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Brief review: Regulatory T cell development in the thymus

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Abstract

Development of a comprehensive regulatory T cell compartment in the thymus is required to maintain immune homeostasis and prevent autoimmunity. Here, we review cellular and molecular determinants of T_{reg} cell development in the thymus. We focus on the evidence for a self-antigen focused T_{reg} cell repertoire, as well as the APCs responsible for presenting self-antigens to developing thymocytes. We also cover the contribution of different cytokines to thymic T_{reg} development and the cellular populations that produce these cytokines. Finally, we update the originally proposed "two-step" model of thymic T_{reg} differentiation by incorporating new evidence demonstrating that T_{reg} cells develop from two T_{reg} progenitor populations and discuss the functional importance of T_{reg} cells generated via either progenitor pathway.

Introduction

Adaptive immunity evolved as a powerful defense mechanism to eliminate foreign pathogens and eradicate transformed cells. This system relies on two chief capabilities-extensive repertoire diversity and the ability to discriminate "self" versus "non-self" (1). In T cells, diversity is derived from random rearrangements of the TCR alpha and beta loci (2, 3). However, diversity comes at a cost, as some of these rearrangements will generate self-reactive T cells capable of initiating pathogenic immune responses. The thymus acts as a training ground for T cells and plays a role in ensuring a diverse, "non-self" focused, TCR repertoire capable of eliminating pathogens. The process of generating a diverse TCR repertoire also leads to the development of many autoreactive T cells. Many of these autoreactive T cells are eliminated via clonal deletion in the thymus. However, many self-reactive T cells do escape clonal deletion and, when left uncontrolled, elicit detrimental autoimmune diseases. While several mechanisms evolved to control autoimmune responses, a specialized subset of suppressor $CD4^+$ T cells, termed regulatory T cells (T_{reg}), plays a particularly important role in maintaining immune homeostasis.

Over the past 20 years tremendous progress has been made in the identification and understanding of T_{reg} cells. This relatively small population, ~1% of developing CD4 single positive thymocytes and ~10-15% of $CD4^+$ T cells in secondary lymphoid organs, is responsible for maintaining immune homeostasis and is crucial for survival (4–9). T_{reg} cells are an incredibly diverse population with regard to both TCR repertoire and function. T_{reg} cells regulate numerous physiologic processes, including maternal-fetal conflict (10–17), germ cell tolerance (18), stem cell differentiation in the skin (19), muscle repair (20), adipocyte homeostasis and function (21–25), and retinal inflammation (26). In addition, T_{reg}

cells also regulate effector immune responses in disease states such as germinal center reactions (27, 28), inhibit overzealous T cell responses during infection (29–34), enhance effector T cell differentiation and memory formation to pathogens (35–37), inhibit tumor immunity (38, 39), and promote tolerance to environmental and commensal antigens (40–42). The burden of regulating these diverse processes has led the field to propose two broad functional classes of T_{reg} cells defined by their ontogeny- peripheral- (pT_{reg}) and thymic- (tT_{reg}) derived T_{reg} cells. In this review we focus on tT_{reg} cell development.

Why the thymus?

The thymus has been an organ of immense curiosity for immunologists for some time. While initial thymectomy experiments failed to reveal immunological consequences (43), subsequent work revealed a central function in immune responses (44–46). Work as early as 1962 by Jacques Miller suggested a role in immune tolerance, as day 3 thymectomized (d3Tx) mice succumbed to an autoimmune wasting disease by 3 months of age (47). A seminal study in 1969 described that day 3, but not day 7 or later, thymectomized mice developed autoimmunity of the ovary that could be rescued by a thymus transplant (48). Work by Gershon and Kondo subsequently showed that thymocytes could produce dominant tolerance during immune responses to sheep red blood cells and coined the term “suppressor T cells” (49–51). Together, this work suggested the existence of a population of thymus-derived suppressive T cells that had delayed kinetics of thymic export.

Although the concept of immune suppression was clearly correct, early models to explain this process proved unsatisfactory. Most notably, it was suggested that “suppressor T cells” could function via a soluble factor encoded in the MHC locus, I-J (52). However, the I-J locus was eventually found not to encode a unique protein (53). This led many to reject the concept of a unique population of T cells capable of immune suppression (54). Despite these controversies, work in the early 1980’s already suggested the presence of a subpopulation of T cells, defined by anti-Lyt-1 (later described as CD5) antibody positivity, that were capable of suppressing autoimmunity in d3Tx mice (55). A seminal study by Sakaguchi in 1995 discovered that $CD25^+$ T cells were necessary and sufficient for suppressing autoimmune responses. The identification of CD25 as a marker of suppressive T cells was critical to add legitimacy to the field (4). A follow-up study connected this concept to autoimmunity observed in d3Tx experiments, as d3Tx prevented accumulation of $CD25^+$ cells in the periphery of mice. Transfer of $CD25^+$ cells into d3Tx mice was able to rescue autoimmunity, while transfer of CD25-depleted splenocytes caused autoimmunity in athymic mice, revealing that thymically derived $CD25^+$ T cells were critical controllers of autoimmunity (56). Groundbreaking studies in humans, suffering from immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX), and scurfy mice, identified a critical role for the transcription factor FOXP3 in T_{reg} cells (6, 7, 57). This led to the generation of a series of reporter mice to track FOXP3 expression in live cells (58–60), enabling functional T_{reg} transfer experiments. Additionally, protocols were developed to detect intracellular FOXP3 by flow cytometry that enabled tracking and quantification of T_{reg} cells in non-reporter mice and humans (61). The identification of CD25 and FOXP3 as useful markers of T_{reg} cells led to an explosion of studies seeking to understand T_{reg} cell development and function.

Two-step model of thymic T_{reg} cell development

The prevailing paradigm of thymic T_{reg} cell development involves a two-step process (62, 63). Step one is driven by strong TCR stimulation in developing CD4 single positive thymocytes. This causes the upregulation of the high affinity IL-2 receptor, CD25, as well as TNF receptor superfamily (TNFRSF) members GITR, OX40 and TNFR2, thereby generating $CD25^{+}FOXP3^{-}$ T_{reg} cell progenitors ($T_{reg}P$). The second step is driven by cytokine-dependent conversion of $T_{reg}P$ into mature T_{reg} cells via upregulation of FOXP3. These $CD25^{+}FOXP3^{+}$ cells are mature T_{reg} cells that emigrate from the thymus and mediate tolerance. More recent studies have implicated an alternative, $CD25^{-}Foxp3^{lo}$ $T_{reg}P$ cell population (64); differentiation of these $T_{reg}P$ depends on the same two-step process (65). In this review we focus on the mechanisms that drive T_{reg} cell development in the thymus and summarize current evidence on how the thymus shapes the T_{reg} repertoire and function to maintain comprehensive immune tolerance.

TCR signals as an instructive cue for thymic T_{reg} cell development

Whether the tT_{reg} cell TCR repertoire is enriched in self-reactive TCRs was initially controversial. For example, one group found extensive overlap between TCRs in T_{conv} and T_{reg} cells and suggested that T_{reg} cells respond to “non-self” antigens (66). Likewise, analysis of AND TCR transgenic mice observed that inducing antigen expression increased T_{reg} cell proportion but not numbers in the thymus, suggesting that engagement of cognate self-antigen was not driving T_{reg} cell development (67). Nevertheless, other studies have provided evidence that the T_{reg} cell TCR repertoire is more self-reactive than its conventional counterpart, and that acquisition of agonist TCR stimulation is important in T_{reg} cell development. This view originated from early experiments observing the presence of $CD25^{+}$ cells in the thymus of wildtype mice, but not those expressing a transgenic TCR specific for foreign antigen (68). This hypothesis was confirmed in later studies showing that TCR transgenics could drive thymic T_{reg} cell development only when the cognate antigen was also expressed in the thymus (69). Further, TCR sequencing experiments on mice with reduced TCR repertoires observed that T_{reg} TCRs are largely distinct from conventional T cell TCRs (70, 71), but overlap with TCRs expressed by pathogenic self-reactive T cells in *Foxp3*$^{-/-}$ mice (72). In addition, a series of experiments observed that intraclonal competition for cognate antigen limits T_{reg} cell differentiation (73, 74) suggesting that interaction with antigen, presumably self-antigen, is important for T_{reg} cell development. Later work used TCR transgenics with varying affinity for OVA and observed a linear relationship between TCR affinity and T_{reg} cell development (75). OVA-specific T_{reg} cells develop in RIP-mOVA thymi with TCRs spanning a broad 3 log fold response range. While lower affinity TCRs can drive T_{reg} induction, TCR affinity and T_{reg} cell niche size are directly correlated with higher affinity TCRs driving increased numbers of T_{reg} cells (75). Further, analysis of Nur77-GFP transgenic reporter mice, in which GFP is expressed coordinately with TCR signal strength, observed that T_{reg} cells were interacting more strongly with self-antigens (76). For example, lower proportions of TCR transgenic thymocytes in chimeric mice led to increased $CD25^{+}$ cell proportions and higher Nur77-GFP signal, confirming that developing T_{reg} cells compete for self-antigen during lineage commitment. TCR signal strength has also been related to the competency of developing $T_{reg}P$ cells to respond to low levels of intrathymic IL-2, suggesting another mechanism that

would bias a T_{reg} cell repertoire towards self-reactivity (65). More recent studies have shown that intermediate dwell times for TCR-peptide:MHC complexes facilitate Treg differentiation, while shorter dwell times preferentially drive positive selection and longer dwell times lead to clonal deletion (77). This evidence collectively suggests that T_{reg} cell interaction with thymically presented antigen, at some elevated threshold (Figure 1a), is necessary for initiating T_{reg} cell development.

APCs

Medullary thymic epithelial cells (mTECs)—Thymic selection is defined by a cellular dilemma - without the presence of specialized cell subsets, such as pancreatic beta cells, how is the T cell repertoire pruned of reactivity to tissue specific antigens (TSA) uniquely encoded by these cells? This led to the hypothesis that these specialized self-antigens were in fact expressed at some low level in the thymus, an idea first corroborated by human data correlating thymic insulin expression and susceptibility to the development of diabetes (78, 79). Subsequent work revealed evidence of broad “promiscuous” gene expression in the thymus and attributed mTEC with the sole ability to produce these TSAs (80). These studies also correlated expression of the transcriptional modulator Autoimmune Regulator (AIRE), a gene previously linked to polysymptomatic autoimmunity (81, 82), with the presence of TSA expression in mTECs. This supposition was confirmed in a set of ground-breaking experiments, showing AIRE expression was necessary for tissue specific gene expression in mTECs. Mice that lacked thymic expression of these TSAs had increased numbers of autoreactive T cells in peripheral lymphoid organs, which led to multiorgan immune destruction and generation of autoantibodies (83). Likewise, HEL reactive TCR transgenic T cells underwent clonal deletion when HEL was expressed under the control of the rat insulin promoter, an AIRE responsive locus in mTEC. The proportion of $CD25^{+}$ thymocytes increased; however, since there was no change in absolute number of these cells, the authors dismissed a role for T_{reg} cell development to these antigens (84). These observations led to the hypothesis that the main role of AIRE in central tolerance was due to clonal deletion of tissue specific effector T cells.

While some controversy exists, numerous studies have now defined a role for AIRE-mediated ectopic antigen expression in mTECs in tT_{reg} cell development. Early studies in humans patients with Autoimmune Polyendocrinopathy Candidiasis and Ectodermal Dysplasia, a disorder caused by mutations in *AIRE*, documented a loss of T_{reg} cells and alterations in their TCR repertoire (85). Further, expression of hemagglutinin (HA) via the AIRE promoter in mice led to the development of HA-specific T_{reg} cells, which was dependent on MHC-II expression on mTECs (86). However, a follow-up study in AIRE-OVA mice produced a counterpoint to this hypothesis, as MHC-II knockdown on mTECs caused an increase in OVA-specific T_{reg} cell development (87). This finding suggested that low levels of high affinity antigens drive tT_{reg} differentiation, while higher expression of these same antigens resulted in clonal deletion. In addition, another study observed AIRE-dependent prostate-reactive T_{reg} cell development in the thymus (88). Interestingly, analysis of the TCR repertoire of T_{conv} and T_{reg} cells in wildtype and $Aire^{-/-}$ mice found that cells normally directed towards the T_{reg} cell lineage were instead found in the T_{conv} lineage in $Aire^{-/-}$ mice (89), suggestive of T_{reg} cell agonist selection via AIRE driven antigens. A

similar phenomenon is observed in human patients harboring *AIRE* mutations in which TCRs normally found in T_{reg} cells are found in the T_{conv} compartment (90). In addition to AIRE, the transcription factor FEZF2 also regulates expression of TSA in the thymus. *Fezf2*$^{-/-}$ mice also developed multiorgan autoimmunity, but the spectrum of organs targeted was distinct from *Aire*$^{-/-}$ mice (91). *Fezf2*$^{-/-}$ mice have fewer T_{reg} cells in the thymus and an altered TCR repertoire, reiterating a role for TSA expression in T_{reg} cell development. These results point to a crucial role for mTEC-derived TSA in central tolerance and T_{reg} cell development.

Recently, a distinct stromal cell involved in initiating type II mucosal immune responses, the Tuft cell, has been identified in the thymus. Tuft cells were found to resemble mTEC and produce IL-25, a major inducer of IL-4 production (92, 93). Tuft cells contribute to the Hassall's corpuscle, a structure in the thymus previously associated with T_{reg} cell generation in humans via licensing thymic dendritic cells (DC) to produce CD80 and CD86 via TSLP stimulation (94). Interestingly, we observed that mice lacking the transcription factor POU2F3, which is required for Tuft cell development, have reduced numbers of FOXP3^{lo} T_{reg}P suggesting that Tuft cells can influence T_{reg} cell differentiation (95). Although the mechanism for this remains unclear, it may be due to IL-25 production or the expression of unique TSAs by Tuft cells such as taste receptors (93).

Dendritic cells—The thymic DC compartment consists of conventional DC, including SIRPα^{+} and CD8α^{+} DC, and plasmacytoid DC (pDC) (96). Earlier studies suggested that DC favor clonal deletion over T_{reg} cell development (86, 97). However, experiments using MHC-II$^{-/-}$ bone marrow chimeras clearly implicated a role for bone marrow-derived DC in both clonal deletion and T_{reg} cell induction (98). Other data using *in vitro* models of T_{reg} cell development also observed efficient T_{reg} generation by conventional DC, and to a lesser extent pDC (98–100). While the role of DCs in T_{reg} development has become clearer, the antigens they present, required for inducing tolerance, remain blurry. This is due to the paradox that tolerance to AIRE-driven antigens are frequently dependent on DCs (101). Mechanistic insight to this paradox was revealed in studies documenting antigen transfer from AIRE-expressing mTEC to medullary DC (102, 103). Interestingly, AIRE^{+} mTEC^{hi} cells produce the chemokine XCL1 that recruits thymic CD8α^{+} DCs to the medulla, and *Xcl1*$^{-/-}$ mice exhibit defects in T_{reg} generation (104). CD8α^{+} DC are the dominant cross-presenting thymic DC subtype; thus, in addition to producing intrathymic antigens (105), AIRE also mediates recruitment of APC populations to the thymic medulla required for efficient T_{reg} induction. Subsequent work used TCR sequencing and TCR transgenics derived from TCRs isolated from T_{reg} cells to determine the relative contributions of DCs and mTECs on central tolerance (105). This study observed that for some antigens, mTEC and DC played non-redundant roles in T_{reg} cell differentiation and clonal deletion. However, for other antigens, mTEC and DC played redundant roles in T_{reg} cell selection due to transfer of antigen from mTEC to DC. Indeed, more recent studies using a prostate reactive TCR transgenic observed that DC were required to generate T_{reg} cells in the thymus, despite expression of the antigen being AIRE dependent (106). These experiments highlight the complex interconnections between thymic DC and mTEC necessary for broad induction of antigen-specific thymic T_{reg} cells

The contribution of SIRPα$^+$ DC and pDC in T_{reg} cell polarization is particularly interesting as these represent migratory DC populations, capable of trafficking peripheral antigens to the thymus and inducing T_{reg} cell differentiation (96, 107, 108). pDC also survey the gut via a CCR9 dependent mechanism (109), a chemokine receptor also required for pDC thymic localization and induction of central tolerance to peripheral antigens (110). This could represent a mechanism to transport gut-derived environmental or commensal antigens to the thymus. However, the contribution of endogenous peripheral self- or non-self-antigen trafficking to the thymus in T_{reg} development remains an open question.

B cells—The presence of non-transformed B cells in the thymus was observed more than 30 years ago (111). Early studies observed that B cell-deficient animals failed to delete Mtv-9 specific T cells, but reconstitution of these mice with B cells rescued this deletion (112). Further, *in vitro* studies observed efficient deletion of thymocytes by thymic but not splenic B cells (113). More recent studies have confirmed a role for thymic B cells in deletional tolerance to self-antigens (114, 115). For example, B cells induce clonal deletion of KRN autoreactive TCR transgenic T cells (116). The role of intrathymic B cells in T_{reg} cell development is less clear. The first evidence that thymic B cells affect tT_{reg} cell development came from the observation that BAFF-Tg mice had more tT_{reg} cells than WT mice, due to an increase in thymic B cells. However, tT_{reg} cell development was decreased when thymic B cells were derived from hen egg lysozyme specific transgenic B cells, suggesting that a broad, self-reactive B cell repertoire was required to promote tT_{reg} cell development (117). Using *in vitro* differentiation models, it was also observed that B cells isolated from the thymus were able to polarize CD4$^+$ thymocytes to the T_{reg} cell lineage in a contact, CD80/86, and MHC-II dependent manner (118). These experiments suggested that B cells increase the presence of CD25$^+$ T_{reg}P cells but do not facilitate the subsequent conversion of T_{reg}P cells to mature T_{reg} cells.

T cells reactive to B cell encoded proteins (such as Ig) are deleted by thymic B cells (119–121). There is some evidence that T_{reg} cells may also be generated to BCR antigens (120), although whether this happens in the thymus is unclear. In mouse and humans, AID- and CD40L-deficiency results in autoimmunity that correlates with a decrease in the proportion T_{reg} cells (122). These studies, combined with observations that thymic B cells induce T_{reg} cell development in an MHC-II dependent manner, suggest that thymic B cell-induced T_{reg} cell generation is critical for comprehensive immune homeostasis. Moreover, it was observed that self-antigens drive thymic B cell class-switching, which was required for inducing tolerance to self-antigens and was dependent on AID (123). A thymic B cell licensing process has also been described wherein interactions with T cell-derived CD40L increases antigen presentation on thymic B cells and induces AIRE expression on these B cells (124). This raises the possibility that thymic B cells have a parallel function to mTEC in producing TSA. However, it is still unclear what specificities of tT_{reg} cells are dependent on thymic B cells and whether interactions with thymic B cells preferentially promote T_{reg} cell development via CD25$^+$ or Foxp3^{lo} T_{reg}P cells.

Cytokines in thymic T_{reg} cell development

Prior to the identification of CD25 as a marker for T_{reg} cells there were hints that IL-2 receptor signaling was important for immune tolerance. In 1993, *Il2*$^{-/-}$ mice were generated; these mice had increased numbers of activated T cells and developed colitis-like disease (125). Similar observations were made in *Il2ra*$^{-/-}$ and *Il2rb*$^{-/-}$ mice (126, 127). This was initially puzzling as IL-2 is a known T cell growth factor. Subsequent studies revealed that expression of IL2Rβ specifically in the thymus was sufficient to rescue the autoimmune phenotype observed in *Il2rb*$^{-/-}$ mice, suggesting a role for IL2R signaling during tT_{reg} development (128). These findings were questioned by studies showing development of CD25^{-}FOXP3^{+} T_{reg} cells in *Il2*$^{-/-}$ mice (129–131) and that transfer of T cells from *Il2*$^{-/-}$mice could protect against experimental autoimmune encephalomyelitis (EAE) (132). However, further analysis observed that while *Il2*$^{-/-}$ do develop a small population of CD25^{-} Foxp3^{+} T_{reg} cells, *IL2Rβ*$^{-/-}$ have a larger block in T_{reg} cell development (131, 133). Further experiments observed that IL2Rβ binding cytokines, IL-2 and IL-15, were the major inducers of T_{reg} cell development (131), although IL-7 had limited capacity to induce FOXP3 expression (134, 135). These latter findings reconciled previous reports of T_{reg} cell development in *Il2*$^{-/-}$ mice, suggesting that in the absence of IL-2 other cytokines drive T_{reg} development, although not as efficiently as IL-2. Further, *Stat5*$^{-/-}$ T cells are unable to differentiate into T_{reg} cells, while constitutive activation of STAT5 in STAT5b-CA transgenic mice led to a striking increase in T_{reg} cell differentiation (136, 137). Together, these findings confirm the critical role STAT5 plays in T_{reg} cell development.

Other γC cytokines have also been evaluated for their effect on T_{reg} cell development. IL-4 potently inhibits induced T_{reg} cell generation, and IL-4 blockade increased T_{reg} cell differentiation both *in vitro* and *in vivo* (138). Moreover, IL-4 is unable to induce STAT5 activation in CD25^{+} T_{reg}P cells and *Il4ra*$^{-/-}$ mice show no obvious defect in T_{reg} cell generation in the thymus (134). However, more recent work has observed that IL-4 stimulation of Foxp3^{lo} T_{reg}P maintains FOXP3 expression and upregulates CD25. Further, *Itk*$^{-/-}$ mice, which exhibit elevated IL-4 production, exhibited an IL4Rα-dependent increase in FOXP3^{lo} T_{reg}P and mature T_{reg} cells. Consistent with this observation, BALB/c mice also have increased tT_{reg} cell production that is diminished on the *Cd1d*$^{-/-}$ background (95), which eliminates NKT2 cells responsible for producing excess IL-4 in BALB/c mice (139, 140). Thus, IL-4 may function as a survival factor, or provide a direct differentiation stimulus, for FOXP3^{lo} T_{reg}P. However, the mechanism by which IL-4 promotes tT_{reg} cell development and the significance of this pathway remain unclear.

The cellular sources of cytokines needed for tT_{reg} development remain incompletely understood. T cells and dendritic cells represent the most likely cellular sources of IL-2 for tT_{reg} differentiation. Recent studies have observed that DC-derived IL-2 was particularly important for inducing T_{reg} cell development in *ex vivo* thymic slice models (141). These experiments suggested that DCs create a niche for efficient T_{reg} cell development by providing the antigenic stimulation for T_{reg}P cell generation and the cytokine responsible for driving T_{reg} cell maturation. However, more recent work, using *Il2*$^{fl/fl}$ mice crossed to T cell (*Cd4-Cre*), DC (*Cd11c-Cre*) or B cell (*Cd79a-Cre*) specific CRE-recombinases, observed that T cell-derived IL-2 is necessary and sufficient to drive tT_{reg} cell development (142).

Further, autocrine production of IL-2 was not required for conversion of $T_{reg}P$ into mature T_{reg} cells. It remains unclear what subset of T cells is producing the intrathymic IL-2 necessary for T_{reg} cell development. FOXP3 blocks *Il2* transcription (143), likely precluding $FOXP3^{lo}$ $T_{reg}P$ as producers of IL-2. However, $CD25^+$ $T_{reg}P$ may be competent to produce intrathymic IL-2 as these cells are receiving strong TCR stimulation. Alternatively, IL-2 may also be generated by activated recirculating T cells in the thymus (Figure 1b). Future studies are necessary to pinpoint the specific cellular sources of IL-2 in tT_{reg} cell development.

Generation of IL-7 and IL-15 reporter mice has provided initial insight into the cellular players producing these cytokines in the thymus. Using IL-7-GFP knock-in mice, it was observed that IL-7 is present in both the thymic cortex and medulla. However, on a per-cell basis cortical thymic epithelial cells produced more IL-7 than mTECs (144). The lack of robust IL-7 production in the thymic medulla may explain the negligible effect of IL-7 on T_{reg} development (134). IL-15-CFP reporter-mice produced the opposite result; IL-15 was preferentially found in the thymic medulla (145). Interestingly, IL-15 production was highest in $mTEC^{hi}$ cells, the most robust antigen-presenting subset of mTECs defined by high expression of AIRE. More work is required to understand the cellular sources of IL-15 that may be contributing to tT_{reg} cell development.

Transcriptional regulation

Transcriptional regulation of *Foxp3* and the broader T_{reg} epigenetic signature is essential for proper tT_{reg} cell development. Experiments to reverse engineer the T_{reg} cell transcriptional network surprisingly revealed a highly redundant system (146). It was revealed that FOXP3 alone was insufficient to drive the stable T_{reg} cell transcriptional landscape. However, FOXP3 plus any one of a quintet of other transcription factors - EOS, IRF4, SATB1, LEF1 or GATA1 - was sufficient to solidify the T_{reg} cell transcriptional signature. Deletion of EOS or LEF1 had no effect on T_{reg} development by themselves (147, 148), while the effects of IRF4 or GATA1 deletion on T_{reg} development remain unstudied. However, subsequent studies observed a critical role for SATB1 in tT_{reg} cell development. SATB1 deletion at the $CD4^+CD8^+$ thymocyte stage prevented subsequent establishment of T_{reg} cell super-enhancers and caused inefficient *Foxp3* expression during later T_{reg} cell differentiation (149). Early work suggested that TCR stimulation also facilitates T_{reg} cell epigenetic signatures (150, 151). However, more recent experiments using an *Il2ra* mutant mouse provide evidence that IL-2 signaling is important for initiating the T_{reg} epigenetic signature (152). Specifically, SATB1 positioning throughout the genome was interrupted in developing T cells in *Il2ra* mutant mice. These results suggest that IL-2 signaling is also important for SATB1 to establish the T_{reg} epigenetic signature. Finally, deletion of the transcription factors Nr4a1-3 almost completely blocks tT_{reg} generation (153, 154). Whether Nr4a family members, or other transcription factors, act in concert with SATB1 to establish a permissive state prior to *Foxp3* upregulation remains an open question.

Several studies have shown a crucial role for NFκB activation in T_{reg} cell development. In particular, c-Rel activation is required for T_{reg} cell development (155–158). c-Rel, but not NFκB1, activation downstream of CD28 is required for developing T cells to become $CD25^+$ $T_{reg}P$ (156). However, $Foxp3^{lo}$ $T_{reg}P$ are highly dependent on both c-REL and

NFκB1 expression (95). Moreover, p65 (RELA) deficient thymi also contain decreased amounts of $CD25^+$ $T_{reg}P$ and mature T_{reg} cells (159). RELA and c-REL play partially redundant roles in maintaining T_{reg} cell transcriptional signature and homeostasis, although deletion of RELA resulted in a more severe autoimmune phenotype than deletion of c-REL (159). These findings suggest that NFkB family members may also be important in locking in a stable T_{reg} cell phenotype, although the precise function of each NFκB member during tT_{reg} development in establishing the T_{reg} cell transcriptional signature is still uncertain.

A key step in the development of tT_{reg} cells is stable upregulation of *Foxp3*. Much effort has focused on the factors and regulatory elements that control *Foxp3* expression. Several conserved regulatory regions in the Foxp3 locus have been identified. These include the *Foxp3* promoter, three intronic enhancers (*Cns1-3*) (158) and the *Foxp3* pioneer enhancer element *Cns0* (149). *Cns0* is targeted by the transcriptional regulator SATB1 and acts to poise the *Foxp3* locus for active transcription (149). Later during T_{reg} cell selection, *Cns3* acts as a pioneer regulatory element in the *Foxp3* locus to drive *de novo Foxp3* expression. This pioneer function is dependent on agonist TCR stimulation- and CD28-induced activation of c-Rel and binding of c-Rel to *Cns3* (157, 158). c-Rel targeting to the *Foxp3* locus arranges an enhanceosome including several other transcription factors important for *Foxp3* expression including RELA, NFAT, SMAD and CREB (160). *Cns3*$^{-/-}$ T_{reg} cells are biased towards higher self-reactivity suggesting that c-Rel targeting of *Cns3* is required to sensitize the *Foxp3* locus to TCR stimulation (161). Additionally, *Cns3*$^{-/-}$ thymi are devoid of the less self-reactive $Foxp3^{lo}$ $T_{reg}P$ cell population (95). These experiments suggest that *Cns3* evolved in part to expand the repertoire of T_{reg} cells. Interestingly, deletion of an *Il2ra* enhancer element CaRE4 (162), that has been linked to autoimmune SNPs in humans (163–165), causes a mild block in $CD25^+$ $T_{reg}P$ and mature tT_{reg} development (95). Thus, regulatory regions inside the *Foxp3* locus as well as those outside of *Foxp3* are required for proper T_{reg} cell development. Future studies will need to identify other enhancer elements critical for tT_{reg} cell development and determine the specific role these enhancers play in generating the mature T_{reg} cell repertoire and transcriptome.

Cellular models of thymic T_{reg} cell development

Studies of early T_{reg} cell ontogeny (58) illustrated that CD25 expression precedes FOXP3 expression and the thymic $CD4^+CD25^+$ compartment is comprised of both $FOXP3^+$ and $FOXP3^-$ cells (166). This data provided the first hint that $CD4^+$ $CD25^+$ $FOXP3^-$ thymocytes may represent cellular progenitors for mature $CD25^+$ $FOXP3^+$ T_{reg} cells. Subsequent studies illustrated that $CD25^+FOXP3^-$ thymocytes represent the direct cellular progenitors of mature T_{reg} cells (62, 63). These studies provided a “two-step” model of thymic T_{reg} cell differentiation (Figure 1a). In step one agonist TCR stimulation generates a $CD25^+$ $T_{reg}P$ cell, while in step two IL-2/STAT5-converts $CD25^+$ $T_{reg}P$ into mature T_{reg} cells. Later studies connected these two steps, finding that TCR signal strength correlated with expression of three TNFRSF members, GITR, OX40 and TNFR2, and signaling via these TNFRSF members renders developing $T_{reg}P$ cells much more sensitive to IL-2 (65). Thus, higher TCR self-reactivity imputes a selective advantage for developing $T_{reg}P$ by allowing these cells to compete more effectively for IL-2, thereby biasing the T_{reg} cell repertoire towards self-reactivity.

More recently, an alternative $T_{reg}P$ population was identified, defined by low FOXP3 and lack of detectable CD25 expression ($Foxp3^{lo}$ $T_{reg}P$). Initial reports demonstrated that $Foxp3^{lo}$ $T_{reg}P$ cells efficiently develop into mature T_{reg} cells either *in vitro* to high dose IL-2 (200 U/mL) or *in vivo* in the periphery of mice. However, this paper also suggested that FOXP3 is normally a pro-apoptotic protein and must be counterbalanced by γC cytokine stimulation, such as IL-2, in order for $T_{reg}P$ to survive thymic selection (167). Despite the lack of CD25 expression, $Foxp3^{lo}$ $T_{reg}P$ cells are able to differentiate into mature T_{reg} cells in response to low-dose IL-2 (0.2-1 U/mL) (65, 95) or intrathymic transfer (95, 168). Interestingly, in competitive intrathymic transfer experiments, $CD25^+$ and $Foxp3^{lo}$ $T_{reg}P$ both differentiated into mature T_{reg} cells at similar efficiencies - it remains unclear how $Foxp3^{lo}$ $T_{reg}P$ are capable of such IL-2 sensitivity while lacking CD25 expression. $CD25^+$ $T_{reg}P$ experience greater TCR stimulation, as measured by NUR77-GFP signal intensity, than $Foxp3^{lo}$ $T_{reg}P$ during thymic selection (95, 168). The TCR repertoire of these two $T_{reg}P$ populations overlap significantly with mature T_{reg} cells but much less so with each other (95). These observations suggested that these were unique $T_{reg}P$ populations selected by distinct interactions with self-antigens and contributed unique TCRs to the mature T_{reg} cell repertoire. Remarkably, T_{reg} cells derived from $CD25^+$ $T_{reg}P$, but not $Foxp3^{lo}$ $T_{reg}P$, could protect mice from EAE while T_{reg} cells derived from $Foxp3^{lo}$ $T_{reg}P$ were able to consistently suppress colitis. Collectively, these data provide an updated model of tT_{reg} cell development in which both $CD25^+$ and $Foxp3^{lo}$ $T_{reg}P$ contribute quantitatively equivalently, but qualitatively distinctly, to the mature T_{reg} cell repertoire.

Future considerations

Despite decades of research directed at understanding the development of tT_{reg} cells, many questions remain unanswered. While two cellular progenitors have been described that contribute to the mature T_{reg} cell repertoire, the precursors to each of these populations have not been effectively described. Preliminary reports have identified a $CD122^+$ $GITR^+CD25^-$ $Foxp3^-$ $T_{reg}P$ precursor that can give rise to $CD25^+$ $T_{reg}P$ via a c-REL dependent mechanism (169). However, whether this population also represents the precursors to $Foxp3^{lo}$ $T_{reg}P$ remains unclear. Defining the signals and relevant antigens that commit T_{reg} cell development via either $T_{reg}P$ pathway will be important for understanding the role each pathway plays in immune tolerance.

Cytokine signaling is clearly required for T_{reg} cell generation. However, more nuanced effects of cytokines on T_{reg} selection remain poorly defined. CD25 can be expressed on thymic DC and mTEC (142); however, it is unclear if CD25 *trans* presentation (170) occurs in the thymus and if so what affect this has on T_{reg} cell selection. The role of IL-4 is also unclear; mice of different background produce distinct amounts of IL-4 (95, 139, 140), which could influence tT_{reg} cell TCR repertoire and possibly susceptibility to different types of autoimmunity. Further, certain subsets of thymic APC produce different cytokines, such as IL-2 from DC (141) and IL-15 from mTEC (145). Future studies directed at understanding how distinct cytokines affect T_{reg} development will likely produce interesting insight into how cytokine stimulation affects T_{reg} cell repertoires.

Another mystery in T_{reg} cell development is how T_{reg} cells develop that enforce tolerance to transitory states, such as inflammation, puberty, estrous, or distinct metabolic states. Certainly for B cell immune responses there is evidence of thymus induced T_{reg} tolerance to Ig antigens (119–122), and loss of Tuft cells leads to the development of anti-IL-25 antibodies (93). Further, development of inflammation specific T_{reg} cells has been observed in the thymus (171). Interestingly, testosterone levels regulate AIRE-mediated TSA production (172), which may explain resistance to various forms of autoimmunity in males. Prepubertal males and females have similar levels of testosterone (173); thus, any differences imposed by this hormone likely occur after puberty has initiated in humans. The broad specificity of tT_{reg} cells needed to provide tolerance in transitory states is still poorly understood.

The tT_{reg} pool is composed of recently differentiated cells but also T_{reg} cells that have been retained following development (resident) or have recirculated to the thymus from the periphery (174–176). Studies with *Rag2-GFP* mice demonstrate that older GFP-negative T_{reg} cells progressively accumulate in the thymus as mice age and represent the majority of thymic T_{reg} cells by about 8 weeks of age (176, 177). However, the origin of these T_{reg} cells is debated with some suggesting that they are mostly resident cells that never left the thymus (174) and others proposing that they are primarily recirculating cells (176). It has been difficult to distinguish between these two populations to determine their relative contributions to the tT_{reg} cell pool. Thymus transplantation studies demonstrate that T_{reg} cells migrate from the periphery to the thymus preferentially by comparison to conventional T cells (178). Additionally, mature RAG2-GFP$^-$ T_{reg} cells in the thymus have a similar gene expression profile to splenic T_{reg} cells and their TCR repertoire shows evidence of peripheral modification supporting the possibility that these cells are recirculating (176). Resident and recirculating T_{reg} cells have been shown to compete with developing thymic T_{reg} cells for access to IL-2 and limit their differentiation to the T_{reg} cell lineage (141, 176). The immunological benefit of restricting new T_{reg} cell development is unclear. It is possible that these older T_{reg} cells also compete with thymocytes for antigen, co-stimulatory ligands, and TNFRSF ligands necessary for T_{reg} cell development. The presence of a large population of recirculating or resident T_{reg} cells represents both an opportunity to understand the biological importance of these recirculating T_{reg} cells and a problem, as these RAG2-GFP$^-$ T_{reg} cells contaminate analysis of *de novo* T_{reg} cell development. Cellular phenotypes for “old” contaminating T_{reg} cells have been proposed, including CCR6$^+$CCR7$^-$ (178) as well as CD73$^+$ (95); these markers should be used to exclude “old” T_{reg} cells in studies *de novo* tT_{reg} development.

Finally, despite years of debate, controversy still exists over the relative role of tT_{reg} cells and pT_{reg} cells. The hypothetical requirement for pT_{reg} is at mucosal surfaces (179) where diverse antigens are being surveyed or during pregnancy where ectopic alloantigens are contributed by the male gamete (180). Several studies suggest a role for thymic deletion and T_{reg} cell selection in mucosal tolerance (66, 181–183) while other studies argue for the importance of pT_{reg} generation (40, 179, 184–187). More recent studies have suggested that some populations of thymic T_{reg} cells are required to polarize T_{conv} to pT_{reg} cells, perhaps relating these disparate findings (184, 188). Likewise, T_{reg} cells derived from thymic FOXP3^{lo} T_{reg}P were able to suppress colitis, suggesting tolerance to commensal organisms

can be induced by specific tT_{reg} cell subsets (95). Further experimentation is required to conclusively delineate the unique and overlapping responsibilities of pT_{reg} and tT_{reg} in immune tolerance.

Conclusions

The evolutionary constraints placed on T cell selection in the thymus are immense - exogenous pressure from pathogens places a high priority on TCR diversity, while endogenous pressure requires removal of self-reactive and potentially pathogenic T cells. Thus, T_{reg} cell development represents a mechanism that allows this leaky selection system to persist and focus effector T cell responses on “non-self” antigens. Future studies defining endogenous T_{reg} cell antigenic targets, and the thymic populations required to produce these antigens, will be required to understand the complex processes that govern the selection of a competent repertoire of tT_{reg} cells. Further, understanding the role of antigen specificity of T_{reg} cells in homeostatic, inflammatory, or autoimmune contexts will be crucial in linking thymic selection to peripheral homeostasis.

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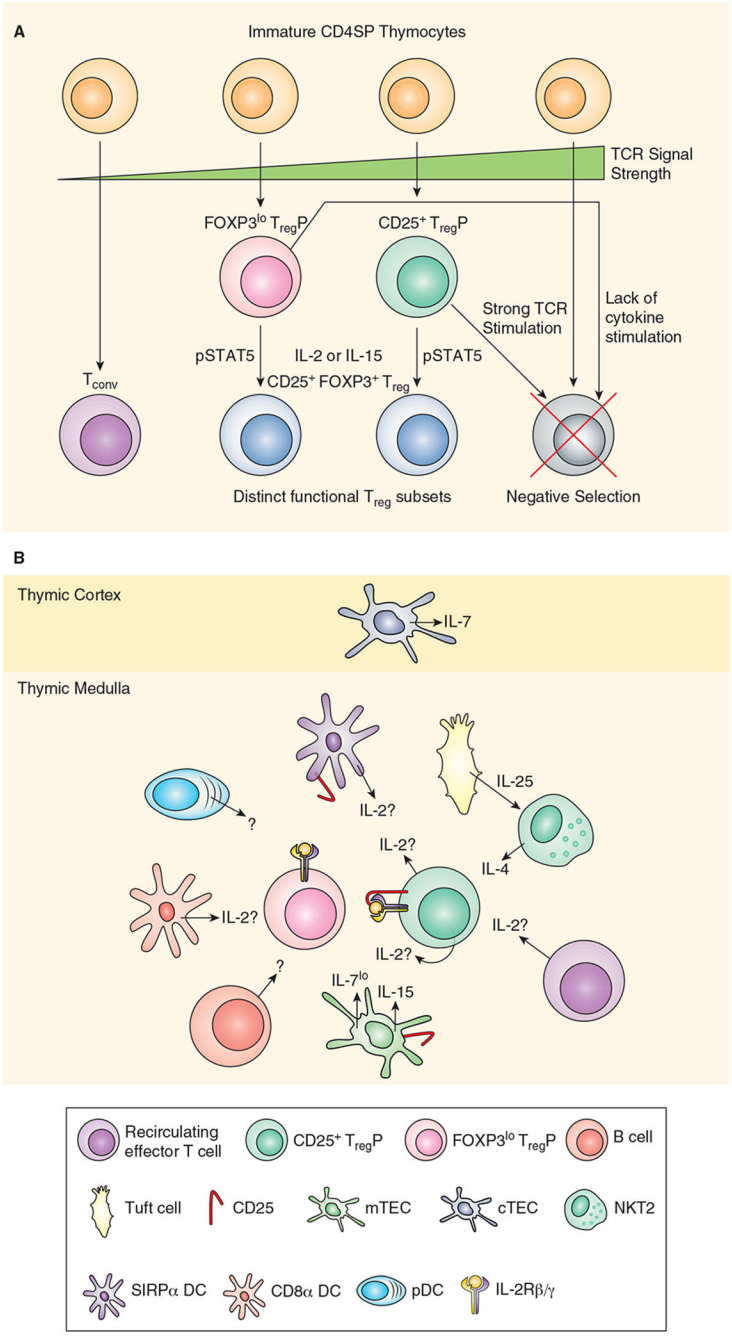

Figure 1.

A, Model of thymic T_{reg} cell development. CD4 single positive thymocytes interact with a range of different affinity for self-antigens presented by thymic APC subsets including, SIRPα$^+$ and CD8α$^+$ DC, pDC, mTEC, B cells and perhaps macrophages. TCR signal strength initiates fate decisions. Weak TCR signaling is required to develop T_{conv}, while strong TCR stimulation drives clonal deletion. Intermediate TCR signaling drives T_{reg} cell commitment; stronger TCR signals lead to upregulation of CD25 generating a CD25$^+$ T_{reg}P while weaker TCR stimulation causes upregulation of FOXP3^{lo}

$T_{reg}P$. Some $CD25^+$ $T_{reg}P$ still undergo clonal deletion, likely due to the high TCR signal strength experienced by this population and FOXP3 expression in $FOXP3^{lo}$ $T_{reg}P$ drives clonal deletion unless counterbalanced by survival signals mediated by engagement of γC cytokines. When either $T_{reg}P$ bind IL-2, or IL-15, this activates STAT5 and completes the differentiation of mature tT_{reg} cells, defined by dual expression of CD25 and FOXP3. *B,* Cytokine producing cells in T_{reg} cell development. Various cells in the thymus contribute cytokines to the thymic microenvironment. cTEC produce IL-7 which may function as a survival factor for developing thymocytes in the cortex. mTEC have been shown to produce IL-15 as well as low levels of IL-7. mTEC also express CD25 which may function to transpresent IL-2 to developing $T_{reg}P$ or deplete local IL-2 from $T_{reg}P$. DC derived IL-2 may be produced by $CD8\alpha^+$ DC or $SIRP\alpha^+$ DC however, $SIRP\alpha^+$ DC also express CD25 which may modulate local IL-2 availability. It is unknown if pDC produce T_{reg} inducing cytokines. Similarly, it is unknown if thymic B cells contribute any cytokines capable of driving T_{reg} differentiation or serve only as a antigen presenting cell. Tuft cells produce IL-25 which acts on NKT2 cells to produce intrathymic IL-4. IL-4 plays a role in promoting survival and/or differentiation of $Foxp3^{lo}$ $T_{reg}P$. Finally, T cells represent the critical source of IL-2 required to drive T_{reg} cell differentiation, however, it is unclear if the IL-2 is being produced by $CD25^+$ $T_{reg}P$ or a subset of recirculating effector T cells.