs at an earlier age) than *IL2Rβ*$^{-/-}$ on the C57BL/6 background. This results in *IL2Rβ*$^{-/-}$ mice on the mixed background having a reduced percentage of Tregs relative to that seen in *IL2Rβ*$^{-/-}$ mice on the C57BL/6 background. Given the expression of both functional IL-7Rα and IL-2Rβ on developing Tregs, we predicted that these two cytokine receptors might both be capable of driving Treg development. Consistent with our previous report, we found that although total numbers of T cells are greatly reduced in *IL7Rα*$^{-/-}$ mice, the percentage of Tregs relative to other T cell subsets was not affected (Fig. 5*A*) (33). Thus, IL-7Rα signaling is not required for Treg development. However, it remains possible that IL-2Rβ and IL-7Rα can act redundantly to drive Treg development. To test this possibility, we compared Treg differentiation in *IL7Rα*$^{-/-}$ vs *IL2Rβ*$^{-/-}$ *x IL7Rα*$^{-/-}$ mice.

Fig. 3-4. Treg development in *IL7Ra*$^{-/-}$ and *IL2Rβ*$^{-/-}$ *x IL7Rα*$^{-/-}$ mice.

A. Thymus and spleen were harvested from 4-5 week old LMC, *IL7Rα*$^{-/-}$, and *IL2Rβ*$^{-/-}$ *x IL7Rα*$^{-/-}$ mice. Cells were stained with antibodies to CD4, CD8 and Foxp3 to identify Tregs. Shown are flow cytometry plots of thymus (top panel) or spleen cells (bottom panel) gated on CD4^{+} T cells. A representative example of 6 independent experiments is depicted. **B.** Shown are bar graphs representing total numbers of CD4^{+}Foxp3^{+} Tregs in the thymus (left panel) and spleen (right panel). Error bars represent standard error of the mean; n= 6 *IL7Rα*$^{-/-}$ and 6 *IL2Rβ*$^{-/-}$ *x IL7Rα*$^{-/-}$ mice; p-values were calculated using two-tailed students t-test.

Fig. 4


A.
LMC
IL7Rα-/-
IL2Rβ-/- x IL7Rα-/-
3.5
96
3.3
96.1
0.45
94.4
THYMUS
10
89
25
70
0.1
96
SPLEEN
FOXP3
CD4
B.
THYMUS
CD4+ Foxp3+ cells (x10^3)
14
12
10
6
2
p = 0.009
IL7Rα-/-
IL2Rβ-/- x IL7Rα-/-
SPLEEN
CD4+ Foxp3+ cells (x10^4)
12
10
6
2
p = 0.06
IL7Rα-/-
IL2Rβ-/- x IL7Rα-/-

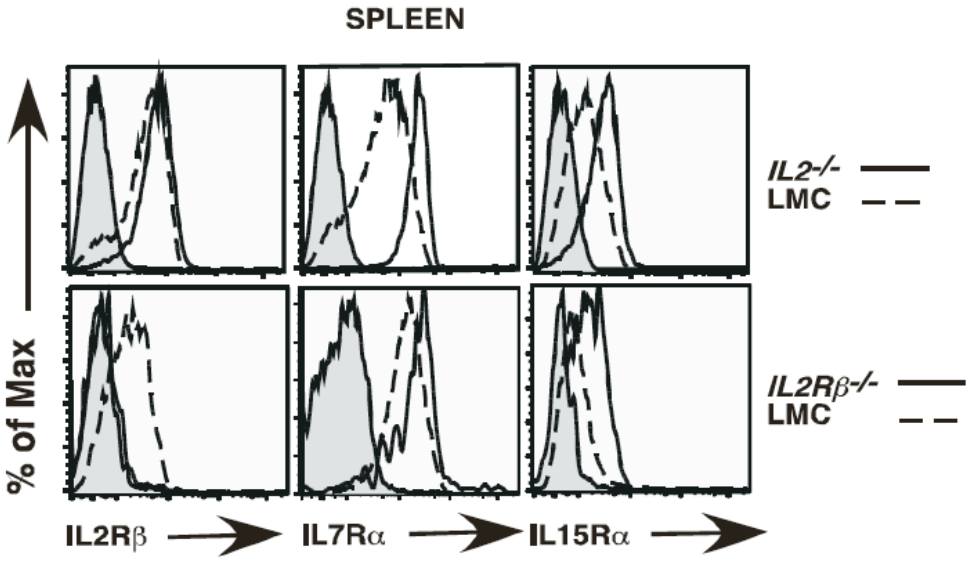
We found that *IL2Rβ*$^{-/-}$ *x IL7Rα*$^{-/-}$ mice showed a significant decrease in Treg numbers when compared with *IL7Rα*$^{-/-}$ mice ($p = 0.009$, Student's *t* test) (Fig. 5, *A* and *B*). Importantly, the numbers of Tregs found in *IL2Rβ*$^{-/-}$ *x IL7Rα*$^{-/-}$ mice (thymus = 195 +/- 75; spleen = 686 +/- 136) were comparable to that which we observed in age-matched *γc*$^{-/-}$ mice (thymus = 80 +/- 22; spleen = 1500 +/- 651) in our previous studies (Fig. 5*B*) (33). These experiments demonstrate that IL2Rα and IL7Rα -dependent cytokines are the only *γc*-dependent cytokines required for Treg development.

IL-7Rα and IL-15Rα are expressed at quite low levels on mature splenic Tregs. Thus, it is rather surprising that splenic Tregs are maintained in young *IL2*$^{-/-}$ mice. To examine this further, we stained CD4^{+} Foxp3^{+} Tregs from LMC and *IL2*$^{-/-}$ mice for the expression of IL-7Rα and IL-15Rα. We found that CD4^{+} Foxp3^{+} Tregs in *IL2*$^{-/-}$ mice expressed significantly higher levels of both the IL-7Rα and IL-15Rα chains (Fig. 6). We considered two explanations for these findings. First, it is possible that, in the absence of IL-2, any splenic Tregs that express higher levels of IL-7Rα or IL-15Rα have a competitive advantage and are selectively expanded. Alternatively, it is possible that IL-2/IL-2Rβ –dependent signals actively down-regulate IL-7Rα or IL-15Rα expression. To distinguish between these two possibilities, we stained the few CD4^{+}Foxp3^{+} Tregs in *IL2Rβ*$^{-/-}$ mice for IL-7Rα and IL-15Rα expression. Once again, we observed increased expression of both IL-7Rα and IL-15Rα on Tregs from *IL2Rβ*$^{-/-}$ vs LMC mice (Fig. 6). In *IL2Rβ*$^{-/-}$ mice, IL-15Rα expression provides no competitive advantage. Thus, this latter finding strongly suggests that IL-15Rα down-regulation,

Fig. 3-5. Expression of IL-2Rβ, IL-7Rα and IL-15Rα on $CD4^+Foxp3^+$ Tregs from *IL2*$^{-/-}$ and *IL2Rβ*$^{-/-}$ mice.

Splenocytes were isolated from 4-5 week old LMC, *IL2*$^{-/-}$ and *IL2Rβ*$^{-/-}$ mice and stained with antibodies for CD4, CD8, and Foxp3 to identify splenic Tregs. Shown are $CD4^+Foxp3^+$ gated cells stained for IL-2Rβ (left panels), IL-7Rα (middle panels) and IL-15Rα (right panels). Gray histograms represent staining of $CD4^+Foxp3^+$ cells with isotype control antibody. Solid lines represent histograms of $CD4^+Foxp3^+$ from *IL2*$^{-/-}$ (top panel) or *IL2Rβ*$^{-/-}$ (bottom panel) mice; broken lines represent staining of $CD4^+Foxp3^+$ Tregs from LMC mice. A representative example of 3 independent experiments is depicted (n= 6 *IL2*$^{-/-}$ and 3 *IL2Rβ*$^{-/-}$ mice).

Flg. 5



and likely IL-7Rα as well, is due to IL-2Rβ -dependent signals. To investigate this further, we took $CD4^+$splenocytes from *IL-2*$^{-/-}$ mice and stimulated those cells with IL-2. We then examined expression of IL-7Rα and IL-15Rα chains on $CD4^+$ $Foxp3^+$ cells (Fig. 7). We observed down-regulation of the IL-7Rα -chain as early as 8 h after IL-2 stimulation; both the IL-7Rα and IL-15Rα chains were clearly down regulated after 24 h of IL-2 stimulation. Taken together, these studies indicate that IL-2 dependent signals can negatively regulate IL-7Rα and IL-15Rα expression on $CD4^+$ $Foxp3^+$ Tregs.

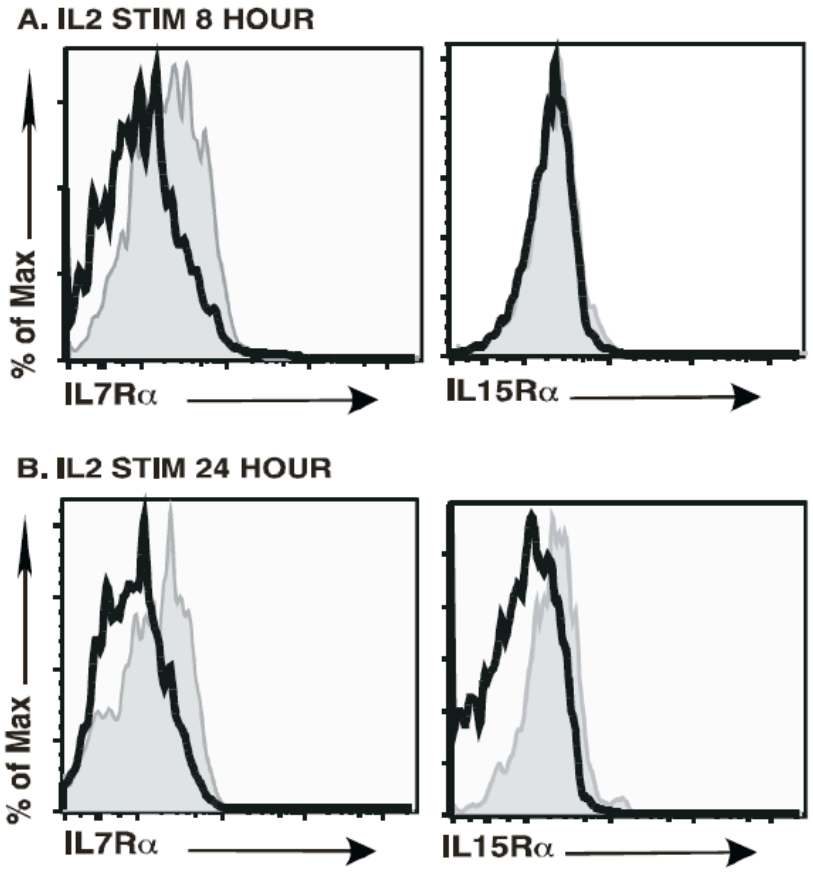
Discussion

These studies identify IL-2, IL-7, and IL-15 as the sole *γc*-dependent cytokines required for Treg development in the thymus. Four pieces of data support this conclusion. First, receptors for these three cytokines are expressed on developing Tregs in the thymus. Second, these cells can respond to IL-2 and IL-7 by inducing robust STAT5 activation. The only other *γc*-dependent cytokine receptors expressed on thymic Tregs are IL-4Rα and IL-21Rα . However, IL-4 and IL-21 did not induce STAT5 activation in $CD4^+Foxp3^+$ thymocytes and only induced minimal STAT5 activation in mature $CD4^+$ $Foxp3^+$ splenocytes. The role of IL-15 is somewhat more complicated. We observed only weak STAT5 induction following ex vivo stimulation of thymic $CD4^+$ $Foxp3^+$ Tregs with IL-15. This may reflect the absence of accessory cells in our ex vivo stimulation cultures that would allow for effective trans presentation of IL-15 to developing Tregs. Third, IL-2 and to a lesser degree IL-7 and IL-15 were capable of inducing the conversion of $CD4^+$ $CD25^+$ $Foxp3^-$ Treg progenitors into mature $CD4^+$ $Foxp3^+$ Tregs. In contrast, TSLP was completely ineffective in this assay. Finally, we

Fig. 3-6. Expression of IL-7Rα and IL-15Rα on $CD4^+Foxp3^+$ Tregs in *IL2*$^{-/-}$ mice after ex vivo IL-2 stimulation.

$CD4^+$ T cells were isolated as described in the methods section and cells were stimulated with 100 units/mL of IL-2 for 8 or 24 hours. Cells were then stained for Foxp3, IL-7Rα and IL-15Rα and analyzed by flow cytometry. Dark unshaded histogram represents IL-7Rα and IL-15Rα expression after IL-2 stimulation for the times indicated. Shaded histogram represents the non stimulated controls. Shown is a representative example of six independent experiments for IL-7Rα expression and two independent experiments for IL-15Rα expression.

Fig. 6


A. IL2 STIM 8 HOUR
% of Max
IL7Rα
IL15Rα
B. IL2 STIM 24 HOUR
% of Max
IL7Rα
IL15Rα

found that *IL2Rβ*$^{-/-}$ *x IL7Rα*$^{-/-}$ mice recapitulated the phenotype reported in *γc*$^{-/-}$ mice, which are essentially devoid of Tregs. In contrast, the reduction in thymic Tregs in *IL4Rα*$^{-/-}$ *x IL2Rβ*$^{-/-}$ mice was no more severe than that seen in *IL2Rβ*$^{-/-}$ mice. Taken together with our previous observation that *IL2*$^{-/-}$ *x IL15*$^{-/-}$ mice have significantly fewer Tregs that *IL2*$^{-/-}$ mice, our findings strongly support the conclusion that IL-2, IL-7, and IL-15, but not other γc-dependent cytokines, can contribute to Treg differentiation in the thymus. We also examined the role of TSLP on Treg differentiation as previous studies have suggested that TSLP plays an important role in human and murine Treg development (127, 130). Our studies rule out a direct role for TSLP in murine Treg differentiation. First, consistent with our earlier observation, *IL7Rα*$^{-/-}$ mice show no reduction in Tregs relative to non-Tregs. Second, we could not detect expression of TSLPRα on thymic Tregs nor induce STAT5 activation following stimulation of these cells with TSLP. Third TSLP was incapable of inducing the conversion of thymic Treg progenitors into CD4^{+} Foxp3^{+} Tregs. These findings demonstrate that TSLP cannot play a direct role in Treg development. It remains possible that TSLP plays an indirect role by acting on other cell types that may be involved in promoting Treg differentiation. However, this function is either not unique to TSLP, or not critical, as Tregs clearly develop in mice lacking the IL-7Rα -chain, which is a critical component of the TSLPR. Although our studies demonstrate that IL-2, IL-7, and IL-15 can redundantly contribute to Treg development and homeostasis, it seems likely that IL-2 is the relevant cytokine in wild-type mice. Specifically, we found that expression of IL-7Rα and IL-15Rα were significantly increased on Tregs in both *IL2*$^{-/-}$ and *IL2Rβ* $^{-/-}$ mice. Ex vivo stimulation of CD4^{+} Foxp3^{+} Tregs from *IL2*$^{-/-}$ mice with IL-2 led to rapid

down-regulation of both of these receptor subunits. Thus, IL-2 plays an important role in rendering $CD4^+$ $Foxp3^+$ Tregs uniquely responsive to IL-2-dependent signals in wild-type mice. This most likely serves to link Treg homeostasis directly to effector T cell activation and IL2 secretion. Effector T cell IL2 production appears to be critical for Tregs to expand in step with activated effector T cells and thereby mediate effective suppression. Supporting this conclusion, *IL2*$^{-/-}$ mice, but not *IL7*$^{-/-}$, or *IL15*$^{-/-}$ mice, show signs of T cell activation and ultimately succumb to lethal multiorgan autoimmune disease. Thus, although IL-7 and IL-15 are capable of sustaining Treg populations in young mice, they are not effective at expanding these cells sufficiently during ongoing immune responses. Finally, these findings also have implications for the use of low-level IL-7Rα expression to identify Tregs (132, 133) as this receptor may be upregulated under conditions of limited IL-2 availability.

Chapter IV

Future Directions

A role for a non-T cell source of IL-2 in Treg development

Abstract

Common γ chain *(γc)*-receptor dependent cytokines are required for regulatory T cell (Treg) development as $\gamma c^{-/-}$ mice lack Tregs. We have recently demonstrated that the only γc-cytokines required for Treg development are IL-2, IL-7 and IL-15 suggesting that these cytokines are redundant for Treg development. A potential confounding issue is that $IL15^{-/-}$ and $IL7^{-/-}$ mice show no defects in Treg development or homeostasis. In studying this issue, we observed that in wild type mice (as opposed to $IL2^{-/-}$), IL-2 is likely to be the only γc-cytokine that can drive Treg development and maintain T cell homeostasis. A key question that remains is what cells are producing the IL-2 needed for Treg development? To examine this, we performed mixed bone marrow chimeras using donor bone marrow from $IL2^{-/-}$ and $TCR\beta^{-/-}$ *x* $TCR\delta^{-/-}$ mice and reconstituted them into $IL2^{-/-}$ *x* $IL15^{-/-}$ *x* $Rag2^{-/-}$ hosts. As expected bone marrow reconstituted into $IL2^{-/-}$ *x* $IL15^{-/-}$ *x* $Rag2^{-/-}$ hosts efficiently generated $Foxp3^{+}$ Tregs. Identical results were obtained when using bone marrow from CD45.1+ wild-type mice plus $TCR\beta^{-/-}$ *x* $TCR\delta^{-/-}$ bone marrow. In contrast, introducing $IL2^{-/-}$ bone marrow alone into $IL2^{-/-}$ *x* $IL15^{-/-}$ *x* $Rag2^{-/-}$ hosts completely failed to generate Tregs. These studies demonstrate that neither T cell nor epithelial cell derived IL-2 is required for Treg development and suggest that a hematopoietic source of IL-2 is sufficient to drive Treg differentiation. Our current studies are focused on characterizing which thymic cell types are involved in this process.

IL-2 was first identified as a T cell growth factor. Subsequently, *IL2*$^{-/-}$ mice were made in which these mice developed autoimmunity such as lymphoproliferation manifesting as lymphoandenopathy, splenomegaly and, ulcerative colitis (124). This was startling in that a loss of IL-2 did not inhibit T cell proliferation as postulated. This was later attributed to the lack of IL-2 to sensitize T cells for activation induced cell death (AICD). However, with the identification of CD25 as a marker for Tregs, it was shown that *IL2*$^{-/-}$ and *IL2Rβ*$^{-/-}$ mice have decreased CD4^{+} CD25^{+} T cells. Indeed, transfer of CD4^{+} CD25^{+} T cells into *IL2Rβ*$^{-/-}$ mice was able to rescue this defect (76). This established a role for IL-2 in regulating Treg homeostasis and function (75, 121, 134, 135).

In addition, work by our laboratory and others has established a role for cytokines in regulating Treg development. Using Foxp3 as a marker for Tregs, we have found that young *IL2*$^{-/-}$ and *IL2Rα*$^{-/-}$ mice have relatively normal numbers of CD4^{+} Foxp3^{+} Tregs. This led us to question whether other cytokines can compensate for Treg development. We have shown that *IL2*$^{-/-}$ *x IL15*$^{-/-}$ and *IL2Rβ*$^{-/-}$ *x IL7Rα*$^{-/-}$ mice have decreased numbers and percentages of CD4^{+} Foxp3^{+} Tregs (Chapter 3 of thesis) (32, 117). In the absence of IL-2, however the IL-7Rα and IL-15Rα can compensate to drive Treg development. Taken together, our results demonstrate that IL-2 receptor signals render Tregs uniquely responsive to IL-2 alone. Thus, in normal wild-type mice IL-2 drives Treg development but in the absence of IL-2, cytokines such as IL-7 and IL-15 can compensate for this developmental defect (Chapter 3 of thesis (117)).

Signals through the TCR/CD28 initiate a Treg progenitor and that cytokine signals such as IL-2 complete this developmental pathway (30, 31). A critical question remains, if IL-2 is essential for Treg development, which cells in the thymic micro-environment are providing the IL-2? It has been proposed that the source of IL-2 might be T-cell derived. Indeed work by Setoguchi et al. have shown that activated T cells in the periphery can make the IL-2 (75). However, it has never been demonstrated that naïve T cells in the thymus can provide the IL-2 to drive Treg development.

Besides naïve T cells, there are several candidate cells in the thymic micro-environment that can potentially produce IL-2: thymic Dendritic cells (tDCs), NK cells, medullary thymic epithelial cells (mTECS) and cortical thymic epithelial cells (cTECSs). With the exception of the NK cells, the linkage of mTECs, cTECs and tDCs in T cells and in particular, Treg development has been proposed (23, 136). In fact, both *B71*$^{-/-}$ and *B72*$^{-/-}$ have decreased Tregs and work by Watanabe et al. have established a role for TSLP in educating human tDCs to direct Treg development (98, 127). There are two types of thymic DCs: plasmacytoid DCs (pDCs) and conventional DCs (cDCs). The cDCs can be further divided into two subsets based on Signal regulatory protein alpha (SIRPα, also known as CD172) and CD8α expression; those arising from a common T/B/NK cell precursor, the intrathymic cDCs, also known as the CD8α^{+} SIRPα^{-}, and those cDCs that arrive in the thymus from the periphery, the extrathymic cDCs, or CD8α^{-} SIRPα^{+} (136).

Work by Proietto et al. has shown that thymic dendritic (tDC) cells can induce $CD4^+$ $Foxp3^+$ Tregs. By in vitro culture and purifying each of the DC subsets and adding $CD4^+$ $CD25^-$ non-Tregs, they were able to show that the extrathymic $CD8\alpha^-$ $SIRP\alpha^+$ and to a lesser extent, the intrathymic $CD8\alpha^+$ $SIRP\alpha^-$ were able to induce the conversion of non-Tregs into $CD4^+$ $Foxp3^+$ Tregs (136). However most of this work was done in vitro culture with cytokines and hence the role of whether tDC derived IL-2 was required for Treg development was not addressed. However, work by Granucci et al. has shown that bone marrow derived DCs can make IL-2. In these studies, bone marrow DCs (derived by in vitro culture for 5 days supplemented with cytokines) were stimulated with live bacteria as a source for lipopolysaccharide and assessed for IL-2 message. By as early as 4 hours, IL-2 message was being made (137). These studies demonstrate that IL-2 can be made by bone marrow derived DCs but the question remain, can tDCs make IL-2? If so, which of the three subsets of tDCs can supply the IL-2 to drive Treg development? The following chapter will show some preliminary evidence that a non-T cell but hematopoietic source of IL-2 is sufficient for $CD4^+$ $Foxp3^+$ Treg development.

Materials and Methods

Mice

CD45.1, IL2$^{-/-}$*, IL15*$^{-/-}$, and *TCRβ*$^{-/-}$ *x TCRδ*$^{-/-}$ were obtained from The Jackson Laboratories. The *IL2 Thy1.1* reporter mice was a kind gift from Dr. Casey Weaver. All the mice were on a C57BL/6 background.

Flow cytometry and FACS analysis

Thymii were harvested and single cell suspensions were prepared, stained and analyzed as previously described (31, 61). Five million cells were used per staining condition. Cells were first pretreated with an Ab that blocks Fc receptor binding (Clone 24G2). Antibodies used were obtained from eBioscience (San Diego, CA): CD3 (2C11), CD4 (RM4-5), CD8 (53-6.7), CD122 (5H4), CD25 (PC61.5), GITR (DTA-1), CD45.1 (A20) and Foxp3 (FJK-16s).

Mixed bone marrow chimeras

Bone marrow cells from *TCRβ*$^{-/-}$ *x TCRδ*$^{-/-}$, *IL2*$^{-/-}$ and WT (CD45.1) mice were harvested and mature hematopoietic cell subsets were depleted using biotinylated antibodies (CD3, CD4, CD8, CD25, B220 (RA3-6B2), CD19 (eBio1D3), Ter119 (TER-119), Gr-1 (RB6-8C5) and NK1.1 (PK136)) by Miltenyi magnetic beads. Cells from distinct mice were mixed at a 1:1 ratio and introduced into sublethally irradiated *IL2*$^{-/-}$ *x IL15*$^{-/-}$ *x Rag2*$^{-/-}$ host mice by tail vein injection. Mice were analyzed 6-8 weeks after bone marrow reconstitution.

Results

$CD4^+$ $Foxp3^+$ Tregs and $B7.2^+$ $CD11c^+$ dendritic cells co-localize to the thymic medulla

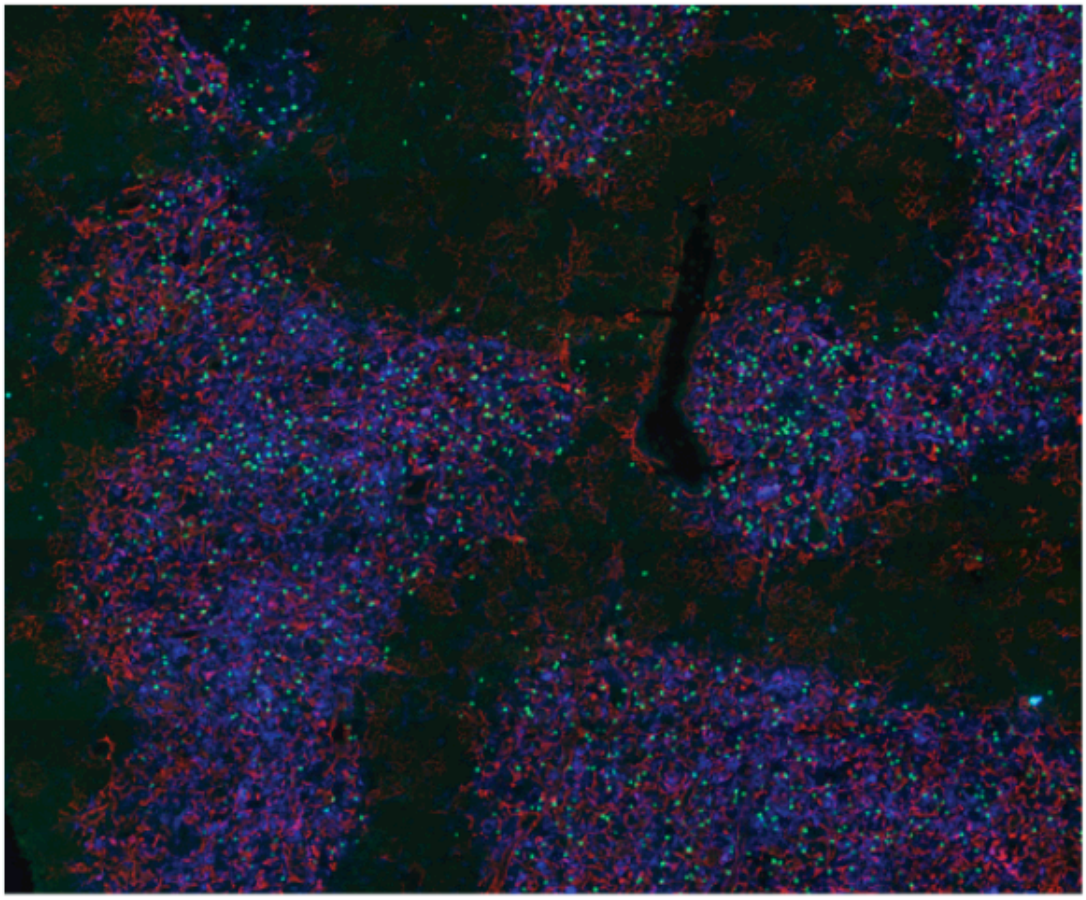
We wanted to examine where in the thymus were the $CD4^+$ $Foxp3^+$ Tregs. We stained for Foxp3 and B7.2 expression and found that both localized to the medulla as shown by Keratin 5 (K5) staining (Figure 1). This result was confirmed by staining a second set of slides for Foxp3 and CD11c (results not shown). These results confirmed that $Foxp3^+$ Tregs and $B7.2^+$ cells were found in the medullary region as previously reported. Due to the proximity of $Foxp3^+$ Tregs and $B7.2^+$ $CD11c^+$ cells, this led us to speculate which cells in the medulla were capable of producing IL-2 in mediating this process. The cells that could possibly produce IL-2 were the tDCs, mTECs or thymocytes.

A non-T cell source of IL-2 can induce Treg development

To investigate whether a non-T cell source of IL-2 was sufficient for Treg development, we made use of *$TCR\beta^{-/-}$ x $TCR\delta^{-/-}$* mice, which have a T cell developmental block at the Double Negative 3 (DN3) stage, but are sufficient in every other cell lineage all of which retain the ability to make IL-2. We postulated that if the only source of IL-2 was limited to hematopoietic cells that could not give rise to T cells, we can induce $CD4^+$ $Foxp3^+$ Treg development. Using these mice, we mixed a 1:1 ratio of bone marrow from either *CD45.1* wild-type plus *$IL2^{-/-}$* or, *$TCR\beta^{-/-}$ x $TCR\delta^{-/-}$* plus *$IL2^{-/-}$* into sublethality irradiated *$IL2^{-/-}$ x $IL15^{-/-}$ x $Rag2^{-/-}$* hosts (Figure 2A).

Fig. 4-1. $CD4^+$ $Foxp3^+$ Tregs and $B7.2^+$ Dendritic cells are localized to the medulla. Shown are 8 μm thick thymi sections from C57BL/6 mice that were stained for Fopx3, B7.2 and Keratin 5 (K5) (Foxp3 = Green, B7.2 = Blue, K5 = Red). $Foxp3^+$ Tregs and $B7.2^+$ cells co-localize to the medulla. A representative example of 2 independent experiments is depicted (n= 3).

Fig. 1



Foxp3	= Green
B72	= Blue
K5	= Red

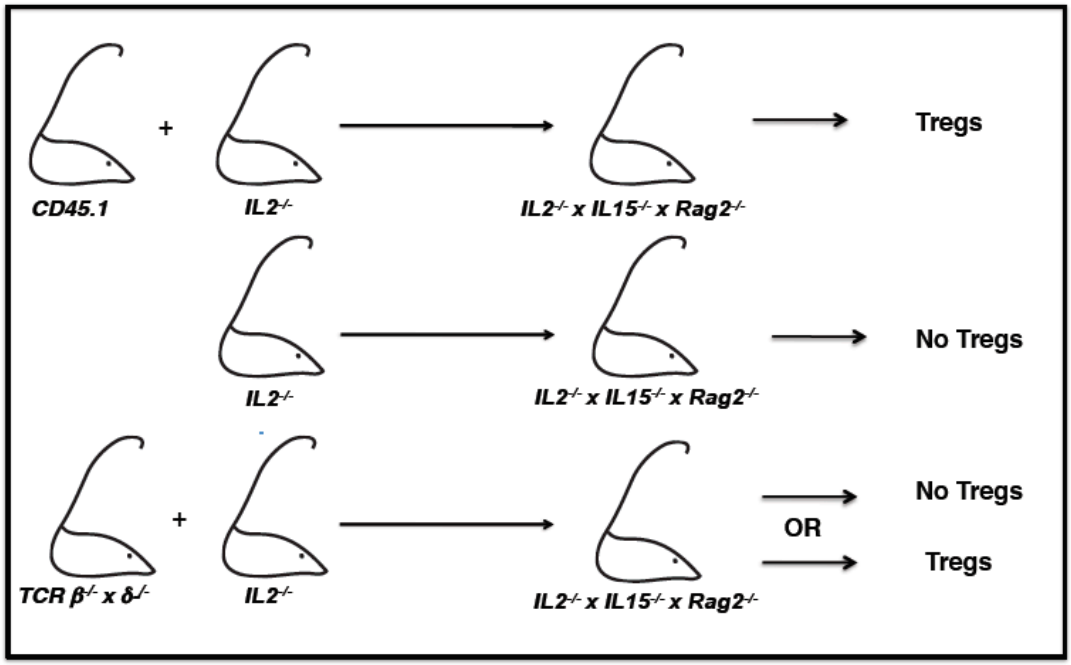
Bone marrow from *IL2*$^{-/-}$ mice was injected via the tail vein into *IL2*$^{-/-}$ *x IL15*$^{-/-}$ *x Rag2*$^{-/-}$ hosts (Figure 2A). In these mice, where there would be no source of IL-2 from the donor or host, we predicted that there would be no CD4^{+} Foxp3^{+} Tregs. As expected, unlike *IL2*$^{-/-}$ alone*, CD45.1* plus *IL2*$^{-/-}$ bone marrow into *IL2*$^{-/-}$ *x IL15*$^{-/-}$ *x Rag2*$^{-/-}$ hosts gave rise to CD4^{+} Foxp3^{+} Tregs. This result was similar when we compared CD4^{+} Foxp3^{+} Tregs from *CD45.1* plus *IL2*$^{-/-}$ to *TCRβ*$^{-/-}$ *x TCRδ*$^{-/-}$ *plus IL2*$^{-/-}$ ($p > 0.5$) (Figure 2B). We found that if the source of IL2 was limited to hematopoietic cells that could not give rise to T cells, CD4^{+} Foxp3^{+} Tregs still developed. In addition, in our mixed bone marrow chimeras, the possible contribution of mTECS to CD4^{+} Foxp3^{+} Treg development was negated since all hosts were on an *IL2*$^{-/-}$ *x IL15*$^{-/-}$ *x Rag2*$^{-/-}$ background. These data suggest that in our model, a T cell source of IL2 is not required for CD4^{+} Foxp3^{+} Treg development. In addition, a hematopoietic source of IL2 such as that might be provided from tDCs are sufficient to drive this process.

Fig. 4-2. A non-T cell source of IL-2 is required for Treg development.

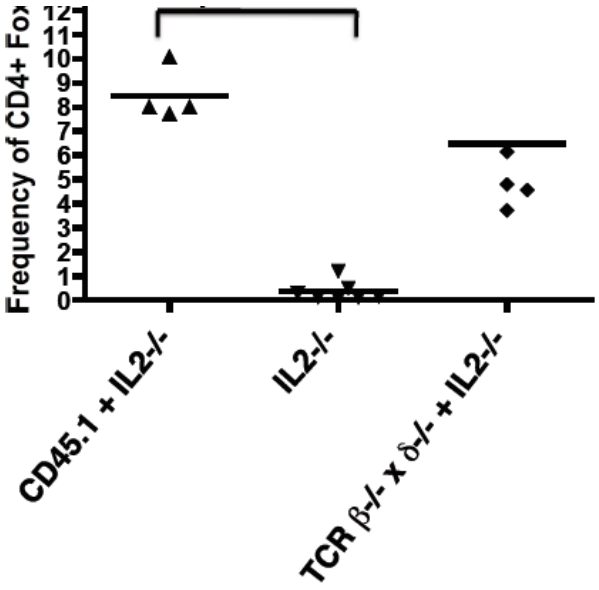
A. Schematic depicting the mixed bone marrow chimeras for *IL2*$^{-/-}$ + *CD45.1, IL2*$^{-/-}$ alone, and *TCRβ*$^{-/-}$ *x TCRδ*$^{-/-}$ + *IL2*$^{-/-}$. **B.** Shown are mixed bone marrow chimeras for *IL2*$^{-/-}$ + *CD45.1* (left), *IL2*$^{-/-}$ alone (middle), and *TCRβ*$^{-/-}$ *x TCRδ*$^{-/-}$ + *IL2*$^{-/-}$ (right). Cells from distinct mice were mixed at a 1:1 ratio and introduced into sublethally irradiated *IL2*$^{-/-}$ *x IL15*$^{-/-}$ *x rag2*$^{-/-}$ hosts. Cells are gated on CD4^{+} Foxp3^{+} Tregs in the CD4SP of thymus. A representative example of 3 independent experiments is depicted.

Fig. 2

A.


CD45.1
+
IL2-/-
IL2-/- x IL15-/- x Rag2-/-
Tregs
IL2-/-
IL2-/- x IL15-/- x Rag2-/-
No Tregs
TCR β-/- x δ-/-
+
IL2-/-
IL2-/- x IL15-/- x Rag2-/-
No Tregs
OR
Tregs

B.


Frequency of CD4+ Fo
0
1
2
3
4
5
6
7
8
9
10
11
12
CD45.1 + IL2-/-
IL2-/-
TCR β-/- x δ-/- + IL2-/-

Discussion

Although these results are preliminary, taken together, they allow several predictions. First, $CD4^+$ $Foxp3^+$ Treg development was evident in the absence of a T-cell source of IL-2. Second, a cell arising from the bone marrow can migrate to the thymus and efficiently induce $CD4^+$ $Foxp3^+$ Treg development. Third, in our model, TECs are not needed to supply the IL-2. Although our results do not definitively exclude a role for T or epithelial cell-derived IL-2 in mediating this process, based on our findings, a hematopoietic source of IL-2 that is not T cell derived is sufficient to promote the development of $CD4^+$ $Foxp3^+$ Tregs.

We have found that naïve T cells from the thymus cannot produce IL-2 (data not shown). However, we have found that $CD4^+$ $CD44^+$ memory T cells in the spleen can make IL-2 (~14%, unpublished results). This would imply that the source of IL-2 in the thymus is not of T cell origin as previously postulated but rather derived from another cell(s) of hematopoietic origin. This would certainly fit well with studies from the Wu laboratory in which they show that a population of migrating $CD8^{lo}$ $SIRP\alpha^+$ tDCs have the capability in vitro to induce $CD4^+$ $Foxp3^+$ Tregs (136). However recent work by Martin-Gayo et al. have demonstrated that thymic plasmacytoid dendritic cells can drive Treg development (138). What remains to be investigated is the relative contribution of each tDC subset as the source of IL-2 for Treg differentiation?

Chapter V

Summary

Regulatory T cells have shown promise in the clinical setting. The functional significance of regulatory T cells is the maintenance of dominant tolerance to self and the prevention of autoimmunity. However, the factors that govern Treg development have not been fully elucidated. This thesis set out to address the molecular mechanism, the cytokine and the accessory cell(s) requirement in the thymic microenvironment that is required for $CD4^+$ $Foxp3^+$ Treg development?

Herein, we show that the PYAP but not the YMNM motif in the CD28 cytoplasmic tail is required for Treg progenitor development. To examine the role of CD28 in Treg progenitor development, we made use of two *Cd28* knock-in mutants with defects in CD28-dependent PI3K (Y170F mutant) or Lck (AYAA mutant) activation, respectively. Using these mice, we performed flow cytometry for $CD4^+$ $Foxp3^-$ $CD25^+$ $CD122^+$ $GITR^+$ Treg progenitors and $CD4^+$ $Foxp3^+$ Tregs. We found that AYAA mutants had decreased $CD4^+$ $Foxp3^+$ Tregs compared to the Y170F mutant mice. More importantly there was a reduction in the percent of $CD4^+$ $Foxp3^-$ $CD25^+$ $CD122^+$ $GITR^+$ Treg progenitors in the AYAA mutants. To examine if this was due to a cell intrinsic defect, we made mixed bone marrow chimeras in which we mixed a 1:1 ratio of either AYAA, Y170F or *Cd28*$^{-/-}$ with cogenically marked $CD45.1^+$ wild-type mice. We found that $CD45.1^+$ wild-type $CD4^+$ $Foxp3^-$ $CD25^+$ $CD122^+$ $GITR^+$ Treg progenitors outcompete AYAA Treg progenitors. In contrast, $CD45.1^+$ wild-type Treg progenitors did equally as well as the Y170F Treg progenitors. These results highlight a role for the CD28 PYAP motif to regulate $CD4^+$ $Foxp3^-$ $CD25^+$ $CD122^+$ $GITR^+$ Treg progenitor development.

The LCK/CD28 pathway has been shown to activate the NFκB pathway. Herein, we examined Treg progenitor development in *c-rel*$^{-/-}$, *nfkb1*$^{-/-}$ and *c-rel*$^{-/-}$ *x nfkb1*$^{-/-}$ mice. We found that in *c-rel*$^{-/-}$ mice unlike *nfkb1*$^{-/-}$ mice, exhibited a dramatic decrease in CD4^{+} Foxp3^{-} CD25^{+} CD122^{+} GITR^{+} Treg progenitors. There was no further decrease seen in *c-rel*$^{-/-}$ *x nfkb1*$^{-/-}$ mice. Mixed bone marrow chimeras with either *c-rel*$^{-/-}$ plus *CD45.1*$^{+}$ wild-type revealed that this result was cell intrinsic as *CD45.1*$^{+}$ wild-type Tregs and Treg progenitors outcompete *c-rel*$^{-/-}$ Tregs and Treg progenitors. These studies suggest that the PYAP motif in the CD28 cytoplasmic domain and C-REL initiate Treg development, step 1 of the Two Step model of Treg development.

IL-2 is the main cytokine in wild-type mice that can promote CD4^{+} Foxp3^{+} Treg development. IL-2Rβ shares the common γ chain with its family members: IL-4Rα, IL-7Rα, IL-9Rα, IL-15Rα and IL-21Rα. Herein, we wanted to establish the relative contribution of each receptor family in CD4^{+} Foxp3^{+} Treg development. We examined the receptor expression on developing and mature CD4^{+} Foxp3^{+} Tregs. We found that the IL-2Rβ was preferentially expressed on developing CD4^{+} Foxp3^{+} Tregs and remained expressed as Tregs migrated into the periphery. In contrast, IL-7Rα was expressed on developing CD4^{+} Foxp3^{+} Tregs, although receptor expression was much lower compared to IL-2Rβ expression. As the CD4^{+} Foxp3^{+} Tregs matured, IL-7Rα expression was down regulated. However, the expression of the IL-15Rα receptor on CD4^{+} Foxp3^{+} Tregs was modest at most on developing and mature Tregs. Previously, we have shown a role for STAT5 in regulating CD4^{+} Foxp3^{+} Tregs development thus, we wanted to establish if receptor expression correlated with STAT5 activation in CD4^{+}

Foxp3$^+$ Tregs. When we stimulated with each respective γc cytokine, we found that STAT5 correlated with respect to receptor expression on developing and mature CD4$^+$ Foxp3$^+$ Tregs. IL-2 induced STAT5 phosphorylation was higher in CD4$^+$ Foxp3$^+$ Tregs compared to IL-7 or IL-15. This validated our previous findings that IL-2 receptor signaling is required for CD4$^+$ Foxp3$^+$ Treg development. Furthermore, examination of *IL2Rβ*$^{-/-}$ *x IL7Rα*$^{-/-}$ knockout mice lacked CD4$^+$ Foxp3$^+$ Treg resembling the phenotype as seen in *γc*$^{-/-}$ mice. Whereas there was no decreased in CD4$^+$ Foxp3$^+$ Tregs in *IL2Rβ*$^{-/-}$ *x IL4Rα*$^{-/-}$ mice when compared to *IL2Rβ*$^{-/-}$ mice alone. Thus, in normal healthy mice, IL-2 is the main cytokine that drives Treg development. However, in the absence of IL-2 such as that found in *IL2*$^{-/-}$ mice, IL-7 and IL-15 can compensate for CD4$^+$ Foxp3$^+$ Treg development, step 2 of the Two Step model of Treg development.

If IL-2 signaling is essential for Treg development what cells are supplying the IL-2 to mediate this process? *IL2*$^{-/-}$ and *IL2Rβ*$^{-/-}$ mice lack CD4$^+$ Foxp3$^+$ Tregs. Herein, to investigate whether a hematopoietic source of IL-2 was sufficient to drive CD4$^+$ Foxp3$^+$ Treg development, we made mixed bone chimeras where IL-2 production was restricted to a non-T cell but hematopoietic source (Chapter 4). We found that in the absence of a T cell source of IL-2, CD4$^+$ Foxp3$^+$ Treg development was efficiently induced. Hence IL-2 production from a hematopoietic source that was not T cell derived was sufficient for CD4$^+$ Foxp3$^+$ Treg development.

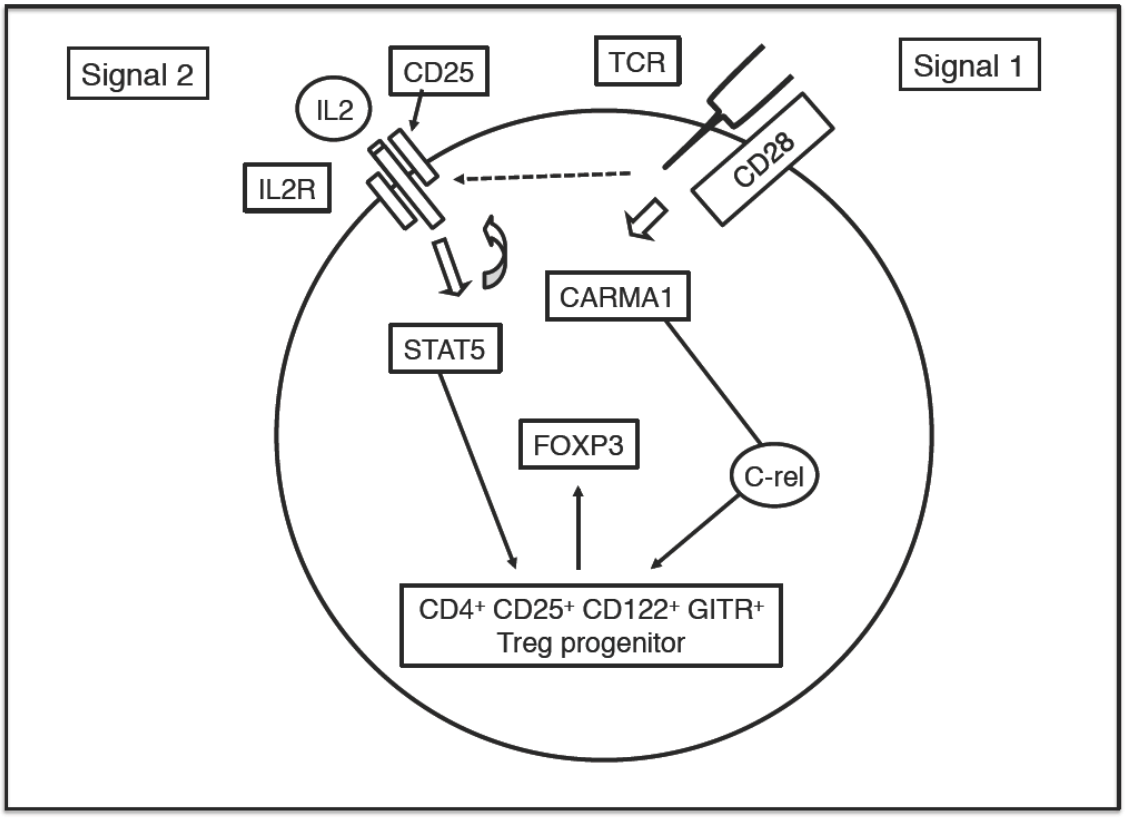
In summary, I would like to leave you with a revised two-step model of $CD4^+$ $Foxp3^+$ Treg development (Figure 5-1). In step 1, a Treg precursor receives a high affinity signal through the TCR. This also requires co-stimulation via the CD28 LCK binding motif. Potentially this recruits PKCθ, which can phosphorylate and activate the Carma1/Bc110/MALT1 (CBM) complex. We think that in developing Tregs, the CBM complex recruits and activates the IKKγ /IKKα /IKKβ complex which allows for the subsequent ubiquitination and degradation of IκB. Degradation of IκB leads to the liberation and nuclear translocation of c-Rel homodimers, or c-Rel/RelA heterodimers. This allows c-Rel to bind to *c-Rel* binding sites in the CNS3 region of the Foxp3 gene (115). We propose that this leads to the up regulation of CD25. Subsequently, in Step 2, IL-2 signaling via STAT5 completes Treg development by turning on Foxp3.

Future studies should focus on the regulation of the IκB family by the IKK complex in regulatory T cells. In addition, these studies should focus on the higher order regulatory network in Treg development such as the recruitment of, in addition to c-Rel, unidentified co-repressors and co-activators at the Foxp3 promoter. However, the development of Tregs would benefit from the understanding of how c-Rel and STAT5 cooperate in directing Treg lineage commitment. Herein, we propose that this might be a two-step process. However, it may be that c-Rel and STAT5 may work in tandem in directing Treg development, however, this needs to be further investigated. The elucidation of these pathways will provide insight into the development of natural Regulatory T cells.

Fig. 5-1. Revised Two-step model of Treg development.

Treg development occurs via a two-step process. In Step 1 (Signal 1), signals through the TCR and CD28 initiate a Treg developmental program dependent on the NFκB signaling pathway. Notably, signals through CARMA1 and C-REL initiate a Treg progenitor in which cytokine signaling such as IL-2 (Signal 2) via STAT5 turns on Foxp3.

Fig. 1


Signal 2
CD25
TCR
Signal 1
IL2
IL2R
CD28
CARMA1
STAT5
FOXP3
C-rel
CD4+ CD25+ CD122+ GITR+
Treg progenitor

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