REVIEW

Spatiotemporal regulation of peripheral T cell tolerance

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The incomplete removal of T cells that are reactive against self-proteins during their differentiation in the thymus requires mechanisms of tolerance that prevent their effector function within the periphery. A further challenge is imposed by the need to establish tolerance to the holobiont self, which comprises a highly complex community of commensal microorganisms. Here, we review recent advances in the investigation of peripheral T cell tolerance, focusing on new insights into mechanisms of tolerance to the gut microbiota, including tolerogenic antigen-presenting cell types and immunomodulatory lymphocytes, and their layered ontogeny that underlies developmental windows for establishing intestinal tolerance. While emphasizing the intestine as a model tissue for studying peripheral T cell tolerance, we highlight overlapping and distinct pathways that underlie tolerance to self-antigens versus commensal antigens within a broader framework for immune tolerance.

Recognition of "altered self"—a fundamental evolutionary innovation of the vertebrate immune system—is based on the binding of T cell antigen receptors (TCRs) to foreign peptides displayed on self–major histocompatibility complex (MHC) molecules. An essential property of this sensing system is the ability to recognize and respond to a diverse array of rapidly evolving intra- and extracellular pathogens while preventing potentially fatal responses against peptides derived from self-proteins. The efficiency of this response against pathogens—afforded by an immense repertoire of clonally distributed TCRs, randomly generated through somatic recombination in developing T cells—demands an equally effective solution for preventing T cell responses directed against both self-antigens and harmless foreign antigens. Tolerance to self-antigens is first established within the thymus, the site where T cells are generated. Thymic (central) tolerance operates as a "first-pass" system, removing self-reactive T cells from the conventional T (T_{conv}) cell repertoire or directing their differentiation into functionally unresponsive cell states or specialized immunomodulatory lineages before their egress into the periphery. Here, the conundrum is one of representation—how to ensure that the entire spectrum of self-antigens, including developmentally restricted or inducible antigens, is displayed to developing thymocytes, a prerequisite for efficient establishment of tolerance. However, negative selection of self-reactive T cells is far from a complete process, with frequent self-specific T cells observed in the periphery of healthy individuals or experimental animals, which, under certain conditions, can be mobilized to mount autoimmune responses (*1*). The incomplete deletion of self-reactive T cells or their diversion to immunomodulatory regulatory T cell (T_{reg}) or intraepithelial lymphocyte (IEL) lineages necessitates peripheral mechanisms that restrain the activation of self-reactive T cells upon encounter with cognate self-antigen.

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Once outside of the thymus, naïve T cells encounter antigens derived from innocuous sources, including commensal microorganisms, dietary antigens, and, during pregnancy, paternal fetal antigens. Of these encounters, the ability to discriminate "harmless" commensal microbes from pathogens, given antigenic overlap, remains one of the most notable feats of the immune system. A breakdown in peripheral tolerance to commensal antigens is thought to underlie the pathogenesis of a broad range of atopic and inflammatory diseases. This notion is supported by a recent report that recurrent TCRs restricted by HLA-B27—an MHC allele with the strongest genetic association with human skeletal and oc… autoinflammatory disorders—are capabl… recognizing peptide motifs shared by commensal microbial antigens and self-antigens and that these T cell clones are expanded at sites of autoimmune inflammation (*2*).

Despite clear parallels between mechanisms of central and peripheral tolerance, each organ poses a distinctive set of challenges with different diversity and abundance of tissue-specific self-antigens and commensal and environmental antigens. Given the tools that exist for the study of peripheral tolerance to gut microbes, including genetically engineered gut commensals as well as the ability to isolate and experimentally manipulate gut microbe–induced T_{regs}, the intestine has proved a fertile area for elucidating mechanisms of peripheral T cell tolerance, as highlighted by the recent discovery of a lineage of tolerogenic RORγt$^+$ antigen-presenting cells (APCs) and their role in instructing intestinal T_{reg} differentiation (*3*–*5*). The extent to which these mechanisms are conserved across tissue sites, as well as between different classes of antigens, remains to be established, however. Within the intestine, first encounters with dietary and commensal antigens occur during defined developmental windows such as the introduction of food proteins during weaning and ordered temporal patterns of microbial colonization. Investigations of peripheral tolerance during this early-life period have yielded insights into its basic mechanisms, which may be harnessed to restore immune balance later in life in the event of a breakdown in immune tolerance.

Here, we review recent advances in investigations of mechanisms of peripheral tolerance. In our efforts to provide an integrated view of this complex network of immunoregulatory cells and molecules, we discuss shared and distinct mechanisms for establishing tolerance to self-antigens versus foreign antigens, with a particular focus on pathways of tolerance to the gut microbiota. While emphasizing the intestine as a model organ for studying peripheral T cell tolerance (Fig. 1), we highlight the importance of particular tissue environments and the developmental stage of the organism in determining tolerance.

Peripheral regulation of T cell reactivity

Mechanisms of peripheral T cell tolerance are traditionally classified as cell intrinsic and cell extrinsic. The former encompass processes of apoptosis and consequent removal of particular T cell clones from the repertoire, induction of unresponsiveness (anergy), or loss of their capacity for inflammatory effector response upon TCR ligation (*6*). Extrinsic mechanisms involve the restraint of T cell responses by specialized immunoregulatory cell types such as T_{regs} or tolerogenic APCs. These distinctions are somewhat blurred because intrinsic mechanisms

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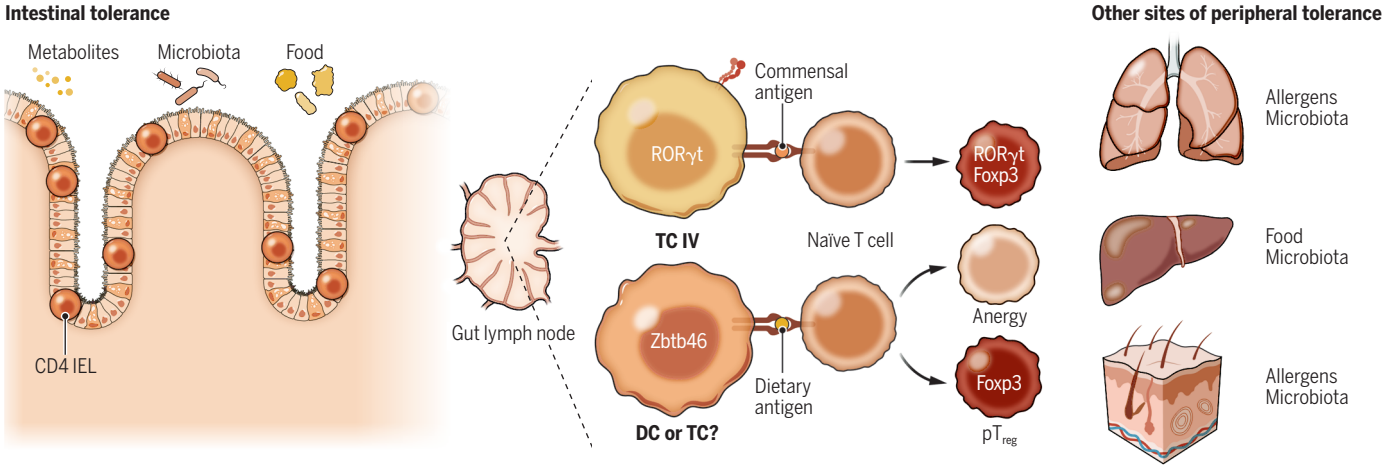

Fig. 1. The intestine as a model tissue for investigation of peripheral tolerance. Identification of the mechanisms underlying intestinal pT_{reg} generation in response to commensal and dietary antigens, including newly characterized tolerogenic APCs, provides a framework for investigation of peripheral tolerance at other tissue sites. Outside of the intestine, it is not clear whether distinct APCs and niche factors, including microbial products, regulate pT_{reg} differentiation, or whether commensal-specific T_{regs} within the skin, lung, and liver are also distinguished by expression of RORγt.

involve signaling through inhibitory receptors that raise the threshold of T cell activation downstream of TCR signaling, rendering T cells sensitive to extrinsic regulation by cell types that express corresponding ligands.

Modulation of T cell reactivity appears to be a major factor that prevents activation of peripheral self-reactive "escapees," at least at steady state. In healthy humans, circulating self-reactive CD8 T cells, although common, are hyporesponsive to ex vivo stimulation with cognate antigen (*7*). Similarly, in mice, peripheral $CD4^+$ T cells specific for a fetal or tissue-restricted self-antigen are characterized by the expression of folate receptor 4 (FR4) and CD73, two hallmarks of anergy (*8*, *9*). These cells are also prone to give rise to T_{regs} upon self-antigen challenge (*8*), suggesting a continuum of cell states that shift from intrinsic to extrinsic mechanisms of T cell tolerance. The extent to which T cell anergy is imprinted in the thymus versus periphery and whether continuous regulation is required to maintain anergy once instructed [e.g., as seen in the tumor microenvironment (*10*)] remain to be elucidated. Beyond tolerance to self-antigens, a recent study of CD4 T cell responses to oral antigens, including dietary protein gliadin (wheat), revealed a dominant population of cells that display overlapping transcriptional and phenotypic features of naïve T cells (*11*). These naïve-like T cells also exhibit hallmark features of anergy, with hyporesponsiveness to systemic immunization as well as increased propensity for peripheral T_{reg} differentiation upon continuous oral antigen exposure. In contrast to the thymus, where clonal elimination is a major mechanism of T cell tolerance, the contribution of deletion to peripheral tolerance is less clear. In this regard, one study that used tissue-restricted promoters to drive expression of Cre-recombinase peptide in the periphery reported that the numbers of Cre-specific CD4 T cells were not impaired, suggesting that deletional tolerance is minimal for antigens that are not expressed within the thymus (*12*). Similar to the thymus, where the strength of TCR signaling is a major determinant of clonal deletion versus diversion to alternative cell fates, it is likely that peripheral deletion may only remove self-reactive T cell clones with the highest-avidity TCRs that happen to escape thymic selection unscathed.

The TCR-dependent activation and differentiation potential of self-reactive peripheral T cells is modulated foremost by the prototypical costimulatory receptor CD28 and coinhibitory receptors programmed cell death 1 (PD-1) and cytotoxic T lymphocyte–associated protein 4 (CTLA-4). CTLA-4 and CD28 converge on two shared ligands, CD80 and CD86, that are expressed by APCs. In addition to competing with CD28 for binding of these ligands and potentially inducing a yet-to-be-defined inhibitory signaling cascade, CTLA-4 on T_{regs} is capable of removing CD80 and CD86 from APCs (*13*–*15*). Induction of PD-1 and CTLA-4 expression on T cells downstream of TCR signaling, coupled with dynamic changes in CD80, CD86, and PD-L1 expression after APC activation, suggests finely balanced signals between these interacting cellular and molecular partners that allow tight temporal and spatial control of T cell self-reactivity and responsiveness and complicate the assignment of an immunostimulatory or immunoinhibitory attribution to APCs based on static analyses of a few select cell-surface molecules. Antibody-mediated CTLA-4, PD-1, or PD-L1 (the ligand of PD-1) blockade, a cornerstone of cancer immunotherapy, has provided strong support for a major role of these pathways in peripheral tolerance in humans. Severe immune-related adverse events (iRAEs) that affect barrier tissues (skin, gut, and lung) and other organs are common in patients treated with PD-1– and CTLA-4–blocking antibodies (*16*). iRAEs seem to represent a breakdown in peripheral tolerance that is due to loss of PD-1–mediated restraint of self-reactive T cells. Cytotoxic CD8 T cells specific for α-myosin heavy chain, for example, are associated with myocarditis observed in $Pdcd1^{-/-}Ctla4^{+/-}$ mice and patients treated with a combination of anti–CTLA-4 and anti–PD-1 antibodies (*17*). Given the preponderance of iRAEs at microbiota-colonized barrier tissues, one would likewise expect a breakdown in tolerance to commensal and dietary antigens. Consistent with this possibility, patients with checkpoint-induced colitis show expansion of colonic lamina propria effector CD8 T cells that are clonally related to tissue-resident memory T cells of an unelucidated specificity (*18*).

Sustained stimulation of antigen-specific CD8 T cells in chronic viral infections and tumors has been linked to a terminal differentiation state termed "exhaustion," which is defined by high amounts of PD-1 expression, certain metabolic changes, and a progressive loss of inflammatory effector cytokine production and proliferative potential (*19*). The term exhaustion has negative connotations, suggesting a loss of functionality rather than a beneficial adaptation that serves to restrain inflammatory T cell responses and protect the host from excessive tissue damage. Through this lens, it is reasonable to consider that sustained antigenic stimulation of T cells—a common feature of infection, autoimmunity, and cancer—could promote their transition from inflammatory effectors into a range of noninflammatory, potentially tissue-supporting, or even immunoregulatory states. In support

of this notion, a recent study demonstrated that in addition to the loss of effector functions, exhausted T cells gain transcriptional and metabolic features of T_{regs}, including the ability to mediate local suppression of CD8 T cell responses by means of CD39-driven extracellular adenosine production (*20*).

A division in labor: Thymic versus peripheral T_{regs}

Foxp3$^+$ T_{regs} represent the principal immunosuppressive arm of the adaptive immune system. Although other T cell subsets—such as Tr1, CD4 IEL, Qa-1–restricted, or KIR$^+$ CD8 T cells (*21*–*24*)—have also been implicated in immune regulation, we limit this section discussion to T_{regs}, whose paramount importance in peripheral tolerance is demonstrated by the early-onset fatal autoimmunity in mice and humans that results from Foxp3 loss of function. A life-long requirement for T_{regs} in the maintenance of peripheral tolerance comes from the similarly aggressive and fatal disease observed not only in microbiota-sufficient but also adult germ-free *Foxp3^{DTR}* mice subjected to sustained ablation of T_{regs} (*25*, *26*). T_{regs} use a variety of effector mechanisms to sustain peripheral tolerance, including direct targeting of activated T cells as well as indirect effects through modulation of APCs, which have been comprehensively reviewed elsewhere (*25*).

Transcriptional and functional heterogeneity has been extensively described among T_{regs}. Arguably the greatest functional division can be attributed to the dichotomy between thymically derived T_{regs} (tT_{regs}) and those derived extrathymically in the periphery (pT_{regs}). The mechanistic studies of pT_{regs} and their origins, transcriptional regulation, and antigenic specificities have revealed distinct facets of tolerance to self-antigens and foreign antigens. tT_{regs}, by virtue of their agonist-driven selection supported by Aire-expressing medullary thymic epithelial cells (mTECs), are enriched for reactivity to self-antigens. Mice deficient in Aire or Aire$^+$ mTECs exhibit decreased T_{reg} numbers in the thymus within the first few weeks of life (*27*, *28*) and exhibit a shift in their tT_{reg} TCR repertoire owing to the retention of T_{reg}-associated tissue antigen–specific TCRs within the CD4 T_{conv} cell population (*28*, *29*). The ensuing autoimmune inflammation, despite normal numbers of T_{regs} within the periphery, suggests that the loss of specific tissue antigen–reactive tT_{reg} clones leads to insufficient control of their self-reactive T_{conv} cell counterparts. Indeed, multiple lines of evidence support the concept of "linked suppression," whereby tT_{regs} most efficiently suppress T_{conv} cells with shared antigenic or tissue specificity (*25*, *30*). Although, at present, no tool exists that allows specific ablation of tT_{regs}, the severe autoimmunity and inflammatory disease in germ-free mice that develops after ablation of Foxp3$^+$ cells, similar to that seen in microbiota-sufficient mice, provides clear evidence that tT_{regs} regulate peripheral tolerance to self (*26*).

Following the discovery that naïve T cells can up-regulate Foxp3 upon activation under particular circumstances to give rise to pT_{regs}, multiple studies have shown that pT_{regs} appear to largely develop in response to commensal microbiota and dietary antigens. Although there is a paucity of available markers to universally distinguish pT_{regs} and tT_{regs}, recent genetic lineage tracing studies of Foxp3$^+$ cells arising de novo within the periphery demonstrated that the differentiation of pT_{regs} can be triggered by microbial antigens in colon-draining lymph nodes and in the colonic lamina propria (*31*). Moreover, colonic pT_{regs} are marked by the expression of orphan nuclear receptor RORγt. Consistent with the role of microbial antigens in driving pT_{reg} generation, germ-free mice are largely devoid of intestinal RORγt$^+$ pT_{regs}, which can be readily induced upon microbial colonization (*32*). Furthermore, naïve CD4 T cells specific for defined commensal antigens give rise to RORγt$^+$ T_{regs} upon adoptive transfer into recipients colonized with the corresponding commensals (*33*–*35*). Thus, RORγt$^+$ pT_{regs} have become synonymous with gut microbe–induced pT_{regs} and represent the dominant Foxp3$^+$ population within the intestine. These microbe-specific pT_{regs} can further differentiate into immunomodulatory CD4 IELs (*24*, *36*), highlighting the importance of the tissue site in determining the mode of immunoregulation.

In keeping with their proven specificity for commensal antigens, RORγt$^+$ pT_{regs} have nonredundant roles in tolerance to commensal microbes, as evidenced by the onset of microbiota-dependent colitis in mice with genetic pT_{reg} deficiencies (*31*, *34*) or disruption of MHC class II (MHCII) antigen presentation on pT_{reg}-inducing APCs (*3*–*5*). Loss of pT_{regs} leads to a reciprocal increase in inflammatory T helper 2 (T_H2) and T_H17 cells and increased recruitment of neutrophils and monocytes, which is thought to underlie the ensuing intestinal inflammation (*3*, *31*, *32*, *37*, *38*). Beyond regulation of proinflammatory T cells, genetic or induced pT_{reg} deficiency leads to mastocytosis (*31*, *39*) as well as an increase in intestinal immunoglobulin A–positive (IgA$^+$) plasma cells (*38*). Within the small intestine and associated lymph nodes, differentiation of pT_{regs} is induced by food antigens (*11*, *40*). Mice deficient in intestinal pT_{regs} are more susceptible to allergic and inflammatory responses to oral antigens (*40*, *41*), further supporting the notion of a functional specialization of pT_{regs} in peripheral tolerance to microbe and food antigens. Although largely studied within the intestine, RORγt$^+$ T_{regs} have also been observed in lymph nodes that drain skin and other sites besides intestine, albeit at lower frequencies. At these sites, RORγt$^+$Foxp3$^+$ cells may represent pT_{regs} generated in response to the local microbial community or colonic pT_{regs}

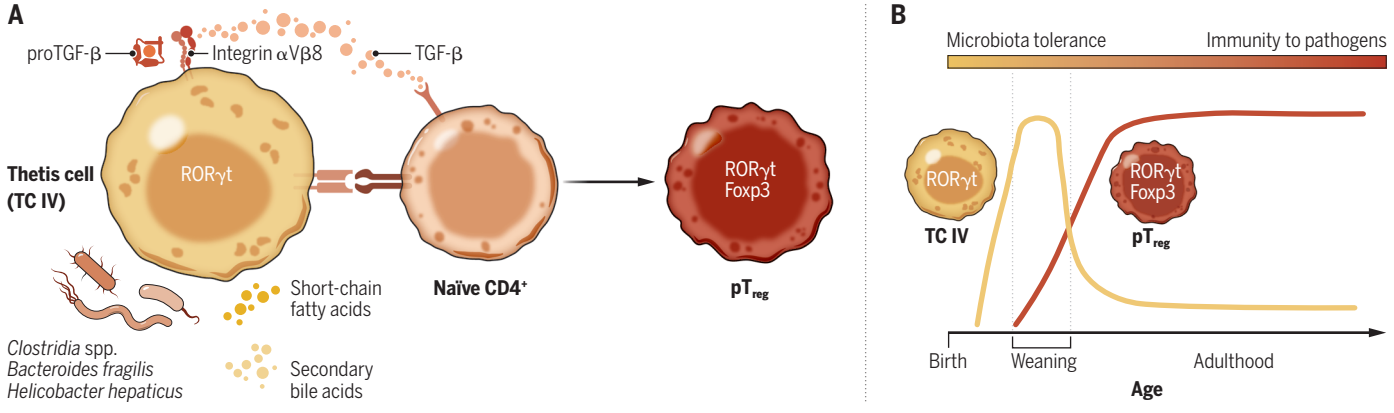

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Fig. 2. Factors that promote pT_{reg} generation. (**A**) Within the intestine, commensal-specific pT_{regs} express RORγt and are instructed by a RORγt$^+$ APC (TC IV) in an ITGB8-dependent manner. A range of factors, including microbial antigens and microbial or dietary metabolites, promote pT_{reg} differentiation. (**B**) A developmental wave of TCs precedes the development of pT_{regs} upon weaning from breast milk to solid food.

that have migrated from the gut. Alternatively, RORγt may be up-regulated upon tT_{reg} activation in the presence of interleukin-6 (IL-6) and transforming growth factor–β (TGF-β) in a manner similar to that which occurs in T_H17 cells. Direct evidence supporting the migration of gut pT_{regs} comes from a recent study in which microbiota-dependent pT_{regs} generated in the gut were shown to migrate to distal sites, such as injured muscle, where they exerted immunoregulatory functions (*42*). Considering the major role that TCR specificity plays in the spatial positioning and function of T_{regs}, there are several possible scenarios for commensal-specific pT_{reg} contribution to tissue tolerance, including cross-reactivity with tissue antigens (*2*), migration of intestinal APCs bearing commensal antigens to distal sites, or low levels of colonization by translocation-competent commensals.

Although a clear role for the microbiota in pT_{reg} differentiation has been firmly established, the exact microbe-dependent signals that drive pT_{reg} differentiation upon antigen recognition are not fully known (Fig. 2). In contrast to tT_{reg} differentiation, where a direct role of TGF-β is still debatable (*43*), pT_{reg} differentiation is dependent on integrin beta 8 (ITGB8)–mediated TGF-β activation and signaling (*3*, *4*, *43*). Beyond specific examples of commensal-mediated modulation of TGF-β (*44*, *45*), a more general role for microbe-directed pT_{reg} differentiation by means of TGF-β signaling has not been established. A multitude of known and still-unidentified factors, some directly linked to microbiota, have been suggested to regulate pT_{reg} differentiation, including retinoic acid, butyrate, and secondary bile acids (*46*–*53*). How these local cues influence expression and activation of RORγt and other transcription factors implicated in microbiota-dependent differentiation of pT_{regs}, including c-Maf (*34*), remains to be determined. These transcription factors contribute to the distinctive pT_{reg} biology, which encompasses both Foxp3-dependent and -independent functions, including (for the latter) the stable expression of IL-10 (*31*, *54*). The recent finding that pT_{regs} are only partially dependent on Foxp3 for their immunosuppressive functions (*31*) sets them apart from tT_{regs}, whose function and fitness are absolutely dependent on Foxp3. These data suggest that Foxp3 acts as a late-acting factor during pT_{reg} differentiation to fine-tune and expand the immunomodulatory capacity of pT_{reg} precursors.

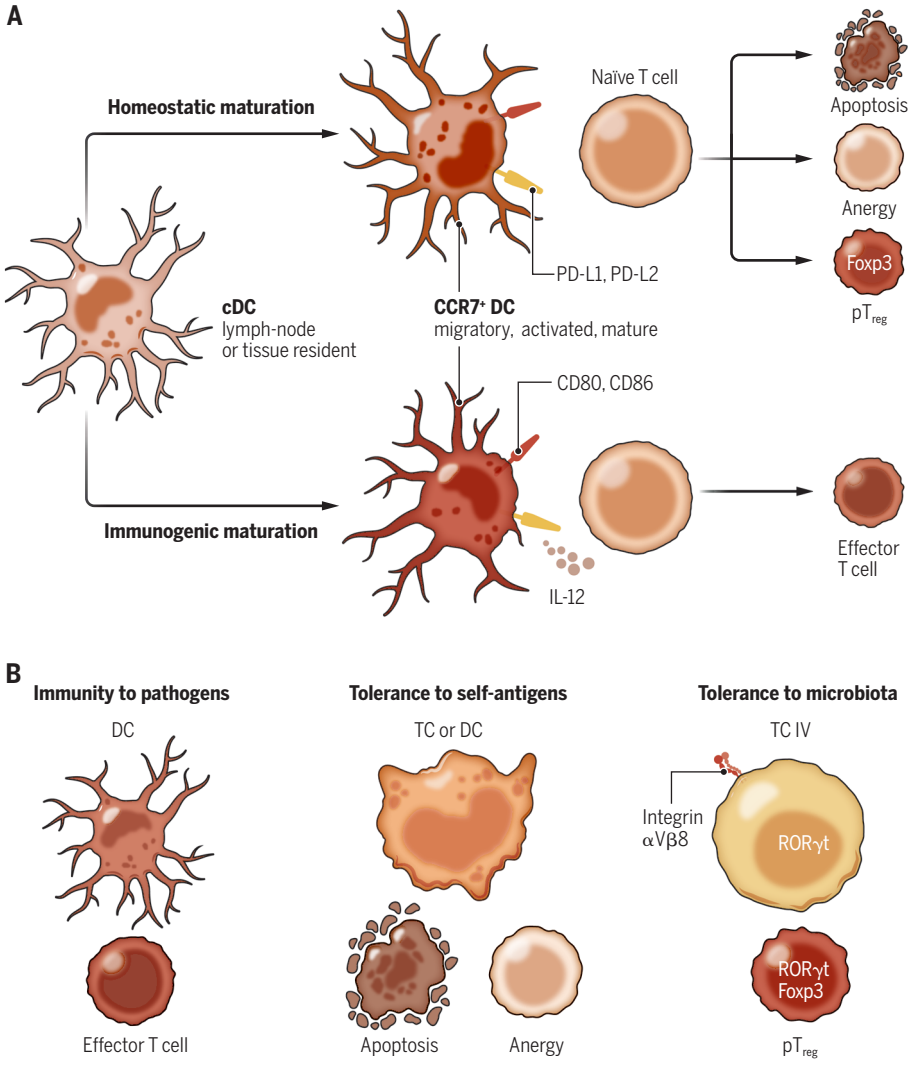

Fig. 3. Two models for tolerogenic APCs. (**A**) Homeostatic DCs are predicted to induce tolerance upon antigen presentation (homeostatic maturation), whereas DCs activated by pathogen-associated signals promote effector T cell differentiation (immunogenic maturation). The distinct "tolerogenic" versus "immunogenic" DC modules that mediate opposing T cell fates, as well as the ability of homeostatic DCs to induce all modes of T cell tolerance, have not been demonstrated. (**B**) Division of labor. Distinct APC subtypes are functionally specialized for induction of tolerance or effector T cell differentiation. Each tissue may use different APC types to direct tolerance to their microbial communities.

Tolerogenic APCs

Considering that APCs are the obligatory instigators of T cell activation and differentiation, the search for APC types or states endowed with a specific ability to induce T cell tolerance or deviation from inflammatory effector fate has been the holy grail of studies of peripheral tolerance. Although the existence of these cells has been widely anticipated, their identities remain poorly defined. Tolerogenic APCs, which, until recently, were assumed by default to be classical dendritic cells (cDCs), are often viewed as a monolithic entity, suggesting a single multipotent cell type with the ability to invoke both cell-intrinsic and -extrinsic mechanisms of peripheral tolerance to the entire spectrum of harmless antigens, including self-antigens and dietary, environmental, and commensal-derived antigens. However, the distinct requirements for pT_{reg} induction, such as TGF-β activation, as well as the recent finding that tolerance to the gut microbiota is instructed by a dedicated tolerogenic $ROR\gamma t^+$ APC, rather than intestinal DCs, point to a framework where distinct types of tolerogenic APCs instruct tolerance to different classes of antigens and induce particular modes of T cell tolerance.

DCs

DCs, the quintessential "professional" APCs, have evolved for highly efficient sampling, processing, and presentation of self-antigens and foreign antigens, alongside the expression of costimulatory and coinhibitory ligands. They are therefore poised to activate naïve T cells and regulate their differentiation fate. In contrast to a dedicated immunosuppressive

T_{reg} lineage endowed with the specific expression of a transcription factor (Foxp3) that ultimately orchestrates their differentiation and immunoregulatory function, a distinct tolerance-inducing DC type has not been identified. Instead, the view at present is that the DC activation state determines a "go" (immunity) or "no-go" (tolerance) decision (*55*) (Fig. 3). According to this model, functionally immature DCs expressing low levels of costimulatory molecules induce a hyporesponsive T cell state or T_{reg} differentiation by default. This model suggests that self-reactivity is limited in a passive manner through lack of a permissive second signal. However, the implicit assumption in this model, namely, that absent infection or sterile inflammation, self-antigens are presented by DCs lacking sufficient CD80 and CD86 levels, is not consistent with the finding that tissue-resident DCs undergo homeostatic maturation and migration to lymph nodes (Fig. 3). DC "maturation"—often used interchangeably with "activation"—refers to a switch from an antigen-acquisition and -processing mode to one of antigen presentation associated with increased expression of surface MHCII and costimulatory molecules (e.g., CD40, CD80, and CD86) (*56*, *57*). DC maturation is also associated with upregulation of chemokine receptor CCR7, which enables their subsequent migration to tissue-draining lymph nodes (*57*), where $CCR7^+$ DCs represent up to 50% of the DC population. Thus, $CCR7^+$ DCs, variably described as "activated," "mature," or "migratory," display a highly conserved transcriptional program that distinguishes them from their $CCR7^-$ counterparts. Paradoxically, among DC subsets, $CCR7^+$ DCs express the highest level of not only costimulatory molecules but also inhibitory molecules such as PD-L1, PD-L2, and CD200 (*58*), suggesting tolerogenic functions, which has led to their recent designation as "mature DCs enriched in immune regulatory molecules" (mreg DCs) (*59*). If exposure of DCs to inflammatory or pathogen-associated signals results in their shift from a tolerogenic to inflammatory response–promoting state, one would expect that $CCR7^+$ DCs would lose their immunoregulatory features in these settings. However, a comparison of unstimulated ("tolerogenic") versus Toll-like receptor–stimulated ("immunogenic") $CCR7^+$ DCs suggest convergent transcriptomes (*60*), including conserved expression of immune regulatory molecules such as PD-L1 and PD-L2. Although $CCR7^+$ DCs exhibit superior in vitro T_{reg} (iT_{reg})–inducing capacity (*59*, *61*)—likely related to their expression of the TGF-β–activating integrin αvβ8 and costimulatory molecules—an in vivo role for homeostatic $CCR7^+$ DCs in pT_{reg} differentiation (or anergy induction) has not been established. Genetic models that allow selective targeting of $CCR7^+$ DCs will therefore be required to discern the functional role of $CCR7^+$ DCs in tolerance versus immunity.

RORγt$^+$ APCs: A recently recognized lineage of tolerogenic APCs

At present, the evidence implicating DCs, which are known to express the transcription factor Zbtb46 and integrin CD11c, in peripheral tolerance stems from studies of ablation of $CD11c^+$ or $Zbtb46^+$ cells or their antigen-presenting capacity using the corresponding drivers of Cre or diphtheria-toxin-receptor expression. In these studies, CD11c or Zbtb46–expressing cells, assumed to be DCs, promoted tolerance to self-antigens (*62*, *63*) as well as oral (*64*) and microbiota-derived antigens (*35*, *65*). However, recent studies have identified a previously unappreciated lineage of APCs, distinguished by expression of the transcription factor RORγt, that share expression of these molecules (*3*–*5*). Furthermore, selective ablation of MHCII in DC precursors showed that antigen presentation by cDCs is dispensable for intestinal pT_{reg} differentiation (*3*–*5*), challenging the long-held view of DCs as the sole APC type responsible for the full spectrum of peripheral tolerance. These same studies also demonstrated that MHCII antigen presentation by $ROR\gamma t^+$ APCs is both necessary and sufficient for pT_{reg} differentiation. In two studies (*4*, *5*), the spectrum of $ROR\gamma t^+$ APCs revealed by single-cell transcriptomics included type 3 innate lymphoid cells (ILC3s) as well as previously described Aire-expressing $ROR\gamma t^+$ cells variably termed $ROR\gamma t^+$ extrathymic Aire-expressing cells (eTACs) (*5*), Janus cells (*4*, *66*), or Aire-expressing ILC3-like cells (*67*). In a third study (*3*), these $ROR\gamma t^+Aire^+$ cells were shown to belong to a broader lineage of cells, named Thetis cells (TCs), that encompasses two transcriptionally distinct $Aire^+$ subsets (TC I and TC III) as well as two $Aire^-$ subsets (TC II and TC IV) (Fig. 4). Although these cells shared markers with both DCs and ILCs, as well as mTECs, specific genetic tracing of ILC and cDC lineages demonstrated that TCs are ontogenically distinct from either one even though the specifics of TC ontogeny remain to be established (*3*, *5*). A unifying finding from the three studies, besides a requirement for MHCII presentation by $ROR\gamma t^+$ APCs, was a requirement for expression of either integrin alpha V (ITGAV) or ITGB8, the two subunits of the TGF-β–activating integrin $\alpha_v\beta_8$. Experiments using mixed chimeras demonstrated that expression of MHCII and either ITGAV or ITGB8 by the same cell was required for pT_{reg} induction (*3*, *4*), consistent with an important role for TGF-β signaling in pT_{reg} differentiation. Among $ROR\gamma t^+$ APCs, only TCs (most notably $Aire^-$ TC IVs), are distinguished by the expression of integrin $\alpha_v\beta_8$, the loss of which led to impaired pT_{reg} differentiation (*3*). The distinctive expression of ITGB8 by TCs, along with the lack of pT_{reg} deficit in mice where MHCII deficiency was restricted to ILC3, argues against a role for ILC3 in pT_{reg} generation. The finding that tolerance to the gut microbiota is instructed by a dedicated tolerogenic $ROR\gamma t^+$ APC, rather than intestinal DCs, highlights a specialized mechanism of protection. This is in contrast to the alternative error-prone scenario, whereby DCs, in an environment-dependent manner, execute both tolerogenic responses to commensals and immunogenic responses to pathogens. A key question that emerges from these findings is whether TC IVs have a role in instructing pT_{reg} differentiation and tolerance to commensal microbes at other barrier sites, such as the skin and lung, and overall pT_{reg} generation, for example, in response to dietary antigens within the intestine or to paternal fetal antigens.

"Studies of early-life immune development have revealed distinct waves of tolerogenic immune cells emerging within the thymus or periphery."

The discovery of TC-instructed pT_{reg} generation alters the paradigm for pT_{reg} generation, raising the question as to whether TC-mediated tolerance extends to other pathways of peripheral T cell tolerance, for example, clonal deletion of self-reactive T cells. Although the expression of Aire by TCs, as well as their transcriptional overlap with mTECs, is intriguing given the established role of Aire in self-tolerance, neither MHCII antigen presentation by $ROR\gamma t^+Aire^+$ cells nor the cells themselves are required for pT_{reg} differentiation (*4*, *5*). These results suggest a functional division between $Aire^+$ and $Aire^-$ TC subsets, manifesting in distinct roles in tolerance to self-antigens versus commensal antigens. A role for $Aire^+$ TCs in self-tolerance remains to be established, but previous studies examining the role of eTACs have suggested that these cells participate in extrathymic deletional tolerance and in maternal-fetal tolerance (*66*, *68*, *69*). Although low expression of Aire transcripts and an Aire reporter transgene are detectable in $CCR7^+$ DCs (*3*, *66*), only $ROR\gamma t^+Aire^+$ cells express detectable Aire protein (*3*, *70*). This, alongside the broader transcriptional overlap between TCs and mTECs, beyond expression of Aire, raises the possibility of a specialized role for TCs in peripheral

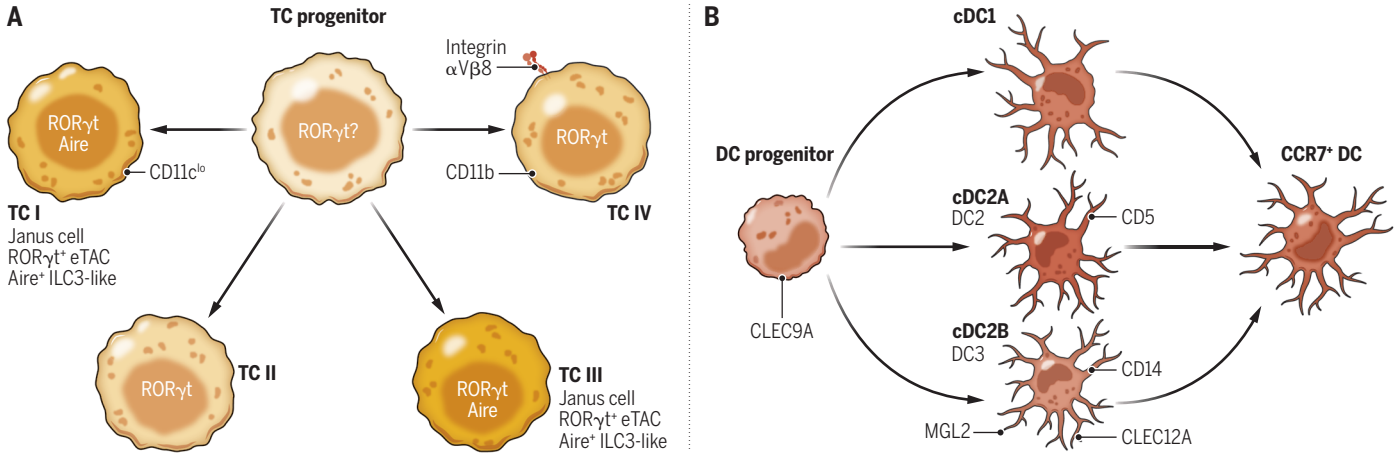

Fig. 4. The expanding spectrum of APCs. (**A**) Newly identified RORγt$^+$ TCs share cDC markers, including CD11c and Zbtb46, but are ontogenically distinct. TCs encompass four subsets that include two Aire-expressing cell types (TC I and TC III), suggesting potential roles in tolerance to self-antigens. Among Aire$^-$ cells, MHCII antigen presentation and ITGB8 expression by TC IVs is required for intestinal pT$_{reg}$ differentiation and tolerance to the gut microbiota. (**B**) DCs arise from CLEC9A$^+$ progenitors. DCs acquire expression of Aire transcript upon differentiation into CCR7-expressing migratory DCs. However, CCR7$^+$ DCs do not express detectable Aire protein.

tolerance to self, paralleling and complementing that of mTECs in central tolerance.

Developmental windows for immune tolerance

Life history of encounters with self-antigens and foreign antigens is not monotonous: Birth, weaning, sexual maturation, and pregnancy place temporal demands on the immune system as a result of the sheer number of new antigens that are encountered. Studies of early-life immune development have revealed distinct waves of tolerogenic immune cells emerging within the thymus or periphery (*3*, *27*, *71*). In addition to quantitative differences in T$_{reg}$ numbers, neonatal tT$_{regs}$ have a distinctive functionality that cannot be compensated by adult tT$_{regs}$ (*27*), suggesting not only layered ontogeny but also developmental stage–specific programs, TCR specificities, and selection in distinct environments that endow early-life tT$_{regs}$ with distinct functional properties. Genetic lineage tracing studies revealed that perinatally generated tT$_{regs}$ persist long after birth, but whether these cells provide specific functions in adulthood remain to be determined.

Within the intestine, the gut microbial community undergoes a profound diversification upon transition from breast milk to solid foods. In addition, this transition is associated with the withdrawal from a wealth of immunomodulatory factors, including growth factors, cytokines, maternal immunoglobulins, and hormones. Failure to establish intestinal tolerance during this weaning period leads to an increased risk of allergic and inflammatory diseases later in life (*71*). Although previously thought of as immature or underdeveloped, the neonatal and infant immune system is highly adapted for establishment of immune tolerance, with the aforementioned layered ontogeny that ensures enriched representation of tolerogenic APCs and T$_{regs}$ during critical windows when the host first encounters dietary and microbial antigens. Adoptive transfer studies of TCR-transgenic naïve T cells expressing a microbe-reactive TCR demonstrated increased propensity for pT$_{reg}$ generation when cells were transferred into mice before weaning (*35*, *72*). Because these adoptive transfer studies were performed with naïve T cells isolated from adult mice, this suggests that the wave of pT$_{regs}$ that emerges upon weaning is driven by extrinsic factors within the developing intestinal niche. The finding that the pT$_{reg}$-inducing APCs, TC IVs, emerge in a developmental wave within the mesenteric lymph nodes that coincides with weaning (*3*) suggests that these cells may define the temporally restricted window for pT$_{reg}$ differentiation and their homeostatic abundance, which in turn will determine the immune-regulatory tone within the intestine. In this regard, an intriguing observation is that pT$_{reg}$ abundance in adulthood is determined by maternally transmissible cues, including breast milk IgA, that are sensed during the first week of life, before the emergence of pT$_{regs}$ (*73*). Similar to the window of opportunity for intestinal tolerance to commensal microbes, neonatal encounters with skin commensal antigens lead to the development of commensal-specific pT$_{regs}$ and tolerance (*74*). Thus, dynamic changes in tolerogenic immune cell populations mirror the developmental demands of the host and perhaps serve to limit an overabundance of dominant immunosuppressive cell types that might compromise effector immunity.

Recent years have seen major advances in studies of peripheral tolerance. Yet these discoveries have also raised a number of intriguing questions: Does each barrier tissue with its particular organization, cellular composition, and microbial community have its own conventions for establishing tolerance, or do certain general rules exist with the same cell types and mechanisms that operate across distinct organs? Analogous to the functional division between tT$_{regs}$ versus pT$_{regs}$, do distinct peripheral APCs instruct tolerance to self-antigen versus foreign antigen? What determines the increased propensity for tolerogenic immune cell fates during early life, and are these same mechanisms used to restore tolerance in adulthood? An increased understanding of the cell types and molecular mediators that establish tolerance will pave the way for future studies that address these questions.

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Spatiotemporal regulation of peripheral T cell tolerance

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