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Regulatory T Cell Development

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Abstract

Foxp3-expressing $CD4^+$ regulatory T (Treg) cells play key roles in the prevention of autoimmunity and the maintenance of immune homeostasis and represent a major barrier to the induction of robust antitumor immune responses. Thus, a clear understanding of the mechanisms coordinating Treg cell differentiation is crucial for understanding numerous facets of health and disease and for developing approaches to modulate Treg cells for clinical benefit. Here, we discuss current knowledge of the signals that coordinate Treg cell development, the antigen-presenting cell types that direct Treg cell selection, and the nature of endogenous Treg cell ligands, focusing on evidence from studies in mice. We also highlight recent advances in this area and identify key unanswered questions.

INTRODUCTION

One of the major functions of the thymus is to furnish the peripheral T cell repertoire with a diverse array of αβ T cells expressing T cell antigen receptors (TCRs) that recognize peptide antigens displayed by host major histocompatibility complex (MHC) molecules. This process generates a diverse anticipatory repertoire of $CD4^+$ and $CD8^+$ T cells with the potential to recognize pathogen-derived peptides displayed by MHC class II (MHC-II) and MHC-I molecules, respectively. This approach to host defense, based on the formation of a repertoire capable of recognizing self-MHC, followed by the clonal selection of rare antigen-specific T cells drawn from a diverse T cell pool, creates a challenging logistical problem, as the immune system must be tolerant to self-peptide/MHC (pMHC) complexes displayed throughout the body. The thymus is also a key site at which two major forms of T cell tolerance are established. In the process of negative selection, some thymocytes exhibiting overt reactivity to self-pMHC ligands are eliminated by clonal deletion, or diversion into innate-like T cell lineages. Additionally, some thymocytes reactive to ligands restricted to MHC-II molecules differentiate into Foxp3-expressing regulatory T (Treg) cells, which function in the periphery throughout life to prevent autoimmune reactions by suppressing the activation and function of conventional T (Tconv) cells. In addition, an expanding body of evidence demonstrates that Treg cells serve unique functions that are distinct from the suppression of autoreactive Tconv cells, including promotion of tissue repair (1, 2), metabolic regulation (3), and hair follicle stimulation (4). In the tumor context, Treg cells are highly enriched in many human and murine tumors, and they are thought to play a key role in restricting antitumor immunity (5). Thus, a lucid understanding of the mechanisms coordinating Treg cell development is crucial for understanding numerous facets of health and disease, including autoimmunity, inflammatory syndromes, and antitumor immunity. Here, we review current knowledge of the immunological forces orchestrating Treg cell development, focusing on mechanistic evidence in mice. We also highlight recent advances and identify key unanswered questions.

A major advance in the understanding of immune regulation came with the identification and characterization of a population of $CD4^+$ T cells, termed Treg cells, that function in a dominant fashion to prevent autoimmune reactions, including organ-specific autoimmunity, systemic autoimmunity, and colitis. Initial evidence of the existence of Treg cells came from early experiments demonstrating that the removal of the thymus in three-day-old mice induced organ-specific autoimmunity, but thymectomy of seven-day-old mice did not (6, 7). This established the existence of a thymus-dependent mechanism that is required for the prevention of autoimmunity and implied that this mechanism was first implemented within an early developmental window. Extensive research using T cell fractionation and reconstitution experiments in rodents demonstrated that regulatory activity could be conferred by phenotypic subsets of $CD4^+$ T cells expressing markers of prior antigen experience. A key advance in this area was the demonstration in mice that regulatory activity could be attributed to $CD4^+$ T cells expressing CD25, the IL-2 receptor α chain (8). Consistent with this, it was shown that day three thymectomy leads to the transient loss of $CD25^+CD4^+$ T cells in the periphery (9), providing a link between the autoimmunity induced by neonatal thymectomy and immune suppression conferred by $CD25^+CD4^+$ T cells.

Despite evidence of the existence of Treg cells, it remained unclear whether these cells represented a population of activated $CD4^+$ conventional T cells or a distinct, stable lineage of cells with specialized regulatory function. In 2003, collective evidence from three reports provided the formal demonstration that the transcription factor Foxp3 uniquely defines Treg cells and is required for Treg cell differentiation (10–12). Prior to these studies, interest in Foxp3 stemmed from genetic studies showing that human subjects with the X-linked autoimmune syndrome immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) harbored loss-of-function

mutations in *FOXP3* (13–15), coupled with genetic studies showing that *scurfy* mutant mice (16), which develop an X-linked autoimmune wasting syndrome that is similar to IPEX (17), harbor a loss-of-function frameshift mutation in *Foxp3* (18). Importantly, previous mechanistic studies in *scurfy* mice had demonstrated that $CD4^+$ T cells are required for disease development and are sufficient to transfer disease to new recipients (19). In addition, in heterozygous $Foxp3^{sf/wt}$ female mice and $FOXP3^{mut/wt}$ human females, in which 50% of T cells express a wild-type allele and 50% express a mutant allele due to X chromosome inactivation, subjects appear phenotypically normal (20, 21), consistent with the idea that Foxp3 plays a role in the establishment of dominant tolerance. Thus, collective evidence suggested that autoimmune pathology observed in Foxp3-mutant mice and humans may be due to a defect in Treg cell fitness or function. The identification of Foxp3 provided a molecular handle to identify, characterize, and manipulate Foxp3-expressing Treg cells and to more fully define the factors orchestrating their development and function. For example, the development of mouse strains expressing the human diphtheria toxin receptor under the dictates of the *Foxp3* promoter enabled experiments involving the inducible ablation of Foxp3-expressing cells in adult animals, which demonstrated that Treg cells are required throughout life to prevent fatal immune dysregulation and autoimmunity (22, 23).

The research highlighted above established that Foxp3-expressing Treg cells represent a distinct lineage of $CD4^+$ T cells that are essential for dominant tolerance and identified the thymus as a major site at which Treg cell development is orchestrated. A critical role for Treg cells in the prevention of autoimmunity implied that clonal deletion is an imperfect process requiring a second tier of regulation. The importance of Treg cells in the prevention of autoimmunity, the regulation of inflammation, and the suppression of antitumor immunity triggered a firestorm of interest in elucidating the fundamental biology of Treg cell differentiation and function. Specifically, when and where do Treg cells develop, what are the signals that induce Treg cell differentiation, and what is the nature of ligands recognized by these cells?

TIMING IS EVERYTHING: KINETICS OF TREG CELL DEVELOPMENT

Early studies of Treg cell developmental kinetics revealed that $CD4^+CD25^+$ cells first appear in the periphery at three days of age and that day three thymectomy leads to transient loss of $CD4^+CD25^+$ cells in the periphery (9, 24). Direct analysis of developmental kinetics came almost 20 years later following the development of Foxp3 reporter mice (25, 26). These mice were particularly important at the time, because robust antibodies for direct intracellular staining of Foxp3 protein were not yet commercially available. Temporal analysis of these mice revealed that Foxp3-expressing $CD4^+$ T cells first appear in the thymus at two days of age, and first seed the periphery at three days of age (25), consistent with observations using the CD25 marker.

Given the initial production of Treg cells in the perinatal period, what are the kinetics of thymic Treg cell production throughout life, and how do Treg cells generated at different ages contribute to immune tolerance? To test the hypothesis that the neonatal period is critical for the establishment of tolerance, Yang et al. (27) utilized an approach in which Treg cells developing in the perinatal period could be inducibly tagged by genetic labeling, permitting tracking and isolation of these cells later in life. These studies demonstrated that perinatally tagged Treg cells are enriched for the capacity to populate nonlymphoid organs and prevent organ-specific autoimmunity caused by deficiency in the transcriptional regulator Aire. Notably, perinatally tagged Treg cells were only able to prevent autoimmunity when isolated from autoimmune regulator (Aire)-sufficient mice, demonstrating a requirement for Aire in establishing a fully competent Treg cell repertoire in the neonatal period, a concept that we discuss below. Mechanistically, the functional properties enriched in perinatally tagged Treg cells could reflect numerous factors, including the

antigen specificities of these cells, the expression of unique homing molecules, or the simple fact that perinatal Treg cells are the first to populate the periphery and may have preferential access to self-ligands and accessory signals promoting their survival, differentiation, and function.

Beyond the neonatal period, to what extent does Treg cell production change later in life? Studies using *Rag2*-GFP reporter mice, in which GFP marks thymocytes that have recently undergone Rag2-dependent TCR rearrangement (28), revealed the striking finding that as mice age, the thymus becomes preferentially populated with Treg cells that have extinguished expression of *Rag2*-GFP (29, 30), indicative of mature Treg cells that are selectively retained or recruited back to the thymus. This raises the fascinating question of whether the accumulation of mature Treg cells, at the expense of newly generated Treg cells, plays a key modulatory role in Treg cell development. Why would extrinsic feedback be necessary to suppress de novo Treg cell generation, rather than an intrinsic waning in the production pipeline? Does the feedback mechanism exhibit antigen specificity? If so, what is the fate of self-reactive precursors that sense self-ligands in the thymus but fail to properly differentiate into Treg cells? One hypothesis, based on data from fetal thymic organ culture, suggests that the accumulation of mature Treg cells in the thymus may represent a negative feedback loop in which mature Treg cells suppress de novo Treg cell development by restricting access to IL-2 (29), although the extent to which such mechanisms operate in vivo remains undefined. In all, collective evidence indicates that robust Treg cell production is initiated shortly after birth and wanes over time, with the thymus accumulating mature Treg cells that have either been permanently retained in the thymus or recruited from the periphery.

FINDING ONE'S NICHE: THYMIC ENVIRONMENTS COORDINATING TREG CELL DEVELOPMENT

Considerable work has focused on defining the regions of the thymus in which Treg cell differentiation is initiated and fully executed, as anatomical location defines the nature of antigen-presenting cells (APCs), pMHC ligands, and accessory signals that are encountered by a developing thymocyte. In general, T cell precursors follow an orchestrated path through the thymus marked by multiple quality control checkpoints. The thymus is composed of an outer cortex, where early T cell development and positive selection occur, and an inner medulla, which hosts the later phases of maturation and T cell commitment to distinct lineages.

At what maturational stage is Treg cell development triggered, and does full Treg cell specification require the integration of multiple signals at different stages? Studies addressing these questions have been confounded by two factors. First, the upregulation of Foxp3 is likely temporally and spatially separated from the initial events that trigger Treg cell differentiation. This notion was supported by the identification of $CD25^{hi}Foxp3^{-}$ Treg cell precursors, which represent developmental intermediates that have been triggered to undergo Treg cell selection but have yet to upregulate Foxp3 (31). At present, there are limited tools to define the precise stage at which thymocytes first perceive the TCR-dependent signals triggering Treg cell selection prior to induction of Foxp3 expression. Treg cell heterogeneity is another issue that has confounded characterization of Treg cell development, as developing polyclonal Treg cells likely represent a composite of Treg cells following different, asynchronous developmental trajectories. Moving forward, these limitations may be overcome through the study of individual Treg cell clones with known antigen specificities, coupled with recent advances in the development of fluorescent timing reporters (32).

Numerous studies have examined the roles of the thymic cortex versus the medulla in fostering Treg cell development. Early studies using mice in which MHC-II molecules were exclusively expressed in the cortex revealed near-normal percentages of Treg cells, suggesting that ligand

presentation in the cortex is sufficient to direct the differentiation of a sizable population of Treg cells (33–35). However, little is known about the TCR diversity, antigen specificity, and functional capacity of Treg cells selected solely on cortically expressed ligands. In contrast, multiple lines of evidence suggest that the thymic medulla is critical for the differentiation of many Treg cells. First, >90% of Foxp3-expressing cells are at the CD4 single-positive stage of development (25, 26, 36), a stage at which cells are known to reside in the medulla. Consistent with this, direct analysis of Foxp3-expressing cells in tissue sections demonstrated that the large majority of Foxp3$^+$ cells are positioned in the medulla or at the corticomedullary junction, which demarcates the boundary where positively selected thymocytes first enter the medulla (25, 29, 33). Second, it has been demonstrated that the development of some Treg cell specificities is dependent on expression of Aire, which is highly expressed by medullary thymic epithelial cells (mTECs) (37, 38). Likewise, inducible ablation of mTECs (39, 40) or selective reduction of MHC-II expression by mTECs (41) induces a substantial reduction in polyclonal Treg cell percentages. Third, genetic deficiencies that disrupt the proper formation of the thymic medulla lead to broad alterations in Treg cell development (42). The disparity in findings regarding the roles of the cortex and medulla may reflect heterogeneity in Treg cell developmental pathways, coupled with differences in the nature of self-ligands triggering Treg cell selection. Thus, it is likely that antigen presentation in both the cortex and medulla contributes to the formation of a replete Treg cell repertoire of appropriate diversity.

GETTING MIXED SIGNALS: SIGNALS REQUIRED FOR TREG CELL DEVELOPMENT

A major question lies in understanding the molecular signals that are required for Treg cell development in the thymus. Given the importance of Treg cells in various aspects of health and disease, it is conceivable that dysregulation of these molecular pathways could lead to inefficient or aberrant Treg cell differentiation, thereby predisposing individuals to autoimmune disease or inflammatory disorders. Evidence consistent with this idea comes from human studies showing that mutations or polymorphisms in genes encoding IL-2, CD25, and CTLA-4 are associated with autoimmune disease, suggesting that deficiencies of Treg cell differentiation or function may underlie disease susceptibility (43–49). As outlined in **Figure 1**, it is now clear that Treg cell development requires at least three major signals: TCR-dependent recognition of pMHC-II ligand, CD28-dependent signaling triggered by the costimulatory ligands CD80 or CD86 (also called B7.1 and B7.2, respectively), and cytokine signaling triggered by sensing of IL-2, IL-15, or IL-7. Here, we review evidence of the critical importance of these pathways and highlight unanswered questions in this area.

TCR sequencing studies revealed that the TCR repertoire expressed by peripheral Treg cells is largely distinct from that of conventional CD4$^+$ T cells, with some degree of overlap (50–52). This suggested that Treg cell differentiation is a TCR-directed process in which the recognition of distinct pMHC-II ligands either directs de novo Treg cell differentiation of distinct T cell clones or promotes the survival or retention of select Treg cell specificities after an initial stochastic Treg cell differentiation process. Two seminal studies analyzed the developmental trajectories of individual Treg cell clones reactive to unidentified self-ligands, which revealed key facets of Treg cell development that were not previously evident (53, 54). Most importantly, these studies showed that the expression of Treg cell–derived TCRs promoted Treg cell differentiation in the thymus, whereas the expression of TCRs derived from CD4$^+$ conventional T cells did not, providing the formal demonstration that Treg cell development is a TCR-instructive process. As with most biological processes, this phenomenon was not black-and-white; the efficiency of skewing to the Treg

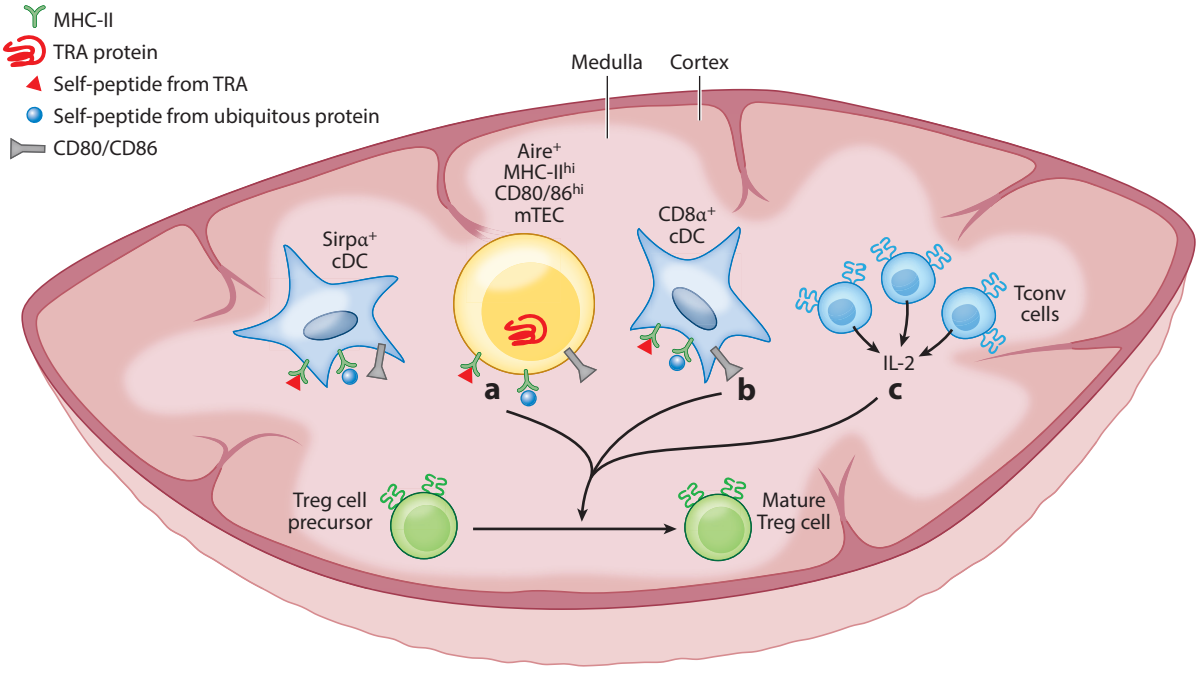


Figure 1

Factors required for Treg cell differentiation in the thymus. The thymic differentiation of Foxp3$^+$ Treg cells requires three types of major signals: (*a*) TCR-dependent recognition of pMHC-II ligand, (*b*) CD28-dependent signaling triggered by the costimulatory ligands CD80 or CD86, and (*c*) cytokine signaling triggered by sensing of IL-2. How these signals are conferred, and whether they must be provided in a defined order, remains an area of active investigation. Positively selected thymocytes enter the medulla, where they encounter an array of APCs displaying self-pMHC-II ligands and accessory signals. Treg cell differentiation largely depends on ligand presentation by three APC types: mature Aire-expressing mTECs exhibiting an MHC-II^{hi}CD80/86^{hi} phenotype, Sirpα$^+$ cDCs, and CD8α$^+$ cDCs (38, 76, 130, 198). Roles for other bone marrow–derived APC types such as B cells and pDCs remain incompletely defined. Aire drives the promiscuous expression of TRA proteins (*red*) by mTECs. mTECs can directly display self-peptides derived from TRA proteins (*red triangles*) via autophagy-dependent mechanisms (101, 137). Alternatively, antigen transfer can result in the presentation of TRA-derived peptides by Sirpα$^+$ and CD8α$^+$ cDCs, which also present peptides derived from ubiquitous self-proteins (*blue circles*). All three of these APC types also express high densities of CD80 and CD86 costimulatory ligands. Notably, it is unknown whether Treg cell differentiation requires simultaneous sensing of pMHC-II and CD80/CD86, or whether these signals can be separated in space and time. Optimal Treg cell differentiation also requires sensing of IL-2, with some compensatory contributions from IL-15, or IL-7. Recent studies demonstrate that paracrine production of IL-2 produced by Tconv cells is uniquely required to support Treg cell selection in the absence of IL-15 (78). Little is known about the location and distribution of IL-2-producing cells in the thymus, and whether developing Treg cell precursors sense IL-2 via stochastic encounters or a directed process. Abbreviations: APC, antigen-presenting cell, cDC, conventional dendritic cell; mTEC, medullary thymic epithelial cell; pDC, plasmacytoid dendritic cell; Tconv, conventional T; TCR, T cell receptor; TRA, tissue-restricted antigen; Treg, regulatory T cell.

cell lineage varied from TCR to TCR. Second, these studies revealed that for Treg-biased clones, the efficient induction of Treg cell selection required the introduction of monoclonal precursors at low clonal frequencies, revealing the existence of a limited saturable resource, or niche, that supports the development of each Treg cell clone. The finding that the niche size, defined as the maximum number of Foxp3$^+$ cells of a given specificity that can populate the thymus at any given time, varied for different Treg cell clones suggested that competition for access to a limited pool of pMHC-II ligands was the likely driver of this effect. Third, it was found that peripheral Treg cell differentiation in lymphoreplete or lymphopenic hosts was negligible, suggesting that for the clones analyzed, the thymus is uniquely permissive for Treg cell differentiation. Collectively, these

MEASURES OF TREG TCR/pMHC-II BINDING AFFINITY

There is considerable interest in defining the affinity and kinetics of pMHC-II ligand binding by Treg cell–expressed TCRs, as binding properties may be a primary determinant of Treg cell development and function. With this in mind, surrogate markers of affinity are commonly utilized to gain insight into the strength with which Treg cells recognize ligand. For example, the Nur77-GFP reporter serves as a useful measure of TCR signal strength in some instances, as the intensity of GFP signal increases following in vitro T cell stimulation with peptide ligands of increasing potency (83). However, in vivo studies demonstrate that the intensity of Nur77-GFP fluorescence wanes over time following cessation of TCR signaling (197). Thus, for Treg cells exhibiting intermediate Nur77-GFP fluorescence intensities, it is not possible to distinguish whether these are Treg cells expressing intermediate-affinity TCRs that are actively engaging ligand, or Treg cells expressing high-affinity TCRs that have not been recently triggered by pMHC-II ligand. Thus, data using surrogate markers of TCR signal strength should be interpreted with caution. Further studies using biochemical measurements of the pMHC-II binding properties of Treg TCRs reactive to natural endogenous ligands, such as those reported by Stadinski et al. (123), are necessary.

studies demonstrate that Treg cell differentiation is directed by the TCR-dependent recognition of pMHC-II ligands. However, as described in the sidebar titled Measures of Treg TCR/pMHC-II Binding Affinity, direct information regarding the ligand-binding properties of endogenous Treg-derived TCRs is limited, necessitating the use of surrogate markers of TCR/pMHC-II affinity.

An additional class of signals required for Treg cell development involves signaling through the T cell–expressed CD28 costimulatory receptor, triggered by recognition of CD80/CD86 ligands expressed by APCs. This principle was first revealed in studies using *Cd28*$^{-/-}$ and *Cd80*$^{-/-}$*Cd86*$^{-/-}$ mutant mice (55). Given the importance of the CD28 pathway in T cell activation, it was expected that *Cd28*$^{-/-}$ or *Cd80*$^{-/-}$*Cd86*$^{-/-}$ mutant mice on the diabetes-susceptible NOD (nonobese diabetic) background would be resistant to diabetes. In stark contrast, it was found that diabetes was exacerbated in such mice. Analysis of mutant mice revealed a paucity of CD4^{+}CD25^{+} Treg cells in the thymus and periphery. Moreover, treatment of wild-type NOD mice with recombinant CTLA-4-Ig fusion protein, which blocks CD80/CD86 ligands in vivo, induced a substantial loss of CD4^{+}CD25^{+} cells and accelerated diabetes development (55). Thus, these early studies demonstrated that the triggering of CD28 signaling by CD80 and/or CD86 ligands is crucial for optimal Treg cell development. Subsequent work has further illuminated the mechanisms by which CD28 signaling impacts Treg cell biology. Tai et al. (56) used mixed chimeric mice to demonstrate that the requirement for CD28 signaling in Treg cell differentiation is cell intrinsic. Additionally, Lio et al. (57) showed that the Treg TCR repertoire is largely unaltered in CD28-deficient mice, suggesting that CD28 does not impact the range of specificities undergoing Treg cell differentiation. Thus, CD28 signaling likely does not function by altering TCR signaling thresholds but instead functions to ensure the optimal differentiation or survival of those thymocytes that have received appropriate TCR-dependent signals (57).

In addition to recognition of pMHC-II and CD80/CD86 ligands, substantial evidence demonstrates that optimal Treg cell differentiation and fitness require sensing of IL-2 or the related common γ-chain cytokines IL-15 and IL-7. Early evidence for this came from observations that CD4^{+}CD25^{+} T cells were largely absent in IL-2-deficient mice (58, 59) as well as mice lacking the IL-2Rα chain (58) or IL-2Rβ chain (60). These findings were refined in subsequent studies leveraging newly developed approaches for identifying Foxp3-expressing cells, which defined general principles about the role of IL-2 in supporting Treg cell differentiation and function. First, IL-2 plays a major role in supporting Treg cell development and maintenance, as injection of

neutralizing anti-IL-2 antibodies induced a rapid reduction of Treg cell numbers in the thymus and periphery (61, 62) and the development of organ-specific autoimmunity in different mouse strains (62). Second, IL-2 contributes to optimal Treg cell fitness in the periphery (63–66) but is not absolutely required for Treg cell differentiation due to compensatory contributions from IL-15 and IL-7. This principle was revealed through studies using compound mutant mice that demonstrated that IL-15 and IL-7 signaling can partially compensate for the lack of IL-2 sensing and that Foxp3$^+$ Treg cell development is fully abolished in mice that cannot perceive signals triggered by IL-2, IL-7, and IL-15 (64, 65, 67, 68). Third, the signal transducer STAT5 plays a key role in transducing the IL-2-dependent signals driving Treg cell differentiation, as revealed in studies showing that Treg-specific deletion of STAT5 induces a reduction in Treg cell numbers, and that ectopic expression of a constitutively activated form of STAT5b induces expansion of Treg cells (64, 69, 70).

Given that CD28 signaling is known to promote IL-2 production in activated conventional T cells, it is important to consider whether the requirements for CD28 signaling and IL-2 signaling are interrelated. In this regard, multiple lines of evidence suggest that the effects of CD28 signaling are distinct from those of IL-2 signaling. It was reported that the frequency of CD25$^+$Foxp3$^-$ Treg cell precursors is diminished approximately twofold in *Cd28*$^{-/-}$ mice (57, 71), suggesting that CD28 may function in an early phase of Treg cell development that precedes IL-2 signaling. In addition, the fact that overexpression of constitutively active STAT5b leads to major shifts in the Treg cell TCR repertoire (69), whereas CD28 deficiency does not (57), suggests that the two signaling pathways function in different ways. Consistent with this, Hinterberger et al. (72) used intrathymic cell transfers in a TCR transgenic system to demonstrate that early CD25$^-$Foxp3$^-$GITR$^+$ Treg cell precursors required CD80/CD86 signals to proceed through Treg cell differentiation, but later CD25$^+$Foxp3$^-$ precursors did not. Thus, this cumulative evidence suggests that CD28 likely functions in the early, TCR-instructive phase of Treg cell development but is dispensable for a subsequent cytokine-dependent phase.

Beyond TCR-, CD28-, and IL-2/IL-15/IL-7 signaling, additional pathways have been shown to promote the survival of developing Treg cell clones by conferring resistance to clonal deletion, including TGF-β receptor signaling (73) and engagement of CD70 ligand by the T cell–expressed CD27 receptor (74). These findings suggest that the strong agonist TCR signals that direct Treg cell differentiation must be counterbalanced by mechanisms to protect developing Treg cells from apoptosis. Lastly, other work has demonstrated that signaling through TNFRSF members GITR, OX40, or TNFR2 augments cytokine responsiveness and facilitates Treg cell differentiation (75).

Whereas the molecular requirements of Treg cell differentiation are now well defined, key questions remain about how the multiple key signals are sensed and integrated by a developing Treg cell precursor. Are key signals conferred simultaneously within a narrow time window, or are they separated in space and time? If the latter, must the signals be perceived in a defined order, with carefully orchestrated kinetics? And how are the multiple signals integrated via progressive changes in transcriptional and epigenetic states? To answer these questions, it is important to define the cellular sources of pMHC-II, CD80/CD86, and cytokine signals in the thymus.

As discussed in the section below, pMHC-II ligands are displayed by a diverse network of APC types, which appear to collaborate to coordinate the development of a diverse Treg cell repertoire. For CD80/CD86 costimulatory signals, it was demonstrated that CD80/CD86 deficiency on radioresistant host cells (i.e., TECs) does not impact thymic Treg cell numbers, whereas deficiency on bone marrow–derived cells is associated with a significant reduction in Treg cell cellularity (76). Beyond this limited information, little is known about the cellular context in which CD80/CD86 signals are conferred. Moreover, it is unclear whether costimulatory ligands must be displayed by the same APC presenting pMHC-II ligand, or whether pMHC-II and CD80/CD86 signals can

be conferred by different cells. Early efforts to define the sources of IL-2 using in situ hybridization revealed the presence of halos of IL-2 expression by cells having T cell morphology (77), suggesting that IL-2 production is localized to regional clusters of cells. In recent work, Owen et al. (78) used a floxed allele of *Il2* to define the cellular sources of IL-2 supporting thymus-derived Treg (tTreg) cell development, performing studies on an $Il15^{-/-}$ background to avoid compensatory effects by IL-15. This survey demonstrated that IL-2 production by T cells was uniquely required to support Treg cell differentiation, whereas IL-2 production by B cells and dendritic cells (DCs) was dispensable. Consistent with this, Hemmers et al. (79) demonstrated that the major producers of IL-2 in the thymus represent CD4 single-positive thymocytes that display hallmarks of self-reactivity, and that at least some of these IL-2-producing cells are Treg cell precursors. In other work, Weist et al. (80) proposed a role for DC-derived IL-2 in promoting the Treg cell differentiation of OT-II transgenic T cells in in vitro–cultured thymic slices. However, it remains unclear whether the experimental system employed recapitulates the natural environments coordinating Treg cell selection in vivo. Thus, available evidence suggests that tTreg cell development in a natural setting is dependent on IL-2 produced solely by T cells within the thymus.

How are these key molecular signals integrated by a developing Treg cell precursor? A common paradigm from early work suggests that Treg cell differentiation is a two-step process in which TCR- and CD28-dependent signals trigger differentiation to a $CD25^{+}Foxp3^{-}$ intermediate, which subsequently proceeds to a mature $CD25^{+}Foxp3^{+}$ Treg cell phenotype following sensing of IL-2 or related cytokines (31). However, two recent studies have challenged this notion, suggesting the existence of a second pathway in which mature $CD25^{+}Foxp3^{+}$ Treg cells develop via a $CD25^{-}Foxp3^{lo}$ intermediate (81, 82). The implications of these findings are discussed in **Figure 2**.

GAINING A SENSE OF SELF: TREG CELL REACTIVITY TO ENDOGENOUS SELF-LIGANDS

As discussed above, TCR-dependent ligand recognition is required for Treg cell differentiation in the thymus. The paradigm that thymus-derived Treg cells exhibit specificity for endogenous self-ligands stemmed from a substantial body of indirect evidence, including experiments showing that TCR-transduced cells expressing Treg-biased TCRs undergo proliferation in lymphopenic hosts (52), and that many Treg cells are proliferative at steady state (52) and express high densities of the Nur77-GFP reporter, a surrogate readout of TCR signal strength (83). While these and other observations (reviewed in 84) suggested that many Treg cells are reactive to ligands derived from self constituents, it remained possible that some Treg cells recognize commensal or environmental antigens, rather than self-ligands. Thus, a complete understanding of Treg cell specificity requires identification and characterization of endogenous Treg cell ligands.

Early studies examining the antigenic signals driving Treg cell development utilized engineered systems in which model antigens are transgenically expressed in mice, and the developmental fate of antigen-specific TCR transgenic T cells is assessed. These studies showed that the recognition of strong agonist ligands in the thymus promoted Treg cell differentiation (85–93). Notably, a universal feature of these studies was that expression of these engineered antigens also induced clonal deletion of a major fraction of TCR transgenic cells, with a minor fraction of surviving cells exhibiting a $CD25^{+}Foxp3^{+}$ phenotype. This led to the notion that recognition of MHC-II-restricted agonist ligands in the thymus induces both clonal deletion and Treg cell development. As discussed in the sidebar titled Model Antigens and the Study of Treg Cell Development, it is important to consider whether expression of model antigens accurately mimics expression of natural endogenous ligands driving Treg cell selection, and whether clonal deletion is a common

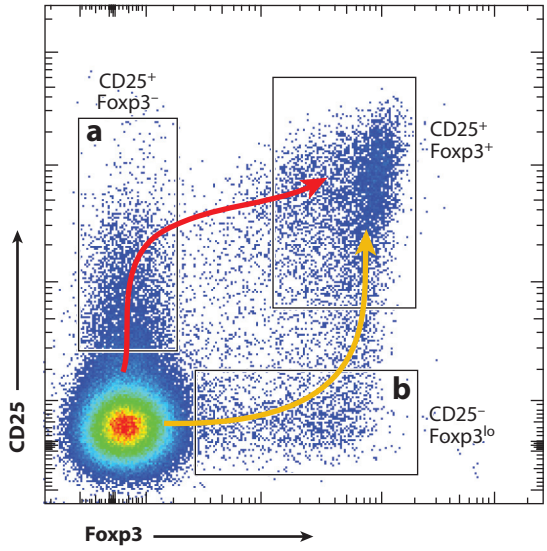


Figure 2

Potential pathways of thymic Treg cell development. For the three major signals required for Treg cell differentiation in the thymus (**Figure 1**), key questions are how these signals are sensed and integrated by a developing precursor, and whether these signals must be perceived in a defined order. As illustrated in the figure, flow cytometric analysis of developing thymocytes plotting CD25 versus Foxp3 expression reveals a clear population of mature $CD25^+Foxp3^+$ Treg cells, as well as minor populations of $CD25^+Foxp3^-$ cells and $CD25^-Foxp3^{lo}$ cells (81, 82). Two major developmental pathways have been proposed: (*a*) *Development through a $CD25^+Foxp3^-$ intermediate*. It has been demonstrated that purified $CD25^+Foxp3^-$ thymocytes can progress to mature $CD25^+Foxp3^+$ Treg cells following intrathymic transfer to MHC-II-deficient hosts and following in vitro culture with exogenous IL-2 or IL-15 (31). These findings suggest that $CD25^+Foxp3^-$ cells represent Treg cell precursors that are poised to express Foxp3 upon cytokine exposure in the absence of additional antigenic signals (31) (pathway indicated by *red arrow*), and support a two-step model of Treg cell differentiation characterized by an early TCR-instructive phase to generate $CD25^+Foxp3^-$ intermediates, followed by a subsequent cytokine-dependent consolidation phase to yield mature $CD25^+Foxp3^+$ Treg cells. (*b*) *Development through a $CD25^-Foxp3^{lo}$ intermediate*. More recently, an alternate developmental pathway has been proposed in which Treg cells can also progress through a $CD25^-Foxp3^{lo}$ intermediate (pathway indicated by *yellow arrow*), and differentiation of these cells into mature Treg cells can also be induced via coculture with exogenous IL-2 (81, 82). The proposed existence of a second developmental pathway raises key questions about Treg cell development moving forward. First, why would the early events of Treg cell differentiation drive some cells into $CD25^+Foxp3^-$ intermediates, whereas other cells differentiate into $CD25^-Foxp3^{lo}$ intermediates? And why can both of these intermediates progress to mature $CD25^+Foxp3^+$ Treg cells upon exposure to IL-2, as demonstrated in References 81 and 82? Second, are the two pathways the result of a stochastic, unordered encounter with the TCR-, CD28-, and cytokine-dependent signals driving Treg cell differentiation? An alternate hypothesis for consideration is that there is a single linear pathway of Treg cell differentiation in which precursors zig-zag from $CD25^+Foxp3^-$ to $CD25^-Foxp3^{lo}$ to $CD25^+Foxp3^+$ Treg cells. This idea is consistent with developmental timing analyses of Owen et al. (81) indicating that $CD25^+Foxp3^-$ cells are at an earlier developmental stage than both $CD25^-Foxp3^{lo}$ and $CD25^+Foxp3^+$ cells. Moving forward, it will be key to identify Treg cell clones that uniquely progress through $CD25^+Foxp3^-$ intermediates and those that uniquely progress through $CD25^-Foxp3^{lo}$ intermediates, to further define these proposed pathways.

alternate fate for Treg cell–biased specificities. In this regard, a major puzzle lies in understanding the factors that determine whether a self-reactive MHC-II-restricted thymocyte will undergo Treg cell differentiation or clonal deletion. Potential factors have been extensively reviewed recently (94, 95) and are illustrated in **Figure 3**.

MODEL ANTIGENS AND THE STUDY OF TREG CELL DEVELOPMENT

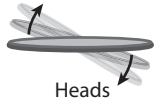
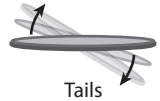
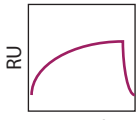
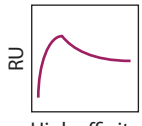
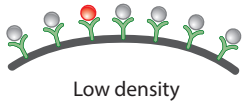
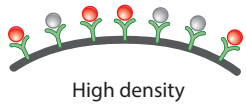
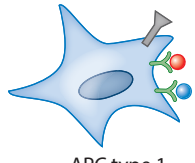
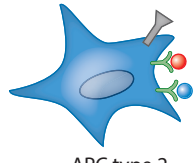
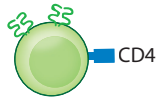
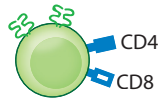
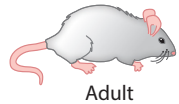
Classically, principles of T cell tolerance have been interrogated using engineered systems in which a model antigen is transgenically expressed in the thymus, and the impact on antigen-specific T cells is assessed. It is important to consider whether these model antigens accurately mimic the natural biology of endogenous Treg cell ligands, with respect to expression patterns, processing and presentation efficiency, APC types, and TCR-pMHC-II binding properties. For example, in transgenic mice expressing the ovalbumin (OVA) protein under the dictates of the rat insulin promoter, OVA-specific TCR transgenic T cells undergo extensive clonal deletion, with only a minor fraction of cells surviving and adopting a Treg cell phenotype (85–93). Notably, this effect differs from many TCR transgenic mice expressing Treg-derived TCRs reactive to natural ligands, for which clonal deletion is negligible (53, 54, 117). Likewise, whereas endogenous Aire-dependent transcripts are expressed by a small percentage of mTECs (110, 111), the expression of model antigens driven by *Aire* regulatory elements (86, 166) drives antigen expression on a large fraction of mTECs, which may be supraphysiological. Moving forward, it will be important to validate findings in model antigen systems by studying Treg cell clones reactive to endogenous ligands.

What is known about the nature of self-pMHC-II ligands that are displayed to developing thymocytes? Analysis of peptides eluted from MHC class II molecules from the thymus using mass spectrometry revealed that the majority of peptides are derived from widely expressed proteins (96–98), including nuclear, cytosolic, secretory, and transmembrane proteins. The sources of these peptides are incompletely defined, but they likely reflect a mixture of endogenous antigens expressed by thymic APCs themselves, coupled with exogenous antigens taken up from the blood or interstitial fluid of the thymus. For example, intracellular proteins can be processed and presented on MHC-II in the thymus via the autophagy pathway (99, 100), and when this pathway is disrupted, T cell selection and self-tolerance are perturbed (101). In addition, injection of antigenic protein in the blood can induce antigen uptake by thymic DCs and subsequent Treg cell selection (102, 103), suggesting that the blood may serve as a source of self-ligands presented in the thymus. Collectively, these studies suggest that the most prevalent self-peptides displayed by APCs in the thymus are derived from abundant proteins that are widely expressed throughout the body. However, as described below, low-density ligands also play a significant role in central tolerance.

In this regard, one of the most fascinating aspects of T cell selection centers on the concept of promiscuous gene expression (PGE) in the thymus. Early evidence of this phenomenon came from studies of transgenic mice that revealed unexpected transgene expression in the thymus driven by the insulin promoter and other promoters thought to be tissue-restricted (104). Since these initial observations, extensive research has demonstrated that PGE is not simply an artifact of transgenesis but instead represents a highly orchestrated process that is critical for the establishment of immune tolerance.

Inspired by observations of transgenic mice, Derbinski et al. (105) demonstrated that transcripts encoding numerous tissue-restricted antigens (TRAs) are expressed by stromal cells within the thymus, and they identified a critical role for mTECs in coordinating PGE. Subsequent identification and characterization of Aire, a transcription factor that is preferentially expressed by mTECs, provided a molecular basis for PGE in the thymus. A role for Aire in immune tolerance was implied by genetic studies in humans, which demonstrated that human subjects with loss-of-function mutations in AIRE develop autoimmune polyglandular syndrome 1 (APS-1), an autoimmune disease characterized by mucocutaneous candidiasis, autoimmune destruction of the parathyroid and adrenal glands, and hypogonadism (106, 107). In 2002, Anderson et al. (108) showed that Aire-deficient mice developed organ-specific autoimmunity due to defects in

radioresistant stromal cells and revealed that Aire functions in part by driving the PGE of hundreds of genes, many of which encode peripheral TRAs. Mechanistically, Aire and its associated factors are thought to act on stalled polymerases at chromatin-accessible regions to promote transcription (109). Interestingly, the transcription of distinct target genes in individual mTECs occurs sporadically in a largely monoallelic fashion, with a given transcript only expressed by a small percentage of all mTECs (110, 111). However, the collective population of mTECs can direct the PGE of thousands of transcripts, encompassing a large fraction of the peripheral transcriptome (112). The fact that a given gene is only transcribed by a small percentage of

		TCR-dependent signals induce:	
		Treg cell development	**Clonal deletion**
A	Stochastic	 Heads	 Tails
B	TCR/pMHC-II binding properties	 Intermediate affinity or t1/2	 High affinity or t1/2
C	Ligand density	 Low density	 High density
D	APC type	 APC type 1	 APC type 2
E	Duration of TCR signaling	 Short duration	 Long duration
F	Maturational stage	 More mature	 Less mature
G	Age-dependent effects	 Neonatal	 Adult

(*Caption appears on following page*)

Figure 3 (*Figure appears on preceding page*)

Potential factors driving the alternate fates of Treg cell development versus clonal deletion. During maturation in the thymus, thymocytes exhibiting overt reactivity to self-pMHC-II ligands can undergo Treg cell differentiation or clonal deletion. The factors that determine these alternate fates remain incompletely defined. The figure illustrates six potential factors that are not mutually exclusive and are potentially interrelated. (A) Stochastic: Cell fate is determined stochastically, with some cells undergoing Treg cell development and others removed by deletion. (B) TCR/pMHC-II binding properties: Outcome is determined by the affinity or half-life of TCR binding to pMHC-II. For example, modest interactions could favor Treg cell development, whereas stronger interactions could favor deletion (88, 123). (C) Ligand density: Fate is impacted by the density of pMHC-II ligands displayed by the collective population of thymic APCs, with low-density ligands promoting Treg cell selection and high-density ligands triggering deletion (95). (D) APC type: The identity of the APC displaying pMHC-II and accessory factors impacts outcome, with a given APC type promoting Treg cell selection and a different APC type driving deletion. (E) Duration of TCR signaling: The duration with which a thymocyte senses TCR-dependent signals, impacted by the factors described in rows B–D, determines outcome. For example, transient TCR signaling triggered by engagement of sparse ligands could promote Treg cell differentiation, whereas sustained TCR signaling induced by widely presented, high-density ligands could favor deletion. (F) Maturational stage: The outcome of encounter with pMHC-II ligands may be impacted by the maturational state of the thymocyte. For example, less mature thymocytes at the $CD4^+CD8^+$ stage could be prone to deletion, whereas more mature $CD4^+CD8^-$ thymocytes could be more likely to undergo Treg cell differentiation. (G) Age-dependent effects: The collective impact of factors A–F may change throughout life. For example, the early neonatal period may be permissive for Treg cell selection, whereas later periods may favor deletion (123). See References 94 and 95 for a more extensive discussion of these concepts. Abbreviations: APC, antigen-presenting cell; pMHC, peptide/MHC; TCR, T cell antigen receptor.

mTECs at any given time implies that the ligand density of Aire-dependent peptides is likely to be very low, a concept that may explain the limited antigenic niches supporting the differentiation of many Treg cell clones. Lastly, it is important to highlight two key unanswered questions regarding PGE by mTECs. First, it was demonstrated that for some TRA-encoding transcripts, PGE occurs in an Aire-independent fashion, suggesting that mTECs engage additional mechanisms to promote PGE (113). A potential candidate is the factor Fezf2, which has been suggested to drive TRA expression by direct binding to DNA elements near target genes (114). However, a role for Fezf2 in coordinating the development of distinct TRA-specific Treg cells has yet to be shown. Second, it is clear that Aire functions to promote transcription on prepoised, chromatin-accessible sites (115). Thus, it will be critical to identify the factors that function upstream of Aire to set the table for Aire-dependent transcription.

At the time of these seminal inquiries, promiscuous expression of self-peptides in the thymus, driven by both Aire-dependent and Aire-independent mechanisms, was largely thought to promote tolerance by driving clonal deletion of developing thymocytes exhibiting reactivity to these self-peptides. However, a series of later studies demonstrated that Aire is required for thymic differentiation of select Treg cell specificities (37, 38, 116, 117), which are estimated to comprise ~25% of the thymic Treg cell repertoire and ~5% of the peripheral Treg cell repertoire in different studies (37, 38). TCR profiling of recurrent Tconv cell clones infiltrating the prostates of Aire-deficient male mice revealed that dominant Tconv clones did not represent specificities that evaded clonal deletion in the absence of Aire. Instead, these infiltrating cells represented clones that are normally skewed to the Treg cell lineage in wild-type mice but are misdirected into the $Foxp3^-$ Tconv compartment in settings of Aire deficiency (37). Work from Yang et al. (27) showed that Aire-dependent Treg cells generated early in life were required for the prevention of organ-specific autoimmunity and that early transfer of Treg cells alone was sufficient to prevent development of autoimmune pathology in Aire-deficient recipients. Collectively, these studies demonstrated a major role for Aire in driving the generation of a substantial fraction of Treg cells, which is required for prevention of organ-specific autoimmunity.

The search for endogenous Treg cell ligands has been complicated by the immense diversity of the self-proteome, coupled with technical limitations in identifying rare self-peptides displayed by MHC-II molecules at low density. By focusing on Aire-dependent Treg cell specificities reactive to prostate-associated antigens, Leonard et al. (118) identified two endogenous self-peptides recognized by recurrent Treg cell clones. Notably, the two peptides were derived from a single prostatic protein, Tcaf3, which was previously identified as a major autoantigen targeted by antibodies under settings of immune dysregulation (119). This finding, although limited in scope, suggests that organ-specific Treg cells may be focused on a limited number of antigenic determinants in a given regional site. In other work, Hassler et al. (120) used pMHC-II tetramers to demonstrate that approximately 30% of endogenous $CD4^+$ T cells reactive to peptides derived from the myelin-associated proteolipid protein are skewed to the $Foxp3^+$ Treg cell lineage. Additional studies reported that peptides derived from the myelin oligodendrocyte glycoprotein and insulin B proteins are recognized by Treg cells in autoimmune models of experimental autoimmune encephalomyelitis and diabetes, respectively (121, 122). Since these peptides represent key targets of Tconv cells driving autoimmune pathology in these contexts, it is unclear whether these represent specificities that are naturally skewed to the Treg cell lineage.

A recent report from Stadinski et al. (123) provided a more expansive view of the antigen specificities of naturally occurring Treg cell clones. By generating a large panel of immortalized cell lines expressing TCRs expressed by Treg cells from neonatal mice, they showed that 20–35% of these TCRs conferred overt reactivity to splenic DCs. Interestingly, the number of reactive clones was further increased using splenic DCs that had been activated by innate signals, suggesting that some Treg cells are reactive to self-ligands that are differentially displayed by activated DCs. By screening a large peptide library composed of self-peptides reported to bind to the $I\text{-}A^b$ class II molecule, the group identified 17 self-peptides recognized by Treg cell clones that were derived from widely expressed self-proteins involved in diverse biological processes.

In sum, available evidence suggests that the development of a complete Treg cell repertoire requires thymic display of peptides derived from widespread self-proteins and promiscuously expressed tissue-restricted self-proteins. With this in mind, what is the nature of APCs that display these self-ligands to developing thymocytes to coordinate Treg cell selection?

FINDING ONE'S IDENTITY: ANTIGEN-PRESENTING CELLS COORDINATING TREG CELL DEVELOPMENT

As introduced above, the thymus functions to establish a competent peripheral pool of naive $CD4^+$ and $CD8^+$ T cells while directing self-reactive thymocytes to alternate cell fates of clonal deletion, differentiation into innate-like lineages, and Treg cell selection. These fates are coordinated by a network of cell types that generate antigens, display self-pMHC ligands to developing T cells, and provide accessory signals and contextual cues that specify cell fate. Given that both Treg cell development and clonal deletion are common fates of thymocytes in the medulla (124), it is crucial to define the factors that determine whether a given self-specific thymocyte will undergo Treg cell differentiation or clonal deletion (see **Figure 3**) and determine whether distinct APC subsets are specialized to orchestrate Treg cell development. In addition, given that Treg cell differentiation requires the provision of pMHC-II ligand, CD80/CD86 costimulation, and cytokine signals, it is important to consider whether distinct APC subsets are specialized to confer one or more of these signals in the proper spatial and temporal contexts. The thymus is populated by numerous MHC-II-expressing cell types, including $Sirp\alpha^+$ and $CD8\alpha^+$ conventional DCs (cDCs), plasmacytoid DCs (pDCs), B cells, macrophages, and both cortical TECs (cTECs) and mTECs (41, 125). Coculture experiments demonstrated that many APC types (including TECs, $Sirp\alpha^+$

and CD8α$^+$ cDCs, B cells, and macrophages) can induce Treg cell differentiation in vitro (76, 125, 126), suggesting that diverse APCs can confer the necessary signals in reductionist experiments. Is this functional redundancy operative in vivo, given the unique microenvironments encountered by developing thymocytes during maturation? Below, we discuss Aire-independent and Aire-dependent Treg cell selection separately, as the sources of self-ligand recognized by these subsets are likely to differ.

When defining the APCs that coordinate Treg cell development, it is important to consider the process of antigen transfer between different cell types, as this occurs readily in the thymus and can complicate data interpretation. For example, it has been demonstrated that cytosolic or transmembrane proteins can be transferred from radioresistant cells to bone marrow–derived APCs (38, 127–129), but multiple mechanisms of antigen exchange in the thymus have been described and could be operative simultaneously. Thymic APCs may process and cross-present peptides from blood-borne proteins or from proteins acquired from apoptotic bodies of mTECs. Alternatively, intact pMHC-II may be directly acquired from mTEC membranes and presented to developing thymocytes in *trans* by other APC subsets. For example, Perry et al. (127) recently demonstrated that mTEC-derived pMHC-II and other cell-surface proteins can be acquired and presented by thymic DCs via the scavenging receptor CD36. Although nuanced, this concept can have far-reaching implications when determining which APC subsets are required for Treg cell development, and it should be carefully considered in data interpretation.

Of the MHC-II-expressing cell types in the thymus, what is known about their contribution to the selection of a replete Treg cell repertoire? It was shown that the inducible ablation of a large fraction of mTECs resulted in an ~50% reduction in Treg cell numbers (39, 40), highlighting the critical role for mTECs in either antigen production or presentation. Analysis of chimeric mice lacking MHC-II expression by bone marrow–derived APCs revealed a significant reduction in polyclonal Treg cell numbers (130), and the loss of numerous Treg-biased specificities (38), indicating a key role for MHC-II expression by bone marrow–derived APCs. Notably, Perry et al. (38) demonstrated that the development of several Treg cell clones was abolished in mice lacking CD11c$^+$ DCs. Among the thymic DC subsets, analysis of the roles of distinct subsets has been stymied by the lack of robust approaches to inducibly deplete distinct populations, or conditionally delete MHC-II or CD80/CD86 on these subsets. An early report suggested that pDCs can migrate to the thymus from the periphery and impact Treg cell selection (131), but this idea has yet to be substantiated by subsequent work. Lastly, it was reported that the thymus harbors a subset of Aire-expressing B cells that contribute to self-antigen presentation in the thymus, but a role for these cells in Treg cell selection has not been evident (132), and functional experiments indicate that the prevention of autoimmunity does not require Aire expression by bone marrow–derived cells (108, 133).

For Aire-dependent tolerance, thymic grafting and bone marrow chimera experiments defined a critical role for mTECs, which represent the major Aire-expressing cell type in the thymus (108, 133). Immature mTECs undergo a maturational progression associated with upregulation of Aire, MHC-II, and CD80/CD86 (134). mTECs also produce key chemokines that coordinate the accumulation and positioning of other APC types within the medulla. For example, the production of mTEC-derived XCL1 and CCL19/21 is critical for the proper recruitment and positioning of XCR1$^+$ DCs (135) and CD8α$^+$ cDCs (136), respectively. Thus, mTECs represent a key source of multiple factors that are required for Treg cell development, including antigenic peptide, MHC-II, accessory signals, and chemokines. With this in mind, do mTECs function by directly presenting pMHC-II ligands to developing thymocytes, or are most mTEC-expressed proteins or pMHC-II complexes transferred to neighboring APCs, which then orchestrate Treg cell selection?

ORIGIN OF THYMIC SIRPα$^+$ cDCs

For major DC subsets in the thymus, Sirpα$^+$ cDCs are commonly referred to as migratory DCs. This terminology has major implications, because it suggests that such cells originate in the periphery and have the potential to transport peripheral antigens to the thymus. However, additional evidence is needed to better understand the origin of thymic Sirpα$^+$ cDCs. The migratory terminology is largely based on two types of experiments (196): (*a*) In mice in which the circulatory systems were joined for three weeks, ~8% of thymic CD11c$^+$ cells in one mouse represented cells derived from the partner mouse, and these cells were largely Sirpα$^+$ cDCs. While one potential conclusion is that mature partner-derived DCs homed to the thymus via the circulation, it is also possible that blood-borne DC precursors were exchanged, rather than mature DCs. (*b*) Following the intravenous injection of 20 million nucleated blood cells (of undefined DC composition), ~1% of the cDCs recovered from the recipient thymus were of donor origin. However, this may also be a result of the transfer of DC progenitors. Moreover, it is unclear whether injection of a large bolus of cells mimics the natural migratory patterns of blood-borne DCs. Moving forward, advanced lineage-tracing techniques will be needed to address this question.

Addressing this question has been challenging from a technical perspective. Initial work utilized mice in which components of the MHC-II presentation pathway were selectively silenced in mTECs (41). Studies using these mice revealed that a minor fraction of Treg cell specificities were lost or significantly reduced in frequency (38), suggesting that direct antigen presentation by mTECs is crucial for the development of some Treg cell specificities. In contrast, studies using model antigens expressed under the dictates of the *Aire* promoter suggested that clonal deletion, not Treg cell differentiation, is the most common outcome of direct MHC-II-restricted peptide presentation by mTECs (41, 137), complicating interpretation. As discussed above, the knockdown of MHC-II expression by mTECs is expected to impact both direct presentation by mTECs and pMHC-II transfer to neighboring APCs, making it difficult to distinguish between these alternate pathways.

More recent studies have characterized the developmental requirements coordinating the thymic differentiation of individual Aire-dependent Treg cell–biased clones. In one study, Perry et al. (38) demonstrated that some Aire-dependent Treg cell clones failed to develop in *Batf3*$^{-/-}$ hosts, which exhibit a major reduction in Batf3-lineage CD8α$^+$ cDCs (138). In contrast, Leventhal et al. (76) showed that the differentiation of two Aire-dependent Treg cell clones was not impacted by Batf3 deficiency and that the polyclonal thymic Treg cell repertoire was largely unaltered in *Batf3*$^{-/-}$ mice. Interestingly, the selection of one Aire-dependent Treg cell clone required MHC-II expression by DCs, implying that the selection process was dependent on both mTEC-derived antigen and DC-expressed MHC-II. Thus, while Batf3-lineage CD8α$^+$ cDCs may contribute to the selection of some Treg cell clones, the selection of Aire-dependent Treg cells does not appear to be a major nonredundant function of CD8α$^+$ cDCs.

In addition to CD8α$^+$ cDCs, Sirpα$^+$ cDCs are the second major class of cDCs found in the thymus. As described in the sidebar titled Origin of Thymic Sirpα$^+$ cDCs, these cells are commonly referred to as migratory DCs, although the precise origins of these cells remain incompletely defined. To date, the role of Sirpα$^+$ cDCs in promoting Treg cell development in vivo remains largely undefined due to the lack of experimental approaches to constitutively or inducibly ablate these cells. Nonetheless, some circumstantial evidence is consistent with the possibility that Sirpα$^+$ cDCs may play a prominent role in Treg cell differentiation. For example, Sirpα$^+$ cDCs represent the thymic counterpart of peripheral Batf3-independent CD11b$^+$ cDCs, which are thought to be

ANALYSIS OF TREG CELL DEVELOPMENT: TCR REPERTOIRE DIVERSITY

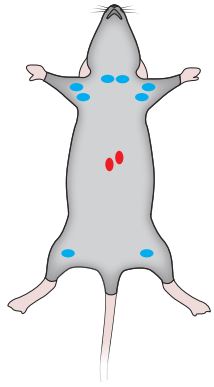
In experiments assessing Treg cell development, the first line of analysis typically centers on quantification of Treg cell frequency and absolute number at various anatomical sites. However, Treg cells can undergo compensatory expansion or contraction in distinct settings, and the Treg cell repertoire represents a diverse array of specificities, including Aire-dependent and Aire-independent clones. Thus, a comprehensive analysis of Treg cell selection should include approaches to assess the TCR repertoire diversity of Treg cell populations. As a case in point, early studies noted that the frequency and absolute numbers of Treg cells were not significantly diminished in the periphery of Aire-deficient mice (133). However, subsequent TCR repertoire profiling of Treg cells in the thymus (38) and periphery (37) revealed that Aire deficiency is associated with substantial shifts in the Treg TCR repertoire, with some specificities lost from the Treg cell compartment and others diverted into the Tconv subset.

more efficient than Batf3-lineage CD8α$^+$ cDCs at presenting MHC-II-restricted antigens (139). Additionally, two studies report that the loss of CD8α$^+$ cDCs in *Batf3*$^{-/-}$ mice is associated with an expansion of both Sirpα$^+$ cDCs and absolute Treg cell numbers (but not clonal diversity) within the thymus (76, 136), suggesting a potential direct relationship between these populations. Studies such as these highlight the importance of analyzing TCR repertoire complexity when studying Treg cell development, discussed in the sidebar titled Analysis of Treg Cell Development: TCR Repertoire Diversity.

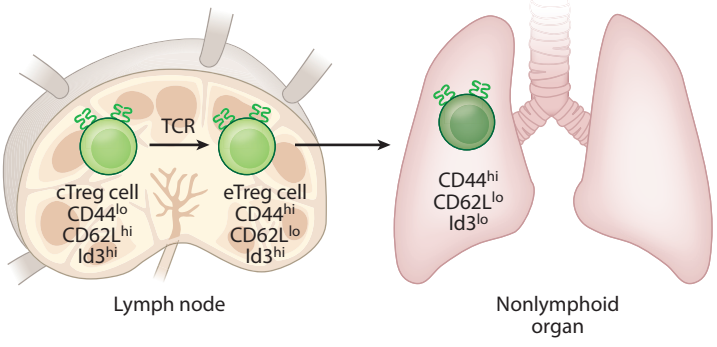
Thus, current evidence suggests that mTECs and DCs collaborate to generate a replete thymus-derived Treg cell repertoire of appropriate diversity (**Figure 1**), comprising a mixture of Aire-independent and Aire-dependent specificities. Thus far, a major requirement for a distinct bone marrow–derived APC subset has yet to be defined, suggestive of broad functional redundancy. Moving forward, addressing this question with higher resolution will require the development of new approaches to constitutively or conditionally deplete distinct APC subsets in the thymus, paired with new ways to conditionally delete MHC-II or costimulatory ligands on different APC populations. In addition, improved approaches are needed to define the positioning, motility, origin, and half-life of key APC populations within the thymus, as well as the mechanisms by which antigenic proteins and pMHC-II complexes are transferred from one cell to another.

Notably, there is substantial evidence that the recognition of self-ligands in the periphery drives additional Treg cell selection and differentiation at extrathymic sites that are critical for Treg cell localization and function (**Figure 4**). In this regard, an expanding body of evidence demonstrates that Treg cells serve diverse peripheral functions that are independent of the suppression of autoimmunity. These functions are extensively reviewed elsewhere (140–142) and include the promotion of tissue repair (1, 2), the stimulation of hair follicle stem cells (4), the modulation of tissue metabolism (3), and the regulation of type 1 inflammation (143, 144). Moving forward, a key goal lies in identifying how this functional heterogeneity is imparted and orchestrated in the periphery, as such knowledge may be key in harnessing these facets of Treg biology for clinical benefit. Specifically, what are the signals that coordinate the acquisition of distinct functional states, and are these fates specified in the thymus, secondary lymphoid organs, or nonlymphoid tissues? Initial insight into this question came from the recent work of Li et al. (145), who demonstrated that the acquisition of a unique Treg cell signature associated with residency in the visceral adipose tissue (VAT) was first triggered in the spleen but required additional differentiation upon arrival in the VAT.

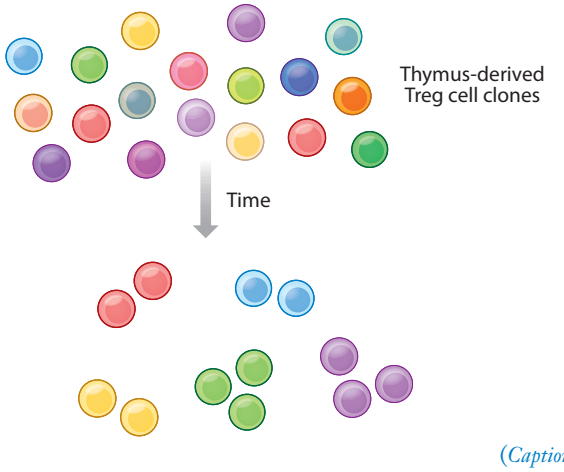
a Asymmetric Treg cell distribution in regional lymph nodes



b Differentiation to an effector Treg cell phenotype



c Selective expansion/retention of distinct Treg cell clones



(*Caption appears on following page*)

Figure 4 (*Figure appears on preceding page*)

Peripheral selection of thymus-derived Treg cells. For Treg cells generated in the thymus, the recognition of self-ligands in the periphery drives additional facets of Treg cell biology. (*a*) Asymmetric Treg cell distribution in regional lymph nodes: The recognition of self-ligands in the periphery drives the asymmetric distribution of Treg cell clones in various regional lymph nodes (199), and the local enrichment of organ-specific Treg cells capable of suppressing organ-specific autoimmunity (76, 119, 200–202). Local enrichment of a given Treg cell clone in a regional lymph node is illustrated in red, with background frequencies in distal lymph nodes indicated in blue. (*b*) Differentiation to an eTreg cell phenotype: Upon arrival in the periphery, tTreg cells exhibit a quiescent $CD44^{lo}CD62L^{hi}Id3^{hi}$ cTreg cell phenotype (203). Over time, a fraction of cTreg cells differentiate into proliferative eTreg cells displaying a $CD44^{hi}CD62L^{lo}Id3^{hi}$ phenotype (203). This differentiation is a TCR-dependent process that is critical for subsequent downstream regulatory functions (76, 204, 205). eTreg cells are enriched for the capacity to populate nonlymphoid organs, a process that is associated with the downregulation of the transcription factor Id3 (206). (*c*) Selective expansion/retention of distinct Treg cell clones: We hypothesize that over time, eTreg cell differentiation drives the selective expansion or retention of those tTreg cell clones (denoted in various colors) that actively engage self-ligands, and they are therefore the most useful for immune regulation. This would predict that over time, the peripheral Treg cell repertoire would be characterized by clonal expansion of select clones and an overall reduction in clonal diversity. In addition, we hypothesize that sustained recognition of self-ligand in the periphery may drive affinity selection of the pool of tTreg cells reactive to a given self-pMHC-II ligand, leading to enrichment of clones expressing TCRs with optimal ligand-binding properties. Abbreviations: cTreg, central Treg; pMHC, peptide/MHC; eTreg, effector Treg; TCR, T cell antigen receptor; tTreg, thymus-derived Treg.

SEEKING STABILITY: THE TREG CELL EPIGENETIC PROGRAM

Given that Foxp3 expression defines Treg cells and is critical for their function, and that perturbations in Treg cell differentiation can lead to autoimmunity, it is of considerable interest to define the stability of Treg cells in health and disease. An initial report using mice in which Foxp3-expressing cells could be genetically fate mapped using a YFP reporter revealed the existence of $Foxp3^-$ T cells that had previously expressed Foxp3 (146), suggesting that Treg cells may exhibit some degree of instability. However, it remained unclear whether these cells were bona fide Treg cells that had lost expression of Foxp3, or cells that had transiently transcribed from the $Foxp3^{Cre}$ locus driving the genetic fate mapping. The notion of Treg instability was further supported by reports using cell transfer experiments, which suggested that some Treg cells can lose Foxp3 expression and undergo reprogramming to adopt T helper–like functions in distinct contexts (147, 148). In contrast, other studies in which Treg cells could be genetically labeled in adult mice and subsequently tracked revealed that Treg cells exhibited a high degree of stability at steady state and in different inflammatory contexts (149). A potential explanation for these disparate findings came from subsequent work that revealed the presence of a minor population of nonregulatory cells that exhibit promiscuous and transient expression of Foxp3, suggesting that the labeling or fractionation of Foxp3-expressing cells at a given point in time may not perfectly distinguish stable $Foxp3^+$ cells from nonregulatory Tconv cells (150).

In-depth studies of the factors regulating Treg cell identity and stability have revealed an important role for epigenetic modifications in the *Foxp3* locus in the establishment of a stable Treg cell program. These concepts have been extensively reviewed in recent literature (151, 152) and are discussed only briefly here. Broadly, it is clear that expression of Foxp3 protein is not sufficient to confer Treg cell identity but must be paired with the appropriate epigenetic landscapes (153). One of the key epigenetic features of Treg cells is a unique signature of demethylated CpGs (154). One Treg cell–specific demethylated CpG region lies within the conserved noncoding sequence 2 (CNS2) element of the *Foxp3* locus. Foxp3 binding to CNS2 is important for establishing stable, heritable Foxp3 expression in Treg cells and depends on demethylation of CNS2 (155). This hypomethylation pattern begins during early stages of thymic development and is critical for

stabilizing the Treg lineage (153, 154, 156, 157) by providing a scaffold for other critical transcription factors (158). Importantly, this DNA demethylation pattern distinguishes bona fide Treg cells from unstable Treg cells that transiently upregulate Foxp3. For example, Treg cells induced in vitro using TGF-β exhibit high expression of Foxp3 protein but lack the Treg cell–specific demethylation signature (154).

PARTING WAYS: DISTINCT NATURE OF THE TREG CELL AND TCONV CELL TCR REPERTOIRES

As alluded to above, TCR profiling using engineered mice with a quasi-diverse TCR repertoire demonstrated that the repertoire of TCRs expressed by Treg cells is diverse and is largely distinct from that of Tconv cells, with a small fraction of shared TCRs (50, 51, 159–161). These data suggest that Treg cells recognize a complex array of antigenic ligands and that Treg cell differentiation is a TCR-instructive process. At least two scenarios can be invoked to explain this bifurcation. First, it is possible that a distinct set of self-pMHC-II ligands drives robust skewing of all antigen-specific cells into the Treg lineage, leading to the observed differences in TCR repertoires. Alternatively, it is possible that for a given self-pMHC-II ligand, unique TCR-pMHC-II binding properties direct some antigen-specific clones into the Treg cell subset, leaving other antigen-specific clones in the Tconv compartment. Studies using pMHC-II tetramers bearing self-peptides have provided key insight into this question, demonstrating that for any given self-pMHC-II complex, the skewing of antigen-specific cells to the Treg cell lineage is incomplete (118, 121–123, 162, 163). Thus, for many endogenous self-pMHC-II ligands, both clonal deletion and skewing to the Treg cell lineage are incomplete, establishing a scenario in which Treg and Tconv cells of matched specificity coexist within the endogenous repertoire. This raises key questions about what prevents the activation of self-specific Tconv cells under homeostatic conditions or during infection, and the role of antigen-specific Treg cells in controlling their Tconv cell counterparts. Another interesting phenomenon is revealed by studies showing that for pMHC-II tetramers bearing any given foreign peptide, the immune repertoire harbors identifiable populations of both Treg cells and Tconv cells, a finding that holds true for both mice and humans, and for innocuous foreign as well as pathogen-derived peptides (86, 87, 163–166). This raises fundamental questions about the extent to which Treg cells reactive to foreign peptides regulate the adaptive immune response and pathogen persistence during infection.

GETTING OUT AND ABOUT: PERIPHERAL TREG CELL DEVELOPMENT

Beyond Treg cell specification in the thymus, it is clear that peripherally induced Treg (pTreg) cells can also differentiate at extrathymic sites. This phenomenon was first evident in studies in which $CD4^+CD25^{hi}$ cells could readily be recovered from lymphopenic mice that received purified $CD4^+CD25^-$ donor T cells by intravenous transfer (8, 167), a phenomenon that was later confirmed by numerous studies using Foxp3 staining to identify pTreg cells. A clear understanding of Treg cell biology requires determining whether pTreg cells serve unique functions in immune regulation that are distinct from those conferred by tTreg cells, and whether dedicated mechanisms exist to coordinate pTreg cell differentiation. As described below, consensus has coalesced around the idea that pTreg cells reactive to environmental antigens, including those derived from commensal microbiota, dietary constituents, and fetal antigens, can play an important role in maintaining immune tolerance and homeostasis at sites of the body that interface with the external environment. Here, we discuss available evidence supporting this idea and highlight key gaps in knowledge moving forward.

Why is it critical to understand the developmental origins of Treg cells, distinguishing tTreg cells from pTreg cells? First, it is likely that the site of origin reflects the nature of antigenic peptides recognized by Treg cells, with the prediction that tTreg cells are likely to recognize self-peptides and pTreg cells are likely to recognize foreign peptides derived from exogenous sources or self-ligands not encountered during thymic development. Second, since pTreg cells develop from Foxp3$^-$ precursors, pTreg cell differentiation may divert CD4$^+$ T cells away from alternate T helper states, thereby profoundly altering the local immune environment. For example, mounting evidence suggests that pTreg cell differentiation in the gut is interrelated and antagonistic with the differentiation of pathogenic Th17 cells (168). Third, pTreg cells have considerable clinical potential, as it is conceivable that antigen-specific pTreg cell differentiation could be induced therapeutically to quell ongoing autoimmune disease or inflammatory disorders. However, in order to do so effectively, it is critical to define the mechanisms coordinating pTreg cell differentiation and the nature of antigens that promote this process.

Building on early work examining the induction of polyclonal pTreg cells in lymphopenic mice, it was shown that CD4$^+$ TCR transgenic T cells reactive to exogenous peptides could be induced to undergo pTreg cell differentiation in wild-type mice using various approaches involving the provision of antigen in the absence of innate signals (169–172). However, it remained unclear whether pTreg cell differentiation is a common process that makes substantial contributions to the mature Treg cell pool at steady state and in contexts of inflammation, infection, and cancer. To address this question, there has been considerable interest in identifying markers that can be used to reliably distinguish Treg cells of thymic and extrathymic origin. Initial reports suggested that the transcription factor Helios (173) and cell surface receptor Neuropilin-1 (174, 175) are uniquely expressed by tTreg cells, but subsequent reports demonstrated that these markers can also be expressed by pTreg cells or in vitro–differentiated Treg cells in some instances (176–180). Thus, the lack of reliable markers of thymic or peripheral differentiation has restricted progress in understanding the origin of Treg cells in different contexts, necessitating reductionist studies in which the developmental potential of distinct Treg cell specificities can be assessed at the clonal level (54, 117).

Given the lack of faithful markers of tTreg and pTreg cells, a report from Petzold et al. (177) was noteworthy in that it provided the first quantitative assessment of the relative frequencies of pTreg cells and tTreg cells in the peripheral Treg cell pool of healthy mice. To do this, the investigators leveraged the serendipitous finding that two distinct Foxp3 reporter mice exhibited differential patterns of expression on tTreg cells and pTreg cells. Specifically, it was found that a Foxp3 reporter inserted in the endogenous *Foxp3* locus was faithfully expressed by both tTreg cells and pTreg cells, whereas a second Foxp3 reporter driven from a BAC transgene was exclusively expressed by tTreg cells. Analysis of mice expressing both reporters revealed that pTreg cells comprised 15–25% of peripheral Treg cells in the spleen and lymph nodes of two-month-old mice, and that this fraction increased to an average of 35% in aged mice. Notably, the pTreg cells defined using the double-reporter system exhibited variable expression of both Helios and Neuropilin-1. Moving forward, this double-reporter approach could be used to define the phenotype, transcriptional programs, and functions of tTreg cells and pTreg cells in different immune contexts.

Despite substantial evidence that pTreg cells are prevalent in the peripheral repertoire, it remained unclear whether pTreg cells serve unique functions that cannot be conferred by tTreg cells. The understanding of pTreg cell function was significantly advanced by the identification of a conserved noncoding TGF-β/Smad response element in the *Foxp3* locus, named conserved noncoding sequence 1 (CNS1), that is required for optimal pTreg cell differentiation (155, 181, 182). Using gene-targeted *Foxp3*$^{\Delta CNS1}$ mice lacking this element, Rudensky and colleagues demonstrated that polyclonal and antigen-specific T cells from CNS1-mutant mice exhibit deficiencies

in pTreg cell induction, develop aberrant type 2 immune responses in the lung and gastrointestinal tract, and have increased absorption of allogeneic embryos during pregnancy (155, 182, 183). In parallel work, Schlenner et al. (181) showed that mice harboring a different targeted CNS1-mutant allele exhibited milder phenotypes than *Foxp3*$^{\Delta CNS1}$ mice, including the lack of unprovoked inflammation at mucosal sites and unaltered susceptibility to experimental colitis. The reasons for the observed differences in the two CNS1-mutant mice are unknown, but they could be due to variations in microbiota or the genomic elements that were targeted for deletion. Nonetheless, studies using CNS1-deficient mice provided key evidence that pTreg cells serve critical nonredundant modulatory functions at anatomical sites that interface with the external environment. It should be noted the CNS1-mutant mice do not exhibit widespread organ-specific autoimmunity or immune activation throughout the body, indicating that tTreg cells are sufficient to maintain most aspects of tolerance and immune homeostasis. Consistent with this, a recent study from Holohan et al. (184) examined CNS1 deficiency on a NOD background and revealed no changes in diabetes incidence and time of onset.

As alluded to above, much of what is known about pTreg cell differentiation and function stems from the study of pTreg cells in tissues associated with the gastrointestinal tract. Definitive demonstration that naturally occurring pTreg cells populate the endogenous repertoire and exhibit specificity for microbial peptides came from a landmark study by Lathrop et al. (185). This study demonstrated that the TCR repertoire of colonic Treg cells is distinct from that of Treg cells at other lymphoid sites, suggestive of reactivity to a unique set of antigens within the colon. Clonal analysis revealed that multiple colonic Treg cell TCRs conferred reactivity to unidentified peptide antigens derived from commensal bacteria, and that such TCRs facilitated microbiota-dependent pTreg cell differentiation, with no evidence of tTreg cell differentiation. These studies provided direct evidence that some colonic Treg cells exhibit reactivity to foreign peptides, and undergo extrathymic differentiation, providing solid experimental support for basic concepts of pTreg cell differentiation and specificity. Subsequent work from this group and others has expanded the understanding of commensal microbiota that can promote pTreg cell induction, as well as the mechanisms regulating pTreg cell differentiation (168, 186–189). Building from these findings, a consensus has emerged suggesting that Treg cells in the gastrointestinal tract represent a composite of ROR-γt-expressing pTreg cells reactive to microbe-derived peptides, together with self-specific tTreg cells reactive to self-ligands (185, 190–192).

Multiple studies have identified peripheral Foxp3^{-}CD4^{+} T cell populations that exhibit a propensity to differentiate into pTreg cells, including CD25^{+}Foxp3^{-} cells (193), recent thymic emigrants exhibiting low-density expression of Qa-2 (194), and FR4^{hi}CD73^{hi} cells (195), suggesting that the peripheral repertoire harbors precursor cells that are poised to differentiate into pTreg cells under distinct conditions. Moving forward, it will be interesting to determine whether the pTreg cell precursor populations described above are a single common T cell subset, and whether the principles observed in lymphopenic mice are also operative in wild-type lymphoreplete settings. Lastly, it is important to consider why the immune system would generate a peripheral reservoir of pTreg cell precursors, rather than generating mature pTreg cells. In this regard, it is possible that the identified populations simply represent transitional intermediates that have already been triggered to undergo Treg cell differentiation but have yet to adopt the full phenotype of mature Treg cells.

In humans, the role of pTreg cells in health and disease has been challenging to define. A key advance came from the work of Bacher et al. (164), who leveraged an assay termed antigen-reactive T cell enrichment (ARTE), which enables the enumeration and phenotyping of rare antigen-specific Treg cells and conventional T cells in human blood, regardless of HLA type. Using this approach, it was demonstrated that all human subjects have measurable frequencies of CD4^{+}

T cells reactive to common aeroantigens and that responses are uniformly skewed to the Treg lineage. While the thymic or peripheral origin of these Treg cells could not be assessed, the fact that these cells were reactive to environmental antigens is suggestive of a peripheral origin of these Treg cells.

The collective evidence discussed above demonstrates that pTreg cells generated extrathymically make unique contributions to immune regulation and can target peptides derived from environmental antigens. However, critical gaps in knowledge remain regarding pTreg cell differentiation and function. First, what are the molecular and cellular mechanisms coordinating pTreg cell differentiation, and what distinguishes pTreg cell induction from alternate $CD4^+$ T cell fates, such as ignorance or differentiation to IL-17-producing cells? Second, what is the nature of environmental constituents that trigger pTreg cell differentiation? Why do some constituents promote pTreg cell development, whereas others do not? Third, are pTreg cells maintained for long periods following induction, and does maintenance require the continued presence of cognate antigen? These questions are especially pertinent when considering that the composition of commensal microbiota, dietary constituents, and additional environmental antigens will change throughout life and have the potential to yield an array of peptides that dwarfs the number of Treg cells that can populate a given anatomical site.

CONCLUSION

Vertical strides have been made in identifying the molecular signals that trigger Treg cell development and homeostasis, as well as the internal wiring conferring Treg cell identity and fitness. However, it is evident from the discussions above that the precise molecular and cellular blueprints of Treg cell selection remain inadequately defined. Moving forward, as outlined in the section titled Future Issues, it will be critical to develop new approaches to understand the positioning, motility, origins, and lifespan of thymic APC populations, as well as their functional contributions to Treg cell differentiation and other fate decisions. In addition, the identification and study of other endogenous Treg cell ligands will enable in-depth studies of the developmental trajectories of naturally occurring Treg cell clones. Ultimately, a more complete understanding of Treg cell development in mice will enable interrogation of similar principles in human Treg cell development.

FUTURE ISSUES

1. For Treg cell–biased specificities, does clonal deletion occur coincidently with Treg cell selection?
2. The development of new approaches for the inducible ablation of distinct thymic APC subsets will enable new insights into Treg cell selection.
3. To test the hypothesis that Treg cells follow one of two distinct pathways during differentiation, a clonal analysis is needed to confirm that distinct clones reproducibly follow one path or the other.
4. Advanced imaging approaches are needed to characterize the development of naturally occurring Treg cell clones.
5. How are different APC types positioned in the thymic medulla, and what are their motility and half-life?

6. What are the mechanisms of antigen transfer between different cell types in the thymus?
7. What establishes the chromatin landscape on which Aire acts?
8. Does recognition of cognate pMHC-II by Treg cells in the periphery further impact their selection, maintenance, and function?

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