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Thymic development and peripheral homeostasis of regulatory T cells

Adrian Liston[1] and Alexander Y Rudensky[1,2]

The development and maintenance of regulatory T (T-reg) cells is crucial for determining the level of reactivity in the immune system. Until recently, however, surprisingly little was known about the factors involved in the development of these cells in the thymus or the mechanisms that maintain them in the periphery. Studies have now demonstrated that thymic development of T-reg cells is facilitated by TCRs with increased affinity for self-peptide–MHC complexes. Increased TCR affinity alone, however, is not sufficient to support the development of T-reg cells, and external factors such as CD80 and CD86, ligands for co-stimulatory receptor CD28, and interleukin 2 are required. These factors are also needed to maintain the T-reg cell subset in the periphery.

Addresses
[1] Department of Immunology, University of Washington School of Medicine, Seattle, WA 98195, USA
[2] Howard Hughes Medical Institute, University of Washington School of Medicine, Seattle, WA 98195, USA

Corresponding author: Liston, Adrian (liston@u.washington.edu) and Rudensky, Alexander Y (aruden@u.washington.edu)

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Introduction

In the past few years, the field of T-cell-mediated suppression has been rejuvenated by major advances, including demonstration of the suppressive function of $CD25^+CD4^+$ regulatory (T-reg) cells in several animal and human pathologies, and initial dissection of the mechanism of T-reg function. The wealth of publications on T-reg cells originates from classical experiments using day-3 thymectomy experiments to demonstrate the existence of a T-cell subset with regulatory properties [1,2], and has increased exponentially since identification of the fork-head transcription factor Foxp3 as a master regulator of the development of these cells [3–5].

Because most research has focused on T-reg function, relatively little progress has been made in addressing the developmental issues raised by key studies. The ability of $CD4^+$ single-positive (SP) thymocytes depleted of $CD25^+$ cells to induce autoimmunity [6] has led to a widely accepted view that T-reg cells diverge from the $CD4^+$ T-cell lineage relatively late during thymic development. This observation has raised numerous questions. What factors are required to direct T-reg fate? Why is there $CD25^+CD4^+$ T-reg deficiency in mice before day 3 of life, allowing autoimmunity in mice subjected to neonatal thymectomy [1,2]? What mechanisms define the size and stability of the peripheral repertoire of T-reg cells? Here we review recent studies addressing these questions.

Factors involved in the thymic development of T-reg cells

Self-reactivity of T-reg cells

T-reg cells keep autoimmunity in check by suppressing self-reactive T cells and by limiting the reactivity of T cells specific to environmental antigens that could arguably be considered 'self'. The question of whether T-reg cells are self-reactive themselves, however, has been raised by observations of increased proportions of superantigen-responsive $CD25^+CD4^+$ T-reg cells in mice expressing the corresponding viral superantigen [7].

A model of T-reg cell development based on increased TCR affinity for self-peptide–MHC complexes originally came from studies of TCR transgenic mice by the Caton [8] and von Boehmer [9] groups. In these studies, the proportion of $CD25^+CD4^+$ cells with suppressive properties sharply increased in TS1 TCR transgenic $CD4^+$ SP thymocytes specific for influenza virus hemagglutinin (HA) that were developing in the presence of another transgene encoding HA as neo-self-antigen. By contrast, thymocytes expressing a lower affinity SW TCR did not differentiate into T-reg cells in the presence of differing levels of transgene-encoded HA [8,9]. Other TCR transgenic models, such as the D011.10 TCR, have yielded similar results [10,11].

Analyses of the absolute numbers of T-reg cells developing in hen egg lysozyme (HEL)-reactive 3A9 TCR transgenic mice, however, have found no significant increase in T-reg differentiation when this TCR is coexpressed with six distinct HEL-encoding transgenes expressed under different promoters ([12]; AL and C Goodnow, unpublished). Furthermore, deletion of self-reactive thymocytes is the primary observation when TS1 TCR transgenic mice are crossed to mice expressing HA encoded by another transgene (HA12) [13]. Similarly, in mice coexpressing a transgenic TCR reactive towards pigeon cytochrome *c* together with a transgene encoding

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pigeon cytochrome *c*, the increase in absolute numbers of $CD25^+CD4^+$ T cells was found to be modest [14••]. Interestingly, when the same transgenic TCR is confronted with varying amounts of neo-self-antigen provided by tetracycline-dependent transgene expression, there is a substantial increase in the proportion of these cells without an increase in absolute numbers [15]. This latter result has led to the idea that negative selection favors the relative enrichment of self-reactive TCR in the T-reg population by means of the elimination of higher affinity thymocytes that fail to upregulate Foxp3; this idea contrasts with earlier studies favoring a model in which commitment to the T-reg lineage is instructed by increased affinity of the TCR signal.

The importance of self-reactivity in T-reg generation is harder to ascertain in mice with a polyclonal TCR repertoire, owing to difficulties in both tracing the fate of a single self-reactive TCR during T-cell development and evaluating any skewing towards the T-reg subset. To circumvent this issue, the diversity of TCRs displayed by T-reg cells versus non-T-reg cells has been assessed by sequencing Vα2-containing variable regions of the TCR α-chain (TCRα) from T cells purified from mice expressing a TCRβ transgene and a single copy of the TCRα locus (*Tcra*$^{+/-}$) to exclude the appearance of T cells with dual TCRα chains. In these studies, the TCR repertoire diversity displayed by peripheral $CD25^+$ and $CD25^-$ $CD4^+$ T cell subsets was found to be comparable, but the repertoires were distinct and only partial overlap was observed [16]. Furthermore, extension of this approach to analysis of thymocyte subsets showed that there is much similarity between thymic and peripheral TCR repertoires of $CD25^+CD4^+$ T-reg cells, as well as between thymic and peripheral TCR repertoires of 'non-regulatory' $CD25^-CD4^+$ cells, indicating that a subset of thymocytes with distinct TCR sequences is recruited into the T-reg lineage during thymic development [17••]. Similar results have been obtained in an analogous experimental system that examined TCRα usage in TCRβ transgenic mice expressing a transgene encoding a 'mini-TCRα' transgenic locus [18•].

Two studies suggest that the feature that distinguishes the TCR pool of T-reg cells from that of naïve $CD25^+CD4^+$ T cells is an increase in self-reactivity. First, retroviral expression of cloned TCR from $CD25^+$ T-reg cells, but not naïve $CD25^-CD4^+$ T cells, in a T cell recognizing a defined foreign antigen was found to confer the ability to expand rapidly in lymphopenic mice [16]; importantly, this expansion required a diverse repertoire of self-peptide–MHC class II complexes. Second, in *Foxp3*$^-$ mice suffering from massive lymphoproliferative autoimmune syndrome, activated $CD25^+CD4^+$ T cells, but not naïve $CD25^-CD4^+$ T cells, were found to display a TCR repertoire very similar to that of $CD25^+CD4^+$ T-reg cells in normal wild-type mice [17••].

TCR signaling requirements

To facilitate T-reg development, thymocytes require a means to transmit the information of increased-affinity TCR engagement to the nucleus to initiate the transcriptional program that commits these cells to the T-reg lineage. Recent findings suggest that the TCR signals that lead to T-reg development have a distinct requirement for particular aspects of the TCR signaling pathway as compared with positive selection of naïve T cells. This notion is best illustrated by the recent observation that mice carrying a targeted mutation of a particular tyrosine phosphorylation site in the Y136F variant of LAT (linker of activated T cells) completely lack $Foxp3^+$ T-reg cells in both the thymus and the periphery but show only a partial defect in positive selection [19••]. This mutation specifically abolishes the ability of LAT to recruit phospholipase Cγ1 on TCR-signaling-induced phosphorylation of Tyr136.

In relation to the efficiency of positive selection of $CD4^+$ T cells, the defect observed in the LAT^{Y136F} variant is comparable to that observed in mice carrying partial loss-of-function alleles of *Slp76* (SH2-domain-containing leukocyte phosphoprotein of 76 kDa) and *Zap70* (zeta-associated protein of 70 kDa) identified through screening of mice isolated in N-ethyl-N-nitroseurea (ENU)-induced genome-wide mutagenesis (O Siggs, L Miosge, AL and C Goodnow, unpublished). Unlike the LAT^{Y136F} mutant, however, these mutant alleles show a normal proportion of thymocytes expressing Foxp3 (O Siggs, L Miosge, AL and C Goodnow, unpublished). It is therefore likely that T-reg development has a heightened dependence on a defined pathway of LAT signaling in which phospholipase Cγ1 is the key factor [19••].

Another signaling pathway that is engaged by TCR signaling and has been found to have particular importance for T-reg development is NF-κB activation [20,21]. However, these initial studies left open a question as to what extent the observed deficiency in T-reg cells associated with NF-κB pathway impairment is caused by a cell-intrinsic effect in differentiating T-reg precursor cells versus a deficiency in interleukin 2 (IL-2) production by non-regulatory T cells. The latter possibility has received some support in recent studies [21–23].

Cytokines and cofactors

Although several studies suggest that increased TCR self-reactivity and activation of specific signaling pathways might have a role in the development of T-reg cells, the data also raise the issue of why only some, and not all, thymocytes expressing a given TCR commit to the T-reg lineage. In TCR transgenic systems, this incomplete conversion might involve saturation of a limiting niche, such as availability of the TCR ligand or other factors.

A limiting niche hypothesis does not sufficiently explain the observation of identical TCR sequences in T-reg and

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non-T-reg subsets in three studies that have analysed polyclonal TCR repertoires [16,17••,18•]. These results suggest that thymic development of T-reg cells seems to require two signals: a TCR-affinity-based signal, and a second signal present in the thymus at limiting concentrations. Alternatively, thymic development of T-reg cells might involve pre-existing heterogeneity within thymocytes expressing self-reactive TCR in relation to their potential to upregulate Foxp3 and to commit to the T-reg lineage. Below, we discuss these two non-mutually exclusive possibilities.

A role for IL-2

A key factor known to influence the development of T-reg cells is IL-2. While IL-2 was discovered as a key stimulatory cytokine of T cells, mice deficient in *Il2* or its receptor *Ilr2a* (*CD25*) or *Il2rb* (*CD122*) show signs of severe immunopathology. The lymphoproliferative disease in *Il2*- or *Il2ra*-deficient mice is caused by a defect in *trans*-acting or dominant tolerance, because mice reconstituted with a mixture of wild-type bone marrow and *Il2*- or *Il2ra*-deficient bone marrow do not develop autoimmune lesions [24–26]. It has been proposed that this lack of lesions is caused by a function of IL-2 in the thymic development or peripheral maintenance of $CD25^+$ T cells [27–29]. However, early studies that relied on CD25 as a T-reg marker had difficulty in determining whether IL-2 has a specific role in T-reg biology because CD25 expression is regulated by IL-2 [30].

Since the identification of Foxp3 as a valid marker of T-reg cells, several studies have been able to re-examine whether IL-2 has a role in T-reg development. By using mice that carry a *Foxp3^{gfp}* reporter knock-in allele, it has been demonstrated that $Foxp3^+$ T-reg cells still develop in the thymus — albeit in significantly reduced numbers — in *Il2*$^{-/-}$ and *Ilr2a*$^{-/-}$ mice [31•]. Generation of $Foxp3^+$ thymocytes in the absence of IL-2 signaling has been also confirmed in the above-mentioned HA and HEL 'double' transgenic models of T-reg development.

In one study, HA-reactive TS1 TCR transgenic mice expressing transgene-encoded HA showed only a minor reduction of $Foxp3^+$ thymocytes in the absence of IL-2 or CD25 expression [32•]. In the analogous experimental system using the HEL-reactive 3A9 TCR paired with insulin-promoter-driven HEL, by contrast, the effect of IL-2-deficiency was found to depend on the expression level of HEL-reactive TCR. Specifically, there was a major loss of thymocytes highly expressing 3A9 TCR but only a minor loss of thymocytes expressing low levels of 3A9 TCR (AL, O Siggs and C Goodnow, unpublished). Taken together, these results suggest that IL-2 has a significant but partially redundant role in the differentiation of T-reg cells and has a more profound effect on T-reg precursors expressing higher avidity TCR.

Putative roles for other cytokines

Although the role of IL-2 has been best studied, there is also clear evidence of the involvement of other cytokines in T-reg development. Mice deficient in the common γ-chain IL-2 receptor (IL-2Rγ) have a complete lack of $Foxp3^+$ cells in both the thymus and periphery [31•], and STAT5 has been shown to be essential for the production of $CD25^+$ cells [33,34•]. Similarly, TAK1-deficient mice, which show severe defects in signaling in response to TCR stimulation and common γ-chain cytokines, have a complete absence of $Foxp3^+$ SP thymocytes [35••]. It is not known whether this effect is mediated by a single γ-chain cytokine (other than IL-2 or IL-15) or if it is the cumulative outcome of multiple γ-chain cytokines that support T-reg development.

Two candidate cytokines that are unlikely to have a non-redundant role in T-reg development in the thymus are transforming growth factor-β (TGFβ) and IL-7. In both *Tgfb1*$^{-/-}$ mice and mice with T-cell-specific ablation of a conditional *Tgfbr2* allele induced by CD4–Cre, a normal $CD25^+Foxp3^+$ thymocyte subset has been observed [36–38]. Similarly, in mice lacking IL-7, the proportion of $Foxp3^+$ thymocytes in the $CD4^+$ SP thymocyte subset is not changed [39]. Nevertheless, it is possible that IL-7 might contribute to T-reg development in the absence of IL-2.

Recently, thymic stromal lymphopoietin (TSLP) has been implicated in T-reg development in humans on the basis of its high expression in Hassall's corpuscles — groups of cells that are found in the medullary region of human thymus where most $Foxp3^+$ thymocytes are localized. *In vitro* exposure of thymic dendritic cells to TSLP confers the ability to generate $CD25^+CD4^+$ $Foxp3^+$ T cells on co-culture with $CD25^-CD4^+$ thymocytes [40•]. It seems unlikely, however, that this role of TSLP is evolutionarily conserved, because murine TSLP has a similar effect *in vitro* but TSLP-deficient mice have normal numbers of $Foxp3^+$ thymocytes [41•]. Furthermore, in SCID mice reconstituted with human hematopoietic stem cells human $CD25^+CD4^+$ thymocytes develop despite the inability of murine TSLP to bind to the human TSLP receptor [42]. Thus, additional studies are needed to examine further the role of TSLP in human T-reg development.

CD28 as a necessary cofactor

An essential role for CD28–B7 interactions in T-reg development is suggested by the observations of decreased numbers of peripheral $CD25^+$ T-reg cells in *Cd28*- or *B7.1/B7.2*-deficient non-obese diabetic (NOD) mice, resulting in accelerated development of autoimmune diabetes [43,44]. This effect is partly caused by the role of CD28 signals in maintaining peripheral T-reg cells (see below). More recent studies have shown, however, that CD28 has a role in the thymic development

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of CD25$^+$Foxp3$^+$ cells, because the Foxp3$^+$ thymocyte subset is sharply reduced both in *Cd28*$^{-/-}$ mice [44,45] and in mice treated with antibodies to B7 [44].

A detailed investigation of the role of CD28 signaling in T-reg thymic development by Singer and co-workers [14••] has revealed two distinct effects. First, a lack of CD28 leads to reduced production of IL-2 by Foxp3$^-$ thymocytes and peripheral T cells, resulting in levels that are insufficient to induce upregulation of CD25 and to support T-reg development [14••,44]. Second, in addition to this T-reg deficiency caused by a *trans*-acting defect in IL-2 production, there is a cell-intrinsic requirement for CD28 costimulation in developing T-reg precursors because *Cd28*$^{-/-}$ bone-marrow-derived precursor cells fail to develop normal numbers of CD25$^+$Foxp3$^+$ T cells even when the IL-2 supply is restored ([14••]; and E Bertram and D Sheahan, personal communication). This intrinsic dependence on CD28 costimulation requires the Lck-binding motif, but not the PI3K- or the Itk-kinase-binding motifs [14••], but the mechanisms by which this particular aspect of CD28 signaling influences Foxp3 expression remain unclear.

Developmental issues

A fascinating issue in T-reg cell biology is the nature of the accessory cell that is capable of supporting T-reg development in the thymus. Early work by Laufer and co-workers [46] demonstrated that CD25$^+$ T-reg cells are generated at 60% of normal numbers in K14-Aβ^b mice that express MHC class II molecules exclusively in thymic cortical epithelial cells. As mentioned above, however, Foxp3$^+$ thymocytes in normal thymus are largely limited to the thymic medulla and only few CD4$^+$CD8$^+$ double-positive T cells expressing Foxp3 are present in the cortex [47]. It is unlikely that these rare cortical cells give rise to the bulk of Foxp3$^+$ SP thymocytes present in the medulla, because Foxp3$^+$ CD4$^+$CD8$^+$ double-positive and CD4$^+$ SP cells appear concurrently during development [47]. Furthermore, thymic CD25$^+$ T cells do not seem to have undergone disproportionate proliferation, as measured by labeling and staining for 5-bromodeoxyuridine [7]. The considerable diversity of the TCR repertoire displayed by these cells provides an additional indirect argument against an expansion of cortical T-reg cells in the medulla [17••,18•].

Another issue related to the nature of accessory cells for T-reg development is the time and place of T-reg generation in the thymus during early postnatal mouse development. As mentioned above, the results of early studies of day-3 thymectomized mice suggested that CD25$^+$CD4$^+$ thymocytes are largely lacking in neonatal thymus [1,2]. Recently, this issue has been revisited through the analysis of Foxp3 expression in newborn *Foxp3*^{gfp} mice. Although a substantial number of CD25$^+$CD4$^+$ SP thymocytes were present immediately after birth, only few of these cells expressed Foxp3 [47]. A steady increase in the numbers of Foxp3$^+$ thymocytes was observed in the first 2–3 weeks, and the largest single-day gain in numbers occurred between days 3 and 4 [47]. Because self-reactive TCR is generated randomly and is unlikely to be lacking early in life, the most plausible explanation for this finding is a change in availability of a necessary cofactor, such as those discussed in the previous section.

One model that potentially accounts for both observations is a two-step development of T-reg cells (Figure 1). In this model, the first step of the differentiation process requires the recognition of self-peptide with an affinity above a cut-off threshold — an event that could occur regardless of the selecting cell type and that would prime the cell for receiving additional signals required for T-reg development at the second step. In turn, the second step might involve exposure of the primed cell to IL-2 and B7 or to an unknown cofactor, after which Foxp3 expression is switched on and regulatory capacity is gained. The distribution of both IL-2 and B7 is heterogeneous in the thymus [48–51]. Of relevance, high expression of B7 is limited to the medullary region [49–51]. Thus, the apparent contradiction between the expression of MHC class II molecules in K14-Aβ^b mice in the cortex and the localization of Foxp3$^+$ thymocytes to the medulla can be explained by a division of labor in which step 1 and step 2 are separated anatomically in the cortex and medulla, respectively. In the context of this model of T-reg development, we propose that the necessary cofactor that is limiting in neonatal thymus might be dependent on cross-talk between the thymic epithelium and maturing thymocytes. Evidence for this hypothesis comes from the defective production of CD25$^+$ T-reg cells in mice with abnormal medullary development such as the *NIK*$^{aly/aly}$ [52] and *Traf6*$^{-/-}$ strains [53], and from the well-established role of thymocytes in medulla formation.

Alternative cell fates

Commitment to the Foxp3$^+$ T-reg cell lineage is just one of several alternative cell fates during thymic development, including negative selection, anergy or differentiation into 'conventional' or naïve T cells. Initial models incorporating these alternative fates have assumed that a simple TCR affinity cut-off enables thymocytes with a low-affinity TCR for self-peptide ligands to mature as naïve T cells, thymocytes with an intermediate-affinity TCR to differentiate into T-reg cells, and thymocytes with a high-affinity TCR to become anergic or to be culled through negative selection (Figure 2a). One prediction of such a model would be the identical fate of thymocytes expressing identical TCR. As described above, however, shared TCR usage by T-reg and 'non-T-reg' cells has been observed in both mice with a polyclonal TCR repertoire and mice expressing a single monoclonal TCR. This acquisition of multiple cell fates by thymocytes with identical TCR usage could be caused

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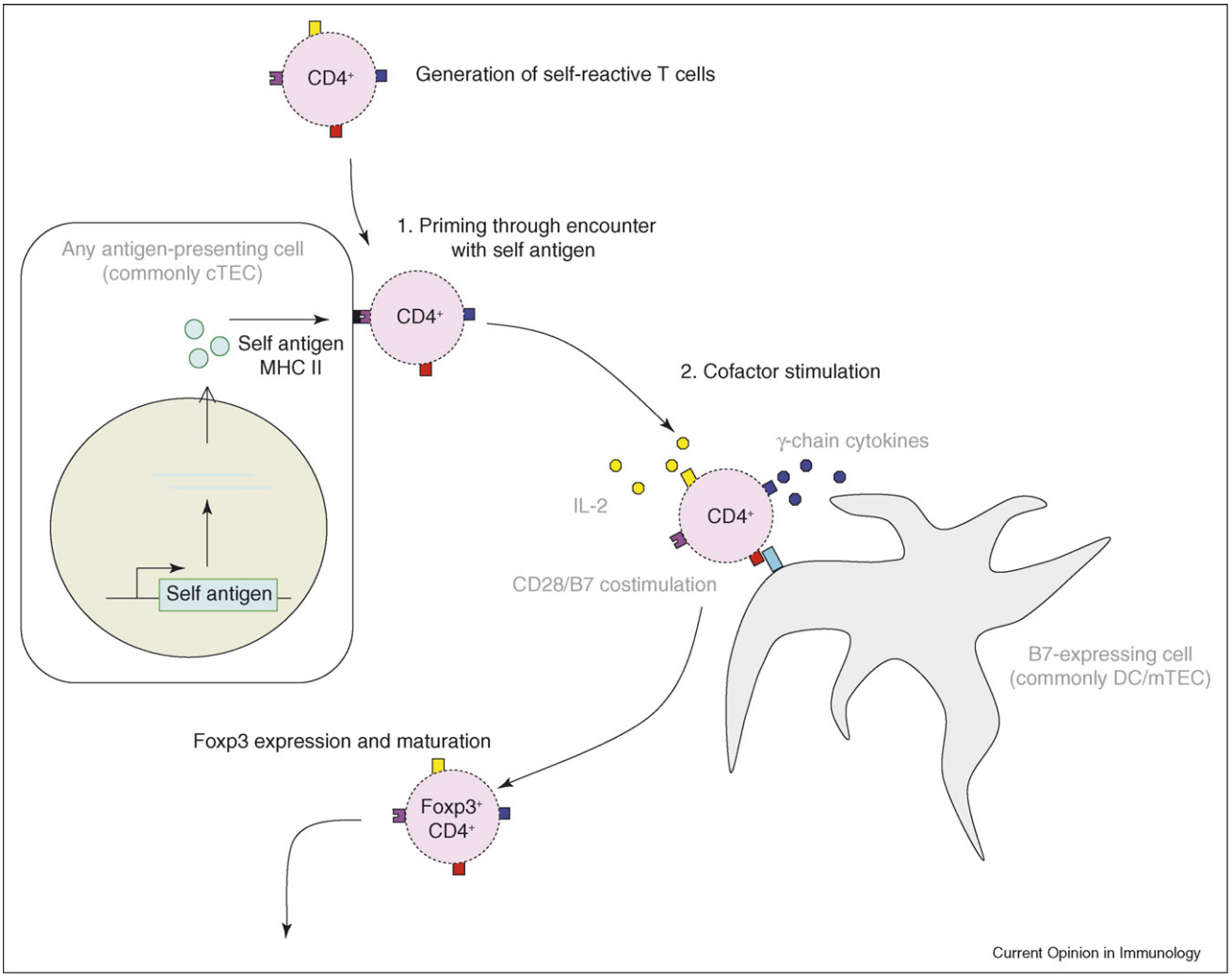
Figure 1



Two-step model of T-reg development. T-reg cells require two events to develop from self-reactive thymocytes: first, encounter with self-antigen; and second, exposure to necessary cofactors including IL-2, common γ-chain cytokines and B7 ligands. In the model shown here, these events are temporally discrete such that a typical conversion might consist of self-antigen stimulation during positive selection in the cortex, followed by cytokine exposure and costimulation from thymic dendritic cells (DC) in the medulla. Abbreviations: cTEC, cortical thymic epithelial cell; mTEC, medullary thymic epithelial cell.

by limited availability of selecting self-peptides or to heterogeneity of precursor thymocytes before encounter with self-ligands. The latter possibility is largely unexplored. Lastly, an 'affinity-only' model might be an oversimplification.

Another model also considers affinity for 'self' to be the primary determinant of T-reg lineage development, but is distinguished from the above models by flexible thresholds, whereby a fate outcome can be influenced by cofactors present in the microenvironment or a stochastic event affecting signal transduction or gene expression. According to this 'flexible-fate' model, in an extreme case the same TCR can be expressed by an activated T cell, a T-reg cell, an anergic T cell or even a T cell with a naïve phenotype, depending on the confluence of extracellular signals and conditions (Figure 2b). A problem inherent to this flexible-fate model is the potential reversal of cell fate decision once the microenvironment (e.g. cytokine milieu) or state of intracellular signaling apparatus changes. On the basis of theoretical considerations, such a problem can be overcome by incorporating mechanisms that reinforce cell fate after the initial decision has occurred.

The irrevocable nature of negative selection is an extreme form of fate reinforcement, but T-reg lineage commitment also seems to have a reinforcement mechanism. The expression of Foxp3 provides a feedback loop, because genetically marked thymocytes transcribing the *Foxp3* gene but lacking expression of the Foxp3

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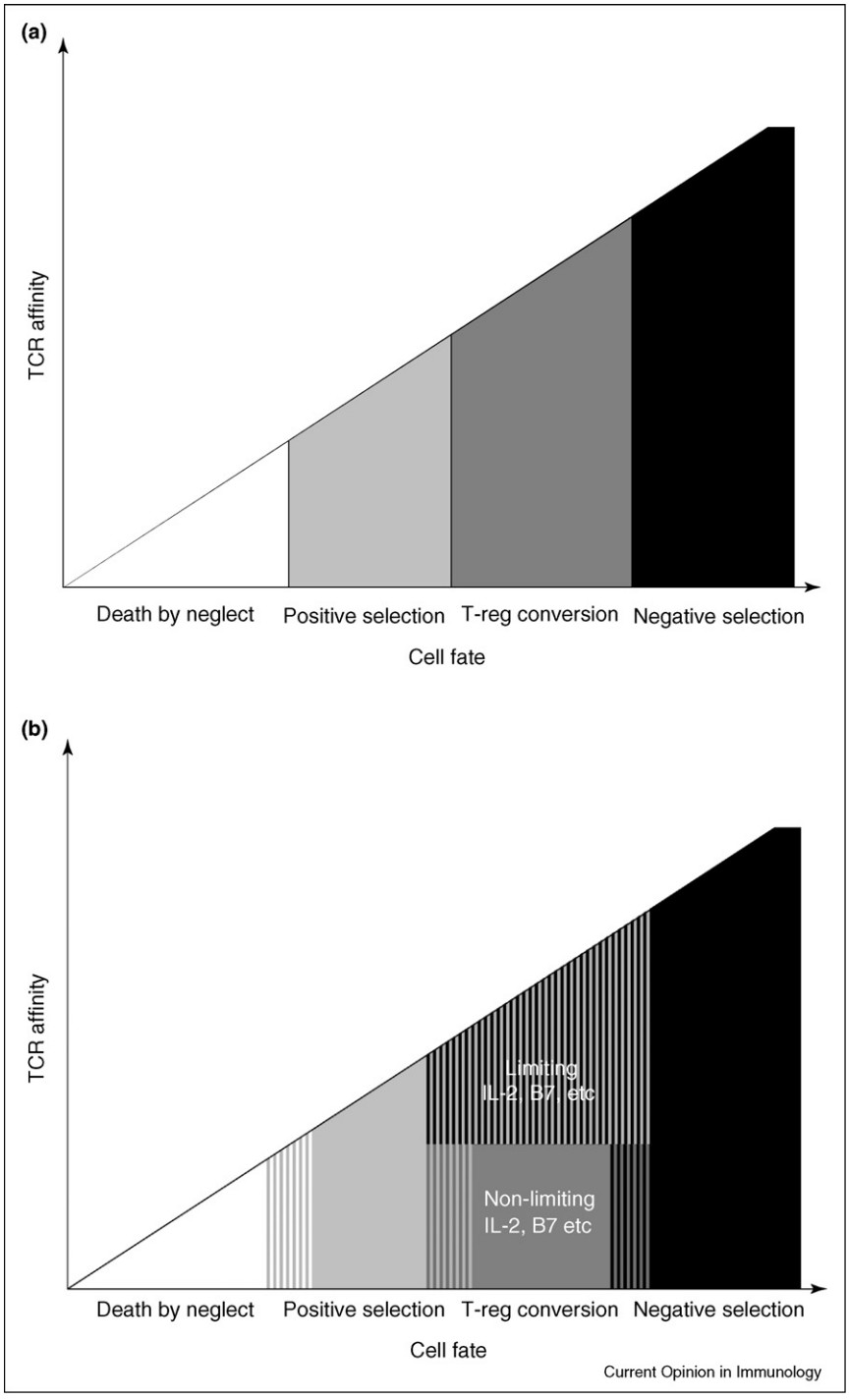
Figure 2



Models of cell fate determination. During thymocyte development, alternative cell fates include death by neglect (white), positive selection into the naïve lineage (light grey), conversion to regulatory cell status (dark grey) and negative selection (black). **(a)** In the strict-affinity model, affinity of the TCR for self-antigen is the sole basis of cell fate. **(b)** To account for differential fates documented for clonal thymocytes, the flexible-fate model allows multiple fates to occur at the TCR affinity boundaries (indicated with stripes). Factors influencing conversion to T-reg cells within this affinity range might include the local concentration of conversion cofactors such as B7 ligands and IL-2. In the absence of conversion cofactors, thymocytes are required to undergo the alternative fates of either maturation as a naïve cell or negative selection.

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protein express lower levels of *Foxp3* mRNA than those observed in Foxp3$^+$ T-reg cells. Furthermore, expression of Foxp3 protein seems to facilitate stable Foxp3 expression (M Gavin and AR, unpublished; see Update). The mechanism is unclear, but it probably involves the upregulation of CD25 and a concomitant rise in IL-2-induced signal, which feed back to increase Foxp3 expression [31•].

In addition to means of reinforcing Foxp3 expression in T-reg cells, another mechanism is expected to prevent the deletion of Foxp3$^+$ T-reg cells upon continuous encounters with self-antigen. In two TCR transgenic models of negative selection, CD25$^+$CD4$^+$ thymocytes have been found to have a ~100-fold increase in intrinsic resistance to apoptosis as compared with CD25$^-$CD4$^+$ thymocytes [12,15]. Likewise, in mice with a polyclonal TCR repertoire, CD25$^+$CD4$^+$ thymocytes show a relative resistance to negative selection induced by viral superantigens [7,54]. Nevertheless, these cells remain sensitive to a sharp increase in TCR signal and can undergo deletion [46].

Peripheral homeostasis of T-reg cells

Despite the importance for balanced immune regulation of tight control over the size of T-reg subset, little is known about the homeostatic control of peripheral T-reg numbers. The homeostatic control mechanisms seem to be distinct from, yet overlapping with, the mechanisms in place for naïve T cells, being less dependent on TCR signaling and dependent on CD28, TGFβ and IL-2, as discussed below.

Peripheral T-reg cells seem to have a lesser dependence on at least some aspects of 'tonic' TCR signaling for their homeostatic maintenance than are naïve 'conventional' CD4$^+$ T cells. In ENU-mutagenesis-derived partial loss-of-function *Slp76* and *Zap70* mouse mutants, the coordinated reduction in absolute numbers of Foxp3$^+$ and Foxp3$^-$ cells that was observed in the thymus is corrected in the periphery for Foxp3$^+$ cells but not Foxp3$^-$ cells (O Siggs, L Miosge, AL and C Goodnow, unpublished). Together with the above-mentioned effect of the LAT^{Y136F} variant on T-reg generation in the thymus, these data suggest that distinct TCR signaling requirements exist for thymic generation and for peripheral maintenance of T-reg cells and 'conventional' CD4$^+$ T cells.

Three factors have been implicated in the control of peripheral T-reg numbers: IL-2, CD28 and TGFβ. In addition to its role in thymic development, CD28 also has a role in peripheral homeostasis of T-reg cells. The reduction in thymic CD25$^+$ cell numbers in the *Cd28*$^{-/-}$ mouse is not corrected in the periphery [43]. Likewise, numbers of peripheral CD25$^+$ T-reg cells in wild-type mice are reduced by treatment with a combination of CD80 and CD86 blocking antibodies [43,44] or on transfer to a *Cd28*$^{-/-}$ host [44]. It is unclear, however, whether CD28 has an indirect function in the setting of the T-reg niche size through regulation of IL-2 production by non-T-reg cells, or a direct function in promoting the proliferation and survival of T-reg cells in the periphery.

TGFβ is also required for peripheral maintenance of T-reg cells because, despite normal thymic Foxp3$^+$ T-cell numbers, reduced peripheral numbers of Foxp3$^+$ T-reg cells have been reported in both *Tgfb1*$^{-/-}$ mice [36] and mice with a T-cell-specific deletion of *Tgfbr2* [37,38]. Because TGFβ signaling deficiency in T cells results in lethal autoimmune lesions, it is possible that the loss of Foxp3$^+$ T-reg cells is an indirect effect of TGFβ signaling deficiency that is due, for example, to excessive proliferation [38].

The peripheral role of IL-2 seems to be more complicated. Whereas absolute numbers and the proportion of Foxp3$^+$ thymocytes are reduced by half, the absolute numbers of peripheral Foxp3$^+$ T-reg cells in 4–5-week-old IL-2- or CD25-deficient mice are close to those in wild-type littermates, although their proportion remains substantially reduced [31•]. A similar observation has been made in 3A9 TCR, insulin-HEL double transgenic *Il2*$^{-/-}$ mice (AL, O Siggs and C Goodnow, unpublished). However, in both cases, immunological tolerance is impaired: expansion of the Foxp3$^-$ effector T-cell population results in lymphoproliferative disease in IL-2- and CD25-deficient mice, and in diabetes in IL-2-deficient double transgenic mice ([31•]; and AL, O Siggs and C Goodnow, unpublished). In the TS1 HA double transgenic mice, by contrast, peripheral Foxp3$^+$ T-reg cells disappear in the absence of IL-2 or CD25 [32•]. Likewise, in a competitive setting in the presence of wild-type CD25$^+$ Foxp3$^+$ cells, *Il2ra*$^{-/-}$ Foxp3$^+$ T-reg cells do not peripherally expand from the low thymic numbers and are present in greatly diminished numbers in the periphery [31•]. These data suggest that, whereas IL-2 has a partially redundant role in thymic T-reg development, it serves as an essential mechanism of the peripheral homeostatic maintenance of T-reg cells.

The role of IL-2 is likely to increase the metabolic fitness and proliferative capacity of peripheral T-reg cells. Indirect support for this notion comes from the finding that pre-incubation of CD25$^+$ T-reg cells with IL-2 allows them to survive on transfer into *CD28*$^{-/-}$ mice [44], whereas *Il2ra*$^{-/-}$ Foxp3$^+$ cells are completely lost when transferred into a lymphopenic host [55]. Furthermore, recent analysis of gene expression in Foxp3$^+$ T-reg cells isolated from *Il2*$^{-/-}$ mice with and without temporal IL-2 reconstitution has directly demonstrated a role for IL-2 signaling in the regulation of cell-cycle- and metabolism-related genes [31•].

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Extra-thymic 'conversion' of naïve T cells to T-reg cells

The peripheral pool of T-reg cells not only includes those differentiated in the thymus but also might include Foxp3$^+$ T-reg cells generated extra-thymically through the 'conversion' of naïve T cells on chronic encounter with antigen present in suboptimal dose or in non-immunogenic form [56,57]. *In vitro* experiments have demonstrated that peripheral T cells retain the ability to turn on Foxp3 expression and suppressive function on TCR cross-linking in the presence of high concentrations of TGFβ or in the presence of suboptimal TCR stimulation [56,58–61]. However, the overall numerical contribution of peripheral 'conversion' to the peripheral T-reg pool and its functional significance are not clear.

The high level of similarity in the TCR repertoire between thymic and peripheral T-reg cells indicates that thymic T-reg cell differentiation accounts for most T-reg cells present in secondary lymphoid organs [17••]. In agreement with these results, Belkaid and co-workers [62] have reported that *Leishmania*-specific T-reg cells present in the skin of infected animals are derived from CD25$^+$ T-reg cells present before infection. Nevertheless, further detailed examination of peripheral T-reg differentiation at different anatomical sites under various conditions is warranted.

Conclusions

Recent studies facilitated the dissection of the requirements for T-reg cell differentiation. In the thymus, T-reg cells seem to develop from thymocytes displaying TCR with a higher affinity for self-antigen in the context of MHC class II. However, TCR signal of a particular strength and duration alone is probably not sufficient for the induction of Foxp3 expression. The cofactors such as CD28 co-stimulation, IL-2 and other cytokines are also necessary for commitment to T-reg cell lineage to occur. The mechanisms by which signals mediated by TCR and cofactors involved in Foxp3 induction interact, and whether they coincide or can be can be temporally segregated, is unknown to date. Once thymocytes acquire Foxp3 expression, the regulatory cell fate is reinforced through a positive feedback loop, increasing Foxp3 expression and cell survival including a partial protection from negative selection. Requirements for peripheral T-reg cell maintenance and for their differentiation from non-T-reg cells in the periphery seem to differ from those in the thymus. Involvement of T-reg cells in control of immunoreactivity to self- and tumor antigens, pathogens and commensal flora warrant further research of molecular and cellular mechanisms of T-reg cell differentiation and homeostasis.

Update

The article cited in the main body of text as M Gavin and AR, unpublished, has now been accepted for publication [63].

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thymocytes in *CD28*$^{-/-}$ mice and *CD28*$^{-/-}$ TCR transgenic mice crossed to self-antigen. By introducing mixed chimeras of *Cd28*$^{-/-}$ bone marrow and *Il2*$^{-/-}$ bone marrow into *Cd28*$^{-/-}$ mice, the authors demonstrate that *Cd28*$^{-/-}$ T cells produce insufficient IL-2 to facilitate the conversion of *Cd28*$^{+/+}$*Il2*$^{-/-}$ cells to T-reg cells. Conversely, when IL-2 availability is rescued by allowing *Cd28*$^{-/-}$ bone marrow to develop in a mixed chimera with wild-type bone marrow, *Cd28*$^{-/-}$ bone marrow is still incapable of producing normal numbers of CD25$^+$ cells. Both the *cis*-acting and *trans*-acting CD28 effects in *Cd28*$^{-/-}$ mice require the Lck-binding domain, but not the PI3K-binding or Itk-kinase-binding motifs.

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