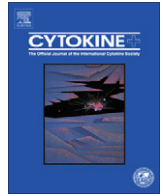




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Review Article

Regulatory T cells in health and disease

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ABSTRACT

Regulatory T (Treg) cells have emerged as a central control point in the modulation of various immune responses, including autoimmune responses and immunity to transplants, allergens, tumors, and infectious microbes. The immune system has evolved complex processes to ensure tolerance to autoantigens while preserving the potential to mount and maintain life-long humoral and cellular immune responses against invading pathogens. In this review, we summarize research showing that naturally occurring (nTreg) and induced Treg (iTreg) cell subsets, and in particular CD4⁺Foxp3⁺ Treg cells, are critical in the control of immune responses in rodents and humans. We also discuss the cellular and molecular factors that affect CD4⁺Foxp3⁺ Treg cell development, homeostasis, and function and consequential immunity to self and non-self antigens. Recent studies have shed light in our understanding of the development of novel methods of autoimmune disease prevention and treatment via enhancing and re-establishing Treg-mediated dominant control over self-reactive T cells in animal models and humans.

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1. Naturally occurring CD4⁺ regulatory T cells

Adaptive immunity requires a finely controlled equilibrium between maintaining peripheral immune self-tolerance while simultaneously preserving the potential to generate protective life-long immunity to a variety of pathogenic microbes [1]. In order to control these two drastically different immunological outcomes, several mechanisms exist, including a spectrum of CD4⁺ Treg cells, to suppress self-reactive T cells that escape thymic clonal deletion, maintain normal intestinal immunity towards commensal bacteria, and attenuate anti-pathogen effector mechanisms from inducing immune pathology [2]. Pioneering studies performed in 1970s indicated that antigen activated suppressor T cells could be induced to downregulate antigen-specific, T cell responses although these studies invariably led to inconsistent and controversial results [3]. The existence of T cell-mediated suppression in the regulation of immune responses to self and non-self antigens is now unquestioned.

There is ample evidence that Treg actively suppress self-reactive T cells, and onset of autoimmunity [1,4]. In 1995, Sakaguchi et al. showed that a novel subset of CD4⁺ T cells constitutively expressing the IL-2 receptor (R) alpha (α) chain (CD25), in normal rodents possess potent immunosuppressive functions *in vitro* and *in vivo* [5]. These CD4⁺CD25⁺ T cells, termed nTreg cells, represent 1–10% of total CD4⁺ T cells in thymus, peripheral blood and lymphoid tissues, and acquire their regulatory capacity during normal

thymopoiesis (Fig. 1). Once they leave the thymus, they survive in the periphery poised for normal surveillance of self-Ags, and prevent potential autoimmune responses [6–7]. nTreg cells are hyporesponsive to TCR-induced proliferation and cytokine production *in vitro* [6–7]. Currently, CD25 continues to be the most useful cell surface marker for nTreg cells in the naïve immune system, although several other markers, like Foxp3 (see Section 2) and CD127, may be more specific [6].

Physical depletion or functional abrogation of nTreg cells from the periphery of normal mice leads to the spontaneous development of various autoimmune diseases, such as gastritis, thyroiditis, orchitis, oophoritis, type 1 diabetes (T1D), and inflammatory bowel disease (IBD), all prevented by infusion of normal CD4⁺CD25⁺ T cells [7–9]. Furthermore, depletion of nTreg cells provokes effective immunity to autologous tumor cells in otherwise non-responding animals, enhances immune responses to invading or commensal microbes, triggers allergic responses to innocuous environmental substances, and breaks feto-maternal tolerance during pregnancy [10]. Thus, these studies conclusively show that these cells are critically important for the regulation of organ-specific autoimmunity, but also suppress a variety of pathological, inflammatory responses to a wide spectrum of non-self antigens.

The TCR repertoire of nTreg cells is as polyclonal as naïve T cells, although nTreg cells express TCRs of higher affinity for self-peptides thus endowing them to be preferentially self-reactive [11]. These nTreg cells do not produce pro-inflammatory cytokines upon antigenic stimulation and thus are not pathogenic despite the apparent higher reactivity for self-antigens. They potently suppress the activation, proliferation, and effector functions of CD4⁺,

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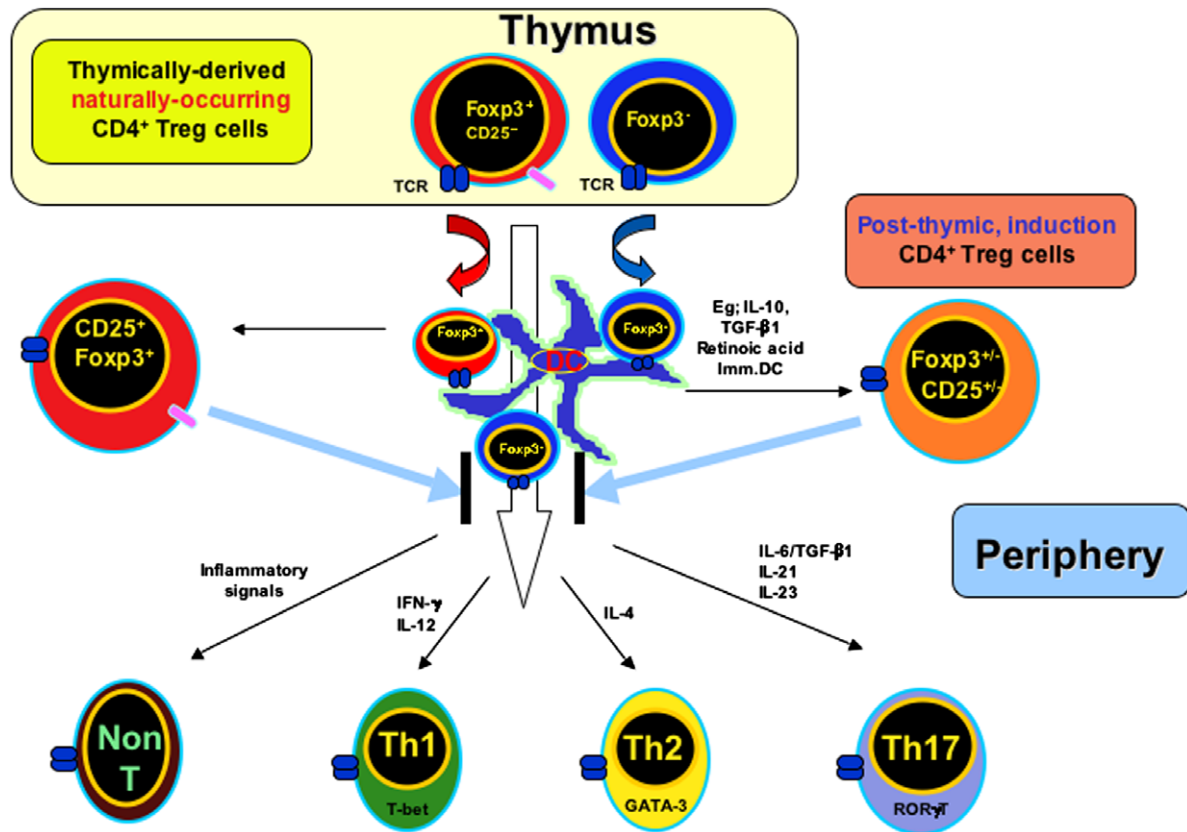


Fig. 1. Control of immune responses by CD4⁺ regulatory T cells. Conventional CD4⁺ T cells [blue] exit the thymus, and upon activation by DC, differentiate into effector Th1, Th2 and Th17 cells which collectively contribute to a variety of peripheral immune responses. Thymic-derived, naturally occurring [red] and peripherally induced [blue] CD4⁺ regulatory T cell subsets downregulate the activation, differentiation and function of Th1, Th2, and Th17 effector cells, as well as non-T cells [green]. While CD4⁺CD25⁺Foxp3⁺ nTreg cells differentiate in the thymus and are found in the normal, na CD4⁺ T cell repertoire, multiple iTreg cell subsets, possibly expressing CD25 and Foxp3, originate from the activation and differentiation of conventional CD4⁺ T cells in the periphery under unique stimulatory conditions. Both Treg subsets conceivably synergize to assure regulation of immune responses.

CD8⁺, NK cells, NK-T, DC, and B cells [12–16]. These unique properties of CD4⁺CD25⁺ nTreg cells make them well poised for controlling potentially pathogenic self-reactive T cells under circumstances favoring immune self-reactivity.

2. Foxp3: key regulator of Treg cell development and function

Foxp3, a forkhead winged helix family transcriptional regulator, is a critical molecular switch for the genetic programming of nTreg cell development and function [17–19]. In mice, the majority of peripheral and thymic Foxp3⁺ cells are CD4⁺CD25⁺, and a genetic deficiency in Foxp3 results in an autoimmune pathology secondary to a loss of CD4⁺CD25⁺ nTreg cells [17,20]. Mere stimulation of murine CD4⁺CD25⁻, Th1 or Th2 cells fails to induce Foxp3 expression [18]. Interestingly, mice overexpressing Foxp3 have an increased development of nTreg cells, and suppresses the development of the lymphoproliferative syndrome and T1D in CTLA-4 deficient and NOD mice, respectively [19,21]. Natural mutations of the *foxp3* gene, as seen in *Scurfy* mice, result in a loss in Foxp3 protein and nTreg cells, and the development of various spontaneous and early onset organ-specific autoimmune pathologies due to hyperactivation of CD4⁺ T cells. The *Scurfy* model is a natural, X-linked recessive mutant with severe lethality in hemizygous males, exhibiting hyperactivation of CD4⁺ T cells, and overproduction of pro-inflammatory cytokines [22–24]. A *Scurfy*-like phenotype is also seen in mice depleted of nTreg cells or with *foxp3* gene deficiency, and mutations of the human Foxp3 gene were subsequently found to be the cause of immune dysregulation,

polyendocrinopathy, enteropathy, X-linked syndrome (IPEX), which is a rare X-linked immunodeficiency syndrome that develops T1D, IBD, allergies, hyper IgE production and many hematological and infectious diseases [22–24]. Infusion of CD4⁺CD25⁺ nTreg cells, but not CD4⁺CD25⁻ T cells, into Foxp3-deficient (*l/l*) or *Scurfy* mice prevents the fatal *Scurfy* syndrome suggesting that Foxp3⁺ nTreg cells are critical for preventing autoimmunity [17,19]. Bone marrow chimera studies have shown that Foxp3 functions in CD4⁺ nTreg cells in a cell-autonomous fashion, although Foxp3 is reportedly expressed in some thymic stromal cells in turn affecting thymic selection [25,26].

Human nTreg cells also express high FOXP3 levels, and the severe autoimmunity that results in IPEX patients demonstrates its central importance in immune homeostasis [22–24]. In contrast to mice, humans express two different splice variants of FOXP3, with the smaller form lacking exon 2, and both variants independently possess the ability to render CD4⁺ T cells anergic [27]. There is currently conflicting views as to whether retroviral-mediated over-expression of is not sufficient to induce potent suppressive activity [27,28]. Importantly, in contrast to murine T cells, human CD4⁺CD25⁻ T cells upregulate expression of FOXP3 upon activation, and non-suppressive, but FOXP3⁺, T cell clones can be observed. Thus, expression of FOXP3 in humans may not be exclusively linked to suppression, and this molecule may have an important role outside the nTreg cell subset. Foxp3 is currently the most specific, reliable molecular marker, irrespective of CD25 expression, for thymic or peripheral nTreg cells in rodents and humans [29].

2.1. *Foxp3/FOXP3 genetic deficiency in mice and humans*

The severe multi-organ autoimmunity that occurs in rodents and humans following *Foxp3* deficiency collectively show the following: first, $CD4^+Foxp3^+$ T cells, irrespective of CD25 surface expression, engage in the maintenance of immunological self-tolerance, and the suppressive activity of $CD4^+CD25^+$ T cells shown in various rodent models of autoimmunity is likely attributed to $Foxp3^+CD4^+CD25^+$ nTreg cells [20–30% of total *Foxp3*-expressing T cells] present within this population; second, the degree of $Foxp3^+$ nTreg cell functional deficiency has a direct effect on the extent of autoimmunity. In addition to provoking autoimmunity that can be elicited by CD25 depletion, a more profound deficiency of $Foxp3^+$ nTreg cells can activate even weak/rare self-reactive T cells thus inducing a wider spectrum autoimmune/inflammatory diseases that are not induced by a less severe deficiency of nTreg cells; third, the susceptibility and spectrum to particular autoimmune diseases is seemingly genetically determined in each host strain of mice [1,10]. Moreover, the longer or more severe the reduction of $CD4^+$ nTreg cells, the higher the incidence of a particular autoimmune disease, and the wider the spectrum of clinically manifested autoimmune diseases in a genetically determined hierarchical pattern. Furthermore, many autoimmune or chronic inflammatory diseases in mice that are induced by severe nTreg depletion resemble human autoimmune/inflammatory diseases clinically and immunologically [10,30]. Therefore, a combination of a profound Treg deficiency and a particular host genetic background is able to produce an autoimmune disease in mice that corresponds to its human equivalent. In the absence of a clear etiological basis for these diseases, these findings may provide insight into their causes, pathogenesis, and eventual treatment design.

2.2. *Foxp3 activity drives nTreg function*

Foxp3 activity is required for the establishment of the molecular programs involved in Treg function [1]. *Foxp3* has been shown to operate as a homo-oligomer, and interacts with a variety of transcription factors including NFAT, AML-1 [acute myeloid leukemia – 1]/Runx1 [runt-related transcription factor 1], the histone acetyl transferase [HAT]/histone deacetyl transferase [HDAC] complex, and NF- κ B. Following its activation, NFAT complexes with AP-1 and NF- κ B and promotes the expression of several genes including *Il2*, *Il4*, *Ctla4* in normal, activated T cells, in turn contributing to T cell activation and differentiation. A recent study has shown that *Foxp3*/NFAT interactions between are dependent on their DNA binding capacity [31,32], and mutations in the forkhead domain of *Foxp3* disrupt the *Foxp3*-NFAT interaction, impair the ability of *Foxp3* to repress *Il2*, activate *Ctla4* and *Cd25*, and abrogate its ability to confer suppressive activity when over-expressed in normal T cells [31,32].

3. Interleukin 2 is essential for $Foxp3^+$ Treg cell function and fitness

IL-2 exerts pleiotropic, and often contradictory effects on a variety of cell types. It facilitates Th1/Th2 differentiation and expands $CD8^+$ memory T cells and NK cells [33]. In contrast, IL-2 promotes Fas/Fas-L dependent, apoptosis in antigen-primed T cells. IL-2 also inhibits the TGF- β 1/IL-6-dependent Th17 cell differentiation of naive T cells [33,34].

Strong evidence shows that IL-2, the ligand for the CD25-containing high-affinity IL-2R, is an important stimulatory signal for the development, function and fitness of nTreg cells [33,35]. As $CD4^+CD25^+$ nTreg cells suppress T cell responses by down-regulating IL-2 synthesis, and paradoxically require IL-2 for their function and fitness, the dynamics of IL-2 production and suppression

remains enigmatic *in vivo* [16]. Mice deficient for IL-2, IL-2R α (CD25), or IL-2R β (CD122) have a drastically reduced pool of nTreg cells and paradoxically die prematurely from a severe lymphoproliferative and autoimmune syndromes (hemolytic anemia and IBD), lymphocytic infiltration of multiple organs, and hyperreactivity to commensal microbes [36]. Similarly, T cell-specific deletion of STAT5A/B, which mediate IL-2R signaling, abrogates the development of nTreg cells [37,38]. Genetic deficiency of CD25 in humans also displays similar clinical and pathological manifestations [39], indistinguishable from IPEX [40]. In IL-2R $\beta^{-/-}$ mice with a IL-2R β transgene that is expressed predominantly in the thymus, $CD4^+CD25^+$ T cell development is restored and autoimmunity is prevented, indicating that an intact IL-2/IL-2R pathway is also required for thymic generation of nTreg [41]. Interestingly, abrogation of the B7/CD28 co-stimulatory pathway results in quantitative and qualitative defects in $CD4^+CD25^+$ nTreg cells in lymphoid tissues, and a consequential induction of autoimmunity [42]. The adoptive transfer of WT nTreg cells can only prevent autoimmunity in mice lacking a functional IL-2R, in contrast to IL-2 $^{-/-}$ mice, and WT Treg cells transferred into neonatal IL-2R $\beta^{-/-}$ mice, undergo rapid and extensive IL-2 dependent proliferation in lymph nodes and spleen [36]. Systemic IL-2 neutralization induces autoimmune gastritis in BALB/c mice, provokes spontaneous autoimmune neuropathy and exacerbates diabetes in NOD mice [43]. These studies show that the lack of nTreg cells contributes to the autoimmune phenotype of IL-2 $^{-/-}$ and IL-2R $^{-/-}$ mice, and that IL-2 is a critical factor for Treg cells [33].

IL-2 is also an important factor affecting the fitness of $Foxp3^+$ Treg cell function. Fontenot et al. examined whether IL-2 was required for the development of $Foxp3^+$ nTreg cells [44]. In this study, mice containing a *Foxp3*-GFP knock-in allele encoding a GFP-*Foxp3* fusion protein were used, thus enabling the identification of $Foxp3^+$ Treg cells by GFP fluorescence [44]. From the analysis of *Foxp3*^{gfp} mice genetically deficient in IL-2 or CD25, IL-2 signaling does not appear to be required for the induction of *Foxp3* expression in developing thymocyte, and both IL-2 $^{-/-}$ and IL-2R $\alpha^{-/-}$ Treg cells are unexpectedly able to suppress T cell proliferation *in vitro*, despite a substantially reduced frequency of these cells *in vivo* [44]. Interestingly, IL-2 signaling was nonetheless necessary for maintaining the expression of genes involved in controlling cell growth and metabolism, possibly indicating that IL-2 signaling may be required for sustaining the homeostasis and competitive fitness of nTreg cells *in vivo* [44].

The requirement of IL-2 in the development, *Foxp3* expression, function acquisition, and peripheral fitness of Treg cells may depend on a number of reasons, including the MHC and non-MHC genetic background, co-stimulatory burden, and degree of lymphopenia in each of the mouse models used [10]. It is also unknown whether compensatory cytokines may drive Treg development or function in the functional absence of IL-2. Interestingly, complete deficiency of $Foxp3^+$ nTreg cells in common γ (γ c) chain deficient mice, in contrast with IL-2R $\alpha^{-/-}$ or IL-2-deficient mice, indicates that other γ c binding cytokines like IL-4, IL-7, IL-9, and IL-15 are also involved in the maintenance of nTreg cells [44]. Interestingly, IL-4, IL-7, or IL-15 deficiency alone does not alter the number of $Foxp3^+$ cells or provoke autoimmunity. Setoguchi et al. showed that IL-2 mRNA and protein expression is the likely product of activated $CD4^+CD25^{low}$ T cells, and to a lesser extent of $CD8^+$, NK, and NKT cells, isolated from normal naive mice [43], although DC-derived IL-2 has been claimed to play a critical role in nTreg cell activation [45].

3.1. *Inflammation drives $Foxp3^+$ Treg cell regulation*

The above findings suggest a negative feedback control mechanism of immune responses initiated by IL-2 produced by activated

non-Treg cells in sites of inflammation, which in turn may drive CD4⁺CD25⁺ Treg cell-mediated suppression of T cell responses. These findings have the following implications for the roles of the IL-2/IL-2R system in Treg-mediated immune self-tolerance. First, IL-2 is not only a cytokine promoting adaptive immune responses but also a mediator of negative feedback control by which activated nonregulatory T cells contribute to the maintenance and activation of CD4⁺CD25⁺ nTreg cells, which in turn limit the expansion of the former. This may occur locally where both nTreg cells and effector T cells are recruited to an APC presenting a particular self- or non-self-antigen, thus contributing to the accumulation and expansion of nTreg cells at the site of inflammation, as observed in the organ/tissue affected in organ-specific autoimmune disease [35]. Second, these results suggest that genetic mutations or variations that modify half-life, synthetic rate or function of IL-2 can be a predisposing factor to organ-specific autoimmunity by potentially abrogating the cellular frequency, function or peripheral homeostasis of nTreg cells. This provides a possible biological explanation for the IL-2 gene polymorphism at the *Idd3* locus that is linked with the susceptibility to T1D and EAE in NOD mice [46]; Sgouroudis et al., unpublished observations). Similarly, there is significant linkage of an allelic variation at the CD25 gene with human T1D [39]. Genetic polymorphisms of the genes encoding other IL-2R chains, or signal transduction molecules like STAT5, might also contribute to determining the susceptibility to T1D and other autoimmune diseases [38]. Third, reduction of IL-2 for therapeutic purposes may elicit autoimmunity under certain circumstances, as evidenced by the ability of cyclosporine A, an immunosuppressant that blocks IL-2 formation in T cells, to cause organ-specific autoimmunity [47]. Finally, IL-2 can be used therapeutically for *ex vivo* expansion and activation of antigen-specific nTreg cells for the eventual cellular therapy of autoimmune disease [35,48].

4. Mechanism [s] of Treg cell-mediated suppression

The mechanism(s) of nTreg cell-mediated suppression is not known [2]. CD4⁺CD25⁺Foxp3⁺ nTreg cells suppress the proliferative and cytokine responses of naive T cells, but also macrophages, DC, NK, NK-T, and B cells, in an antigen non-specific fashion [1,2]. Several mechanisms of Treg-mediated suppression have been proposed. *In vitro* functional studies have shown that activated CD4⁺CD25⁺ nTreg cells may suppress these responses via the secretion of immunosuppressive cytokines by Treg cells, cell-contact dependent Treg/APC or T–T cell interactions, and functional modification or killing of APC [8]. *In vitro*, nTreg cell-mediated suppression adopts a completely cytokine-independent mechanism of suppression not involving the action of immunosuppressive molecules such as IL-10 and TGF-β1, as neutralization of these cytokines does not abrogate suppression, and nTreg cells isolated from TGF-β1- or IL-10-deficient mice remain functional [13,16]. A select number of studies show that activated CD4⁺CD25⁺ T cells mediate their *in vitro* effect via an inactive form of TGF-β1 complexed to its latency associated peptide on the cell surface [48].

The contribution of nTreg-derived cytokines like IL-10 and TGF-β1 in tolerance induction and disease regulation *in vivo* are more complex, and have been implicated in context-dependent and time-dependent fashions in some models. IL-10 contributes to suppression of colitis in mice [49], and Foxp3⁺ Treg cells secreting IL-10 are detectable in lamina propria of the intestine [50]. The reports on the *in vivo* role of TGF-β1 are conflicting. While some studies shown that TGF-β1 mediates suppression of CD4⁺ nTreg cells *in vivo*, other studies show that CD4⁺CD25⁺ nTreg cells are able to suppress intestinal inflammation by a mechanism not requiring nTreg cell-derived TGF-β1 or effector T cell Smad3-dependent

responsiveness to TGF-β [51]. Thus, TGF-β1 may be a direct mediator of immunosuppression or may enhance suppression by sustaining regulatory networks via the post-thymic development of cytokine-secreting Foxp3⁺iT_{reg} cells, and maintaining nT_{reg} cell peripheral homeostasis by bolstering Foxp3 expression. A recent study has shown that Foxp3⁺ nTreg cells preferentially produce IL-35, a novel immunosuppressive member of the IL-12 family [52]. It is unknown whether context-dependent physiological factors regulate the differentiation of nTreg cells or different subsets to suppress by any given mechanism.

Several other mechanisms of Treg-mediated suppression have been proposed to operate, alone or in combination, for the control of immune responses. Upon antigenic stimulation, Treg cells are rapidly recruited via chemokines to Ag-presenting DC where they compete with antigen-specific effector T cells on DC surfaces through a LFA-1 dependent mechanism, and in turn downregulate CD80 and CD86 on DC by a CTLA-4-dependent mechanism [1,12]. Other studies have shown that Treg cells may kill or inactivate responder T cells or APC through cell-to-cell contact by a granzyme- or perforin-dependent mechanism, or through delivery of a negative signal to responder T cells [53]. Potential negative signals include upregulation of intracellular cAMP, which leads to suppression of T cell proliferation and IL-2 secretion, or the generation of pericellular adenosine catalyzed by CD39 (ectonucleoside triphosphate diphosphohydrolase-1) and CD73 (ecto-5-nucleotidase) expressed by Foxp3⁺ Treg cells [1]. Antigen-activated Treg cells may also stimulate DC, via CTLA-4, to form the enzyme indoleamine 2,3-dioxygenase (IDO), which catabolizes tryptophan to kynurenines, which kill T cells [54]. More recently, cytokine competition by Treg cells has also been suggested to induce apoptosis in responding T cells on APC surfaces [55]. Numerous reports have shown that deficiencies of some Treg-related proteins, including IL-10, TGF-β1, granzyme, and IL-35, can abrogate Treg suppression *in vitro* by primarily impacting a unique mode of suppression, however failing to induce autoimmunity *in vivo*, possibly due to compensatory mechanisms [10,52].

5. Tissue localization and homing of Treg cells to sites of inflammation

Studies show that nTreg possess a preferential ability, relative to effector T cells, to home to lymphoid organs and sites of inflammation. DC in the draining lymph nodes present tissue-specific self-antigens, and several studies indicate that Foxp3⁺ Treg specific for tissue self-antigens are accumulate, and get activated, in lymph nodes where tissue-specific self-antigens or foreign antigens are presented [56]. In addition to antigen-specific TCRs, Foxp3⁺ Tregs express a spectrum of adhesion molecules and chemokine receptors enabling them to home to sites of inflammation [2,10,56]. In the peripheral lymph nodes, the majority of Foxp3⁺ Treg cells high levels of CCR7, giving them the capacity to migrating towards the lymphoid-derived chemokines CCL19 and CCL21 [57]. In addition, Foxp3⁺ Treg also express at moderately higher levels than effector T cells CCR4, CCR5, CCR6, CCR8, CXCR4, and CXCR5 [56]. CCR5 was recently shown to direct the homing of IL-10 producing nTreg cells into sites of *L. major* infection where they suppress effector T cell expansion and IFN-γ production, thus promoting the establishment of infection and ensuring the long-term survival of the parasite in the immune host [58]. A similar function for CCR5 was also shown in models of GVH and human tumors (reviewed in [56]). CCR6 is expressed on a distinct subset of mouse and human nTreg cells and CCR6⁺ nTreg cells are enriched in the peripheral blood, rapidly produce IL-10 following Ag stimulation, and accumulate in the inflamed central nervous system [59]. A third of Foxp3⁺ Treg cells express integrin αEβ7 [CD103], which interacts with E-cad-

herin expressed on epithelial cells, and a substantial fraction express CD62L/ α -selectin, which interacts with the vascular addressins CD34, GlyCAM-1, and MAdCAM-1 expressed by the endothelium in lymph nodes and mucosal lymphoid tissue (reviewed in [56]). CCR7⁺CD62L^{high} Tregs, which migrate to the secondary lymphoid tissues, have an enhanced capacity to prevent the development of T1D in NOD mice [57]. Foxp3⁺ Treg cells that express CD103 are memory-like, are more suppressive than CD103[−] Treg cells *in vitro* and *in vivo*, and Foxp3⁺CD103⁺ Tregs are able to control murine colitis in the colonic mucosa [60].

The expression patterns of homing receptors are often comparable to those of effector T cells which home to similar inflammatory sites. For instance, CCR4⁺ Tregs as well as CCR4⁺ effector T cells accumulate in inflamed skin subsequent to antigen exposure [61]. CCR6⁺ Tregs and CCR6⁺ Th17 cells are both elevated in inflamed joints in an animal model of rheumatoid arthritis [62]. Moreover, RA secreted by intestinal CD103⁺ DC induces gut-homing integrin α 4 β 7 on Treg and effector T cells. Treg-specific deficiency of a specific chemokine receptor or its blockade compromises Treg function, for example, in the control of tissue-localized inflammation, local maintenance of pathogen persistence [58]. Overall, the expression of a variety of homing receptors on nTreg cells may endow them with a competitive advantage over effector T cells, and allow them to migrate more efficiently to inflammatory sites and prevent immune responses.

6. Peripheral development of Treg cells from naive T cells

Peripheral, naive T cells, without intrinsic regulatory potential, can acquire Treg function under unique, differentiation signals *in vitro* and *in vivo* (Fig. 1). Functional studies have shown that several iTreg populations, with variable degrees of CD25 expression, may suppress Th1 or Th2 cell-mediated inflammation [7,8]. In general, most iTreg cells arise after deliberate antigen exposure and their definition has been based on their phenotype and, their relative cytokine production capabilities. Th1 and Th2 cells can be considered regulatory by virtues of their cross-antagonizing actions [7,8]. CD4⁺ iTreg cells also include an antigen-specific, IL-10-producing type-1 regulatory [Tr1] cells, which requires IL-10 during its priming phase and mediates its biological effects in an IL-10-dependent fashion. Tr1 cells do not express Foxp3, however, their *in vitro* properties resemble those of Foxp3⁺ Treg cells [63]. Oral feeding of antigen may also induce in gut mucosal sites antigen-specific, Th3 cells, which produce neither IL-4 nor IFN- γ , mediate their suppressive effects via TGF- β [4].

Recent studies have shown that naive T cells can be educated to acquire Foxp3 expression following *in vitro* antigenic activation in the presence of TGF- β 1 [64]. Interestingly, the inflammatory cytokine IL-6 inhibits the acquisition of Foxp3 by activated naive T cells, however TGF- β 1 stimulation in the presence of IL-6 of naive T cells promotes Th17 cell differentiation in mice [65]. Moreover, this process is abrogated by IL-2, which, in turn, induces the differentiation of CD4⁺Foxp3⁺ Treg cells from naive CD4⁺ T cell precursors [34]. Recently, retinoic acid [RA], a secreted product by a particular DC subset in the lamina propria of the gut, inhibits IL-6-driven induction of Th17 cells, but in the presence of TGF- β 1, RA seemingly promotes the differentiation of naive T cells to CD4⁺Foxp3⁺ Treg cells [66–68]. Thus, activation of naive T cells by RA-producing DC in the gut, in conjunction with TGF- β 1 may induce Foxp3⁺ Treg cells, which may tolerize responses to dietary or bacterial antigens.

Although the development of iTreg and thymus-produced nTreg cells are seemingly distinct, it remains uncertain to what extent each subset contributes to the peripheral pool of circulating Foxp3⁺ Treg cells and whether each subset function in similar inflamma-

tory conditions. The nature and stability of the phenotype and function for each subset are also not known.

7. CD4⁺Foxp3⁺Treg cells in the pathophysiology and treatment of human disease

CD4⁺ nTreg cells represent a master-switch of peripheral immune responses, and play a determining role in the balance between tolerance and immunity. There is now compelling evidence in rodents, non-human primates, and humans that abrogation of nTreg cell development and/or peripheral function may permit the preferential activation, expansion and differentiation of such self-reactive T cell in peripheral blood of normal individuals and ultimately cause autoimmune disease *in vivo* [10]. IPEX patients teach us that nTreg cells truly represent a dominant mechanism of self-tolerance in humans. As self-reactive T cells potentially pathogenic for various autoimmune diseases circulate in most individuals, nTreg cell-mediated suppression is a critical mechanism of peripheral self-tolerance. IPEX also teaches us that a monogenic mutation affecting nTreg cells can induce autoimmunity in rodents and humans, thus suggesting that any genetic defect or environmental agent impacting the development, function or fitness of nTreg cells could be a predisposing factor to a variety of autoimmune diseases. For example, polymorphisms in the *Cd25*, *Ptprn22*, and *Ctla4* genes, in addition to predisposing HLA haplotypes, represent significant contributors to the genetic susceptibility of T1D, a phenotype consistent with the fulminant autoimmunity in mice deficient for these genes [1]. Thus, autoimmunity genes, in conjunction with a variety of environmental triggers, may directly impinge the thymic development or peripheral functional homeostasis of CD4⁺Foxp3⁺ nTreg cells, and in turn, contributes to disease susceptibility.

It is likely that a developmental or functional deficiency in nTreg cells cannot, on its own, represent the sole cause of many autoimmune diseases, and other mechanisms probably contribute to the manifestation of clinical autoimmunity. It remains to be seen to what extent a defective nTreg cell compartment contributes to the various human autoimmune disorders, and to what extent nTreg cells can represent a common therapeutic target for all of these diseases. The triggers for autoimmune diseases and the factors that exacerbate their progression have remained largely elusive, despite extensive study. While it is difficult to establish a direct causative link between human disease states and nTreg cells, recent research indicates that a decrease in number and/or function of these cells is associated with autoimmunity in many instances, and strongly suggests that these abnormalities may be a determining factor in disease development. The reasons for why and how these abnormalities arise remain elusive. It is also puzzling that the reported global defects in peripheral nTreg cell function do not lead to widespread autoimmunity, suggesting that a tissue-specific imbalance may also pre-dispose individuals to a given disease. There is also the possibility that nTreg cell defects are indicative, but not causative of autoimmunity. Few studies, however, have shown that some clinically effective treatments actually reverse nTreg cell functional defects, supporting the concept that nTreg cell dysfunction is involved in disease pathology, and that therapeutic manipulation of these cells will be an effective treatment for a variety of autoimmune diseases. Genetic analysis, detection and functional testing of antigen-specific nTreg cells with specific and stable markers, such as Foxp3, will facilitate our understanding at the cellular and molecular levels of how nTreg cells control self-reactive T cells in health, how such mechanism fails in instances of autoimmune disease. The use of antigen-specific nTreg cells may be a future strategy for the treatment and prevention of autoimmune disease.

Tumor-associated immunosuppression is a major obstacle in the generation of potent anti-tumor immunity and the therapeutic efficacy of various immunotherapies. Mice lacking CD4⁺Foxp3⁺ nTreg cells are capable of breaking tolerance to tumor-associated antigens (reviewed in [10]). Thus, CD4⁺Foxp3⁺ nTreg cells promote the establishment of tumor growth and progression, and novel strategies to deplete or attenuate Treg cells locally may represent a valid clinical target for cancer immunotherapy. Similarly, depletion of nTreg may also lead to complete pathogen clearance but may inadvertently prevent induction of long-term immunity and secondary immune responses upon reinfection due to lack of persistence of microbial-specific memory T cells and pathogen persistence [58]. Hence, Treg cells control the quality and tune the magnitude of antimicrobial immune responses to protect the host from pathogenic microbes while avoiding collateral immunopathology or inappropriate responses to commensal microbes. CD4⁺ nTreg can be exploited to tune the intensity of antimicrobial immune responses in acute and chronic infections, and to develop novel immunotherapeutic strategies for various infectious diseases including transient blockade of nTreg homing to elicit potent anti-pathogen immunity.

Identification of biomarkers that can specifically and reliably discriminate between nTreg and effector T cells, as well as nTreg from iTreg cells, will enable us to develop the appropriate reagents [monoclonal antibodies, small peptide agonists/inhibitors] to facilitate our ability to monitor nTreg cellular frequency and function in diseases states or to determine the clinical effectiveness of novel therapeutic strategies destined to modulate nTreg function *in vivo*. New biomarkers may also help to selectively attenuate or enhance Treg-mediated immune suppression. Lastly, the mechanism underlying nTreg cell-mediated suppression still remains highly contentious, with noticeable differences between various *in vitro* and *in vivo* studies in mice and humans. A clearer definition of the cellular and molecular factors underlying the Treg cell development and function will shed light in the development of novel immunotherapeutic strategies which are destined to either potentiate or abrogate nTreg functions and consequently used for the treatment and prevention of various inflammatory disorders.

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