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Naturally arising Foxp3-expressing CD25$^+$CD4$^+$ regulatory T cells in immunological tolerance to self and non-self

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Naturally arising CD25$^+$CD4$^+$ regulatory T cells actively maintain immunological self-tolerance. Deficiency in or dysfunction of these cells can be a cause of autoimmune disease. A reduction in their number or function can also elicit tumor immunity, whereas their antigen-specific population expansion can establish transplantation tolerance. They are therefore a good target for designing ways to induce or abrogate immunological tolerance to self and non-self antigens.

A key issue in immunology is understanding how the immune system is able to discriminate between self and non-self, inhibiting autoimmune responses but allowing effective immune responses against microbial antigens. The immune system has evolved several mechanisms to establish and sustain unresponsiveness to self antigens (immunological self-tolerance), including physical elimination or functional inactivation of self-reactive lymphocytes (clonal deletion and anergy, respectively). There is also substantial evidence that T cell–mediated active suppression of self-reactive T cells is another essential mechanism of self-tolerance[1–4]. Although the idea of T cells that negatively control immune responses is not new for immunologists, there has been great controversy as to whether they actually constitute a functionally distinct cellular entity in the immune system and, if they exist, whether they are important in controlling immunological disorders such as autoimmune disease. Recent years, however, have witnessed resurgent interest in suppressor or regulatory T cells (T_{reg} cells) in many fields of basic and clinical immunology[4,5]. This change is partly due to an improved understanding that the normal immune system endogenously produces as a normal cellular constituent a CD4$^+$ T cell subpopulation that is highly specialized for suppressive function and that an abnormality in the number or function of these cells can be a chief cause of autoimmune and other inflammatory diseases in animals and humans[4].

A cardinal feature of endogenous T_{reg} cells is that most if not all are produced by the normal thymus as a functionally distinct and mature T cell subpopulation and are not induced *de novo* from naive T cells after antigen exposure in the periphery. Most endogenous CD4$^+$ T_{reg} cells constitutively express the CD25 molecule (IL-2 receptor α-chain (IL-2Rα))[6]. In addition, they specifically express *Foxp3*, which encodes a transcription factor, as a key control gene in their development and function[7–9]. With CD25 and Foxp3 as specific molecular markers for detecting and manipulating naturally occurring T_{reg} cells, there is now accumulating evidence that the Foxp3$^+$CD25$^+$CD4$^+$ T_{reg} cell population is actively engaged in the negative control of a variety of physiological and pathological immune responses and can be exploited not only for the prevention or treatment of autoimmune diseases but also for the induction of immunological tolerance to non-self antigens (such as transplantation tolerance), negative control of aberrant immune responses (such as allergy and immunopathology) and enhancement of host defense (such as tumor immunity and microbial immunity)[4]. This review will discuss how naturally arising Foxp3$^+$CD25$^+$CD4$^+$ T_{reg} cells maintain immunological tolerance to self antigens, how their developmental or functional abnormality causes autoimmune disease and how they can be manipulated to control autoimmune responses, to elicit immune responses to 'quasi-self' antigens, as in tumor immunity, or to establish tolerance to non-self antigens, as in organ transplantation.

Thymus-generated natural T_{reg} cells

A crucial experiment for addressing the function of T_{reg} cells in natural self-tolerance is to determine whether their removal from the normal immune system can break self-tolerance, resulting in autoimmune disease. Attempts have been made since the mid-1980s to resolve this issue and identify the purported T_{reg} cells by expression of particular cell surface molecules, such as CD5, CD45RC and CD25 (refs. 4,6,10–12). Such efforts have shown that the CD25 molecule, which is expressed by 5–10% of CD4$^+$ T cells and 5% of CD4$^+$CD8$^-$ mature thymocytes in normal naive mice, is able to operationally if not specifically differentiate naturally present autoimmune-preventive T_{reg} cells from other T cells[6,13–15]. For example, transfer of CD25$^+$ cell–depleted T cell or thymocyte suspensions from normal mice into syngeneic T cell–deficient nude mice results in various autoimmune

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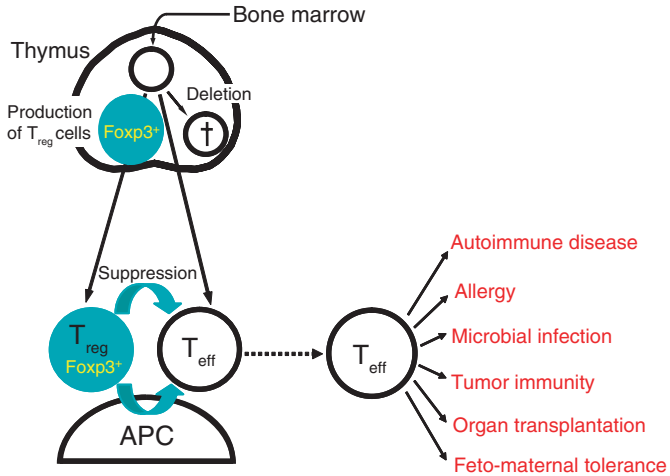

Figure 1 Linkage of central and peripheral tolerance by thymic production of natural $CD25^+CD4^+$ T_{reg} cells that contribute to peripheral self-tolerance. A reduction in $CD25^+CD4^+$ T_{reg} cells or attenuation of their suppressive activity may elicit autoimmunity, tumor immunity, microbial immunity and allergy. In contrast, an increase in the number of T_{reg} cells or augmentation of their suppressive activity may establish transplantation tolerance and maintain feto-maternal tolerance. T_{reg} cells may control effector T cells (T_{eff} cells) either directly or indirectly through APCs. †, death.

diseases in the recipient mice, and transfer of $CD25^+CD4^+$ T cells or thymocytes together with the $CD25^+$ cell–depleted population prevents those diseases[6,14]. Similar transfer of $CD25^-CD4^+$ T cells, especially $CD25^-CD45RB^{hi}CD4^+$ T cells, induces colitis in T cell– and B cell–deficient mice[16]. $CD25^+$ cell–depleted mice also show enhanced immune responses to non-self antigens, such as xenogeneic proteins or allogeneic transplants[6]. In addition, selected strains of normal mice spontaneously develop a similar spectrum of autoimmune diseases after thymectomy during the critical neonatal period (2–4 days after birth) when $CD25^+CD4^+$ T cells become detectable in the periphery; again, transfer of $CD25^+CD4^+$ T cells or thymocytes from normal mice without thymectomy prevents the autoimmunity[13].

These mouse autoimmune and inflammatory diseases are immunopathologically similar to their human counterparts, such as autoimmune gastritis and pernicious anemia, Hashimoto thyroiditis, adrenalitis and Addison's disease, insulitis and type I diabetes, premature ovarian failure with autoimmune oophoritis, and inflammatory bowel disease. Thus, one aspect of peripheral self-tolerance is maintained by $CD25^+CD4^+$ T_{reg} cells, which actively suppress the activation and population expansion of potentially pathogenic self-reactive T cells normally present in the immune system (**Fig. 1**). They also control immunopathology, such as inflammatory bowel disease due to excessive immune responses to commensal bacteria in the intestine. The normal thymus continuously produces T_{reg} cells as a functionally distinct and mature T cell subpopulation, which seems to constitute a distinct cellular lineage contiguous with peripheral $CD25^+CD4^+$ T_{reg} cells (reviewed by Fontenot and Rudensky[17] in this issue). Furthermore, their generation in the normal immune system is in part developmentally programmed.

Evidence for human natural T_{reg} cells in self-tolerance

The X-linked immunodeficiency syndrome IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) is associated with autoimmune disease in multiple endocrine organs (such as type I diabetes and thyroiditis), inflammatory bowel disease, severe allergy including atopic dermatitis and food allergy, and fatal infection[18]. IPEX is caused by mutations in *FOXP3* (*Foxp3* in mice), which encodes the forkhead–winged-helix family transcription factor Foxp3. Mutation of *Foxp3* was first identified as being responsible for an X-linked recessive inflammatory disease in scurfy mutant mice and subsequently for IPEX in humans[19–22].

Foxp3 is crucial in the development and function of natural $CD25^+CD4^+$ T_{reg} cells[7–9]. For example, $CD25^+CD4^+$ peripheral T cells and $CD25^+CD4^+CD8^-$ thymocytes specifically express Foxp3, whereas other thymocytes, T cells, B cells, natural killer cells and natural killer T cells do not[7,8]. Notably, in contrast to the stable expression of Foxp3 in natural T_{reg} cells, activated naive T cells or differentiated T helper type 1 or type 2 cells do not express Foxp3, indicating that its expression is highly specific for natural T_{reg} cells[7–9] (discussed below). Foxp3-deficient mice fail to develop $CD25^+CD4^+$ T_{reg} cells and succumb to scurfy-like inflammatory diseases, which can be prevented by transfer of normal $CD25^+CD4^+$ T_{reg} cells[8]. Furthermore, retroviral transduction or transgenic expression of *Foxp3* in $CD25^-CD4^+$ T cells or $CD8^+$ T cells phenotypically and functionally converts them to natural T_{reg}-like cells; for example, *Foxp3*-transduced $CD25^-CD4^+$ T cells are able to suppress proliferation of other T cells *in vitro* as well as suppress the development of autoimmune disease and inflammatory bowel disease *in vivo*[7–9]. Transduction of *Foxp3* also suppresses IL-2 production but upregulates the expression of T_{reg} cell–associated molecules, such as CD25, cytotoxic T lymphocyte–associated antigen 4 (CTLA-4) and glucocorticoid-inducible tumor necrosis factor receptor (GITR)[7] (**Fig. 2**; discussed below). Thus, *Foxp3* (or *FOXP3* in humans) seems to be a 'master control gene' for the development and function of natural $CD25^+CD4^+$ T_{reg} cells, indicating that the development of natural T_{reg} cells is at least in part genetically and developmentally programmed. It is most likely that in IPEX, disruption of *FOXP3* abrogates the development of T_{reg} cells or alters their function, leading to hyperactivation of T cells reactive with self antigens, intestinal bacteria or innocuous environmental substances, thus causing autoimmune polyendocrinopathy, inflammatory bowel disease and allergy, respectively. These findings substantiate the key contribution of natural T_{reg} cells to the control of immune responses to both self and non-self antigens in humans (**Fig. 1**). Notably, females with a hemizygous defect in *FOXP3*, which produces genetic mosaicism of normal and defective T_{reg} cells because of random inactivation of the X chromosome in individual T_{reg} cells, are completely normal and do not have an intermediate disease phenotype[23]. This indicates that the residual normal T_{reg} cells dominantly control self-reactive T cells in these females, similar to $CD25^+CD4^+$ T_{reg} cell–replenished scurfy mice, demonstrating that the mechanism of dominant self-tolerance is operating physiologically in humans.

Generation and maintenance of natural T_{reg} cells by IL-2

CD25 is a useful molecular marker for operationally differentiating natural T_{reg} cells from other T cells, as discussed above. Every T cell, however, expresses CD25 after activation. Accumulating evidence suggests that CD25 is not merely a marker for the chronically activated state of natural T_{reg} cells but is a crucial molecule for their generation, survival and function. For example, deficiency in IL-2, IL-2Rα (CD25) or IL-2Rβ (CD122) produces similar fatal lymphoproliferative inflammatory disease with autoimmune components (such as inflammatory bowel disease, lymphoproliferation and lymphocytic infiltration into multiple organs), generally called IL-2 defi-

ciency syndrome[24–27] (**Fig. 2** and **Table 1**). Notably, the number of $CD25^+CD4^+$ T cells is selectively reduced in the thymus and periphery of IL-2-deficient mice, regardless of the normal number, subset composition and function of other $CD4^+$ and $CD8^+$ T cells[24,28]. This syndrome can be prevented by inoculation of IL-2-replete spleen cells or thymocytes[28–31]. Transfer of IL-2-deficient bone marrow cells produces multiorgan inflammation in mice deficient in recombination-activating gene(s), whereas transfer of normal bone marrow cells together with those cells inhibits the inflammation by giving rise to $CD25^+CD4^+$ T_{reg} cells[29,30]. Furthermore, administration of neutralizing monoclonal antibody to IL-2 to normal naive mice selectively reduces the number of $CD25^+CD4^+$ T cells in the thymus and periphery[32,33], resulting in the development of organ-specific autoimmune diseases similar to those produced by depletion of $CD25^+CD4^+$ T cells[33]. IL-2 neutralization inhibits the physiological proliferation of $CD25^+CD4^+$ T cells that are presumably responding to normal self antigens[33].

IL-2 is also required for the *in vivo* and *in vitro* activation of T_{reg} cells and for sustaining their CD25 expression[34,35]. The main source of IL-2 responsible for the *in vivo* maintenance and activation of $CD25^+CD4^+$ T_{reg} cells seems to be other T cells, including self-reactive T cells, in the physiological steady state[33]. Thus, IL-2 mediates a feedback control between responder T cells and T_{reg} cells; that is, IL-2 secreted by responder T cells maintains and activates T_{reg} cells, which in turn inhibit IL-2 production in responder T cells.

Thus, IL-2 is a vital cytokine for the thymic generation and peripheral maintenance of natural $CD25^+CD4^+$ T_{reg} cells. CD25 is therefore indispensable for natural T_{reg} cells as a component of the high-affinity IL-2R, because mouse IL-2R requires all three receptor chains (α, β and γ) for IL-2 binding, in contrast to human IL-2R, for which β- and γ-chains can also form an intermediate-affinity receptor for IL-2 (refs. 36,37). This high IL-2 dependency, in addition to Foxp3 expression, is an important feature of natural T_{reg} cells that may differentiate them from other T cells and other types of T_{reg} cells, such as IL-10- or TGF-β-dependent T_{reg} cells[38,39]. Although these findings indicate CD25 is a 'rational' molecular marker for natural T_{reg} cells, some T cells in the $CD25^-CD45RB^{lo}CD4^+$ T cell fraction, which represents less than 10% of $CD25^-CD4^+$ T cells in normal naive mice, also have intermediate expression of *Foxp3* and show *in vitro* regulatory activity[7,40,41]. These $CD25^-CD4^+$ T_{reg} cells become $Foxp3^{hi}$ and $CD25^+$ after T cell receptor (TCR) stimulation *in vivo* and *in vitro* (refs. 7,40 and M. Ono and S.S., unpublished data). An autoimmune-suppressive activity found in the $CD25^-$ T cell population in normal rodents could therefore be attributed to this $CD25^-$ fraction of natural T_{reg} cells[42]. It remains to be determined whether such $CD25^-CD4^+$ T_{reg} cells are derived from $CD25^+CD4^+$ T_{reg} cells that have lost CD25 expression, produced in the thymus as a functionally committed population in parallel with $CD25^+CD4^+$ T_{reg} cells, or have differentiated in the periphery from $CD25^-$ naive T cells, which also have low but detectable expression of Foxp3 mRNA[7,41].

Control of the development and activation of natural T_{reg} cells

There are several key molecules, including CD25 and Foxp3, whose deficiency or functional alteration affects the generation or function of natural T_{reg} cells and thereby causes autoimmune diseases (**Table 1** and **Fig. 2**). These molecules provide important clues to understanding the functions of natural T_{reg} cells in immunological tolerance and immunoregulation (reviewed by von Boehmer[43] in this issue).

An intriguing feature of natural $CD4^+$ T_{reg} cells, whether $CD25^+$ or $CD25^-$, is that they constitutively express CTLA-4, whereas naive T cells express the molecule only after T cell activation[44–46]. This raises the issue of what function, if any, the CTLA-4 molecules expressed by natural T_{reg} cells have in the control of immune responses. In addition to substantial evidence for CTLA-4-transduced negative signaling in activated effector T cells, several findings also support the possible contribution of CTLA-4 to T_{reg} cell–mediated suppression. Administration of monoclonal antibody to CTLA-4 to normal young naive mice over a limited period elicits autoimmune disease similar to that produced by depletion of $CD25^+CD4^+$ T_{reg} cells, without reducing their number[46]. Likewise, treatment with monoclonal antibody to CTLA-4 treatment abolishes the protective activity of $CD25^+CD4^+$ T_{reg} cells in a mouse inflammatory bowel disease model[45]. Furthermore, a lethal lymphoproliferative and autoimmune syndrome that spontaneously develops in CTLA-4-deficient mice is not T cell autonomous but can be inhibited by wild-type T cells[47]. Finally, blockade of CTLA-4 by Fab fragments of monoclonal antibody to CTLA-4 abrogates *in vitro* $CD25^+CD4^+$ T_{reg} cell–mediated suppression in a setting in which T_{reg} cells are prepared from normal mice (and hence constitutively express CTLA-4) and responder T cells are from CTLA-4-deficient mice[45,48]. CTLA-4 blockade also abrogates *in vitro* T_{reg} cell–mediated suppression in humans[49]. These results collectively suggest that CTLA-4 on T_{reg} cells may transduce a costimulatory signal; that is, signals via CTLA-4 and the TCR together activate T_{reg} cells to exert suppression and CTLA-4 blockade prevents T_{reg} cell activation and hence attenuates suppression, causing autoimmune diseases.

Another possible function of CTLA-4 in T_{reg} cells is that it may directly mediate suppression. CTLA-4 on T_{reg} cells triggers induction of the enzyme indolamine 2, 3-dioxygenase by interacting with CD80 and/or CD86 on dendritic cells (DCs)[50]. This enzyme catalyzes the conversion of tryptophan to kynurenine and other metabolites, which have potent immunosuppressive effects in the local environment of the DC[51]. CTLA-4 expressed by T_{reg} cells may also ligate CD80 and, to a lesser extent, CD86 expressed by responder T cells and directly transduce a negative signal to them[52]. These possible functions of CTLA-4 in T_{reg} cell–mediated suppression, however, need to be further substantiated, as CTLA-4-deficient $Foxp3^+CD25^+CD4^+$ T_{reg} cells present in CTLA-4-deficient mice have *in vitro* suppressive activity equivalent to that of CTLA-4-intact $CD25^+CD4^+$ T_{reg} cells from normal mice[46,48].

Signals through CD28 are critical for thymic generation of $CD25^+CD4^+$ T_{reg} cells and their self-renewal and survival in the periphery. The number of $CD25^+CD4^+$ T cells is substantially reduced

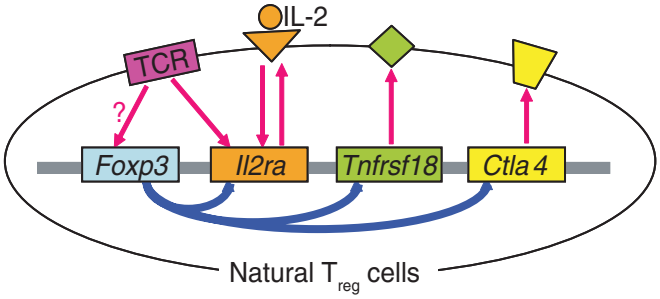

Figure 2 Control of T_{reg} cell–associated molecules in natural T_{reg} cells. Foxp3 seems to control some genes encoding T_{reg} cell–associated molecules (such as CD25, CTLA-4 and GITR). TCR signals and also the binding of IL-2 to IL-2R enhance CD25 expression. Although Foxp3 is stably expressed in mature natural T_{reg} cells, it remains to be determined whether TCR signals control Foxp3 expression in the thymic development of T_{reg} cells.

in the thymus and periphery of CD28- or B7-deficient mice[44,46,53]. Similarly, administration of monoclonal antibody to B7 (CD80 and CD86) or B7 blockade by CTLA-4–immunoglobulin reduces T_{reg} cell numbers in the thymus and periphery of normal as well as thymectomized mice[44]. Notably, CD28 or B7 deficiency or B7 blockade accelerates the development of type I diabetes in nonobese diabetic mice, indicating a predominant effect of T_{reg} cell deficiency on the development of this autoimmune disorder and possible activation of autoimmune effector T cells without CD28-B7 costimulation[44]. It is likely that CD28- or B7-deficiency or B7 blockade may hamper the activation of conventional T cells and consequently their IL-2 production, leading to a reduction in natural T_{reg} cells because of IL-2 deficiency[33,53] (discussed above). Furthermore, a CD28 signal to peripheral $CD25^+CD4^+$ T_{reg} cells, together with a TCR signal, attenuates suppression; for example, strong *in vitro* ligation of CD28 along with TCR stimulation abrogates the anergic and suppressive state of $CD25^+CD4^+$ T_{reg} cells, triggering their proliferation[54,55]. Correspondingly, antigen-presenting mature $CD86^{hi}$ DCs trigger T_{reg} cell proliferation, indicating that activated DCs not only activate naive T cells but also attenuate T_{reg} cell–mediated suppression, thus synergistically provoking effective immune responses to microbes[56,57]. In contrast, when immature DCs with low expression of CD80 and CD86 molecules present self peptides in the absence of DC-maturing 'danger signal', they may activate mainly natural T_{reg} cells and hence sustain self-tolerance. T_{reg} cells may be biased to such a response because of their constitutive expression of CTLA-4 with higher avidity to CD80 and CD86 than CD28, their highly self-reactive TCRs and their apparently 'antigen-primed' state resulting in a low activation threshold[4]. Furthermore, the expression of CD80 or CD86 may differentially activate or deactivate T_{reg} cells; for example, CD80 has much higher affinity for CTLA-4 than does CD86; similarly, CD86 has a higher affinity for CD28 than does CD80 (refs. 58,59).

Natural $CD4^+$ T_{reg} cells, including $CD25^+$ and $CD25^-$ T_{reg} cells, express GITR[60,61]. Other T cells, B cells, DCs and macrophages have low expression of GITR, but increase their GITR expression after activation[60,61]. Although GITR-deficient mice are reportedly normal, administration of agonistic monoclonal antibody to GITR to normal mice elicits autoimmune diseases similar to that produced by T_{reg} cell depletion[60,62] (**Table 1**). *In vitro* studies have shown that crosslinking of GITR, not its blockade, by a specific monoclonal antibody, together with TCR stimulation, abrogates $CD25^+CD4^+$ T_{reg} cell–mediated suppression and also triggers proliferation of T_{reg} cells in the presence of IL-2 (refs. 60,61). How an active GITR signal can abrogate suppression, both *in vivo* and *in vitro*, remains controversial. In the presence of a TCR signal, it may attenuate suppressive activity of T_{reg} cells, activate responder T cells to overcome suppression or render responder T cells resistant to suppression[60,63–65]. Of note, the natural ligand for GITR is expressed by immature DCs, macrophages and B cells and is downregulated in DCs after maturation[64–66] (T. Nishioka and S.S., unpublished data). It remains to be determined how this change in the expression of GITR ligand with DC maturation, together with reciprocal changes in the expression of major histocompatibility complex class II, CD80 and CD86, contributes to the control of immune activation or tolerance.

The number of $CD25^+CD4^+CD8^-$ thymocytes and T cells is substantially reduced in CD40-deficient mice and to a lesser degree in CD40 ligand–deficient mice[67–69]. No autoimmunity develops in CD40-deficient mice however, because CD40-deficiency would impair activation of self-reactive T cells. In contrast, transfer of spleen cells from CD40-deficient mice to syngeneic athymic nude mice results in various autoimmune diseases similar to those produced by the removal of $CD25^+CD4^+$ T_{reg} cells[67]. The transferred T cell populations, which contain a reduced number of T_{reg} cells, could be activated by the recipient CD40-intact antigen-presenting cells (APCs) presenting self antigens. Reconstitution of nude mice with CD40-deficient T cells and normal $CD25^+CD4^+$ T cells indeed inhibits the autoimmune development[67]. Thus, CD40 is critically required at least for the thymic development of natural T_{reg} cells. The CD40–CD40 ligand interaction may also contribute to activation of APCs including DCs and thereby activation of T_{reg} cells.

Toll-like receptors (TLRs) detect microbial infection and have an essential function in the induction of innate and adaptive immunity. A mechanism of TLR-mediated activation of T cell responses seems to be blockade of suppression by T_{reg} cells[70,71]. Stimulation of DCs deficient in the adaptor molecule MyD88 using lipopolysaccharide leads to their maturation and migration to lymph nodes, but not to secretion of TLR-induced proinflammatory cytokines, and fails to elicit primary $CD4^+$ T cell responses, whereas depletion of $CD25^+CD4^+$ T_{reg} cells in this setting restores the T cell response. Cytokines (IL-6 in particular) secreted by TLR-activated DCs render naive T cells resistant to suppression, thus enabling them to respond effectively to invading microbes[70]. In addition, natural $CD25^+CD4^+$ T_{reg} cells are reported to selectively express several members of the TLR family, such as TLR4 (ref. 72). *In vitro* stimulation of $CD25^+CD4^+$ T cells with a high concentration of lipopolysaccharide via TLR4 elicits their proliferation, prolongs their survival, and augments their *in vitro* suppressive activity even in the absence of APCs, indicating that lipopolysaccharide directly acts on TLR4 expressed by T_{reg} cells[72]. It will be useful to determine how stimulation of TLRs on DCs or T_{reg} cells directly or indirectly control T_{reg} cell–mediated suppression. Cytokines such as TGF-β and IL-10 may also be involved in T_{reg} cell–mediated regulation; they mediate suppression, contribute to the expansion and differentiation of T_{reg} cell populations or condition other T cells to become susceptible to suppression[38,39,72–76], as discussed by Fontenot and Rudensky[17] and von Boehmer[43] in this issue.

Table 1 Development of autoimmune disease in gene-deficient mice or in normal mice treated with monoclonal antibody

Condition	Autoimmune disease	Reference
Gene deficiency		
Foxp3	+	8
Il2	+	24,25
Il2ra	+	26
Il2rb	+	27
Ctla4	+	104,105
Tnfrsf18	–	62
Monoclonal antibody treatment		
Anti-IL-2 (N)	+	33
Anti-CD25 (D)	+	46,106,107
Anti-CTLA-4 (B)	+	46
Anti-GITR (A)	+	60

Top, mutant mice; bottom, normal mice. Autoimmune disease developed in experiments in reference 106, but not in those in references 46 and 107. Gene products: *Foxp3*, Foxp3; *Il2*, IL-2; *Il2ra*, CD25 (IL-2Rα); *Il2rb*, CD122 (IL-2Rβ); *Tnfrsf18*, GITR. Monoclonal antibodies: N, neutralizing; D, depleting; B, blocking; A, agonistic.

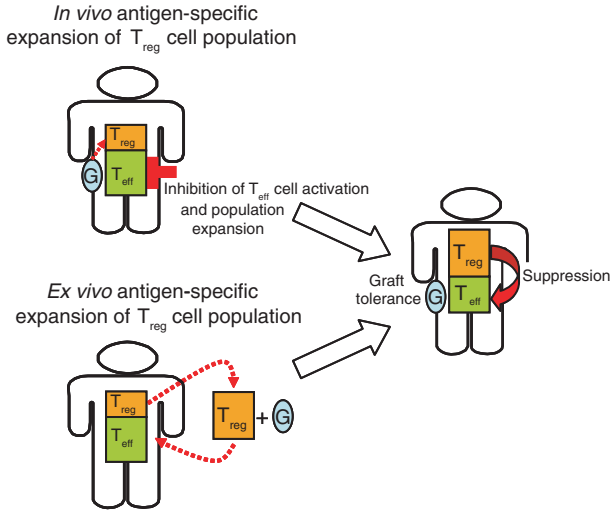

Figure 3 Induction of tolerance to organ grafts through *in vivo* or *ex vivo* antigen-specific population expansion of T_{reg} cells. T_{reg} cells undergo *in vivo* antigen-specific population expansion after sensitization with graft (G) antigens. Graft rejection may be suppressed by selective inhibition of the activation and population expansion of effector T cells during T_{reg} cell sensitization and population expansion, for example, by blocking T cell costimulatory or accessory molecules and, hence, establishing graft tolerance. Antigen-specific T_{reg} cell population expansion can also be achieved *ex vivo* by stimulation of T_{reg} cells with allo antigens in the presence of high doses of IL-2. Transfer of the *ex vivo*–expanded T_{reg} cell population can induce dominant graft tolerance.

Thus, various accessory or costimulatory molecules and cytokines are key in controlling the thymic development, peripheral survival and activation of natural $CD25^+CD4^+$ T_{reg} cells (and responder T cells) and thereby contribute to 'tuning' the intensity of T_{reg} cell–mediated suppression and stable maintenance of self-tolerance (**Fig. 2**). How these signals are integrated in T_{reg} cells remains to be elucidated.

Autoimmunity, tumor immunity and organ transplantation

Natural $CD25^+CD4^+$ T_{reg} cells have several immunological features important for their use in inducing or attenuating immunological tolerance to self or non-self antigens. The TCR repertoire of natural $CD25^+CD4^+$ T_{reg} cells is as broad and diverse as that of $CD25^-CD4^+$ T cells but is skewed more than the latter toward recognizing complexes of self peptide and major histocompatibility complex expressed in the thymus and periphery[54,77]. They can recognize normal self antigens targeted in autoimmune disease[78], tumor-associated antigens[79] and allogeneic transplantation antigens[40]. When stimulated by their respective antigens, they can therefore suppress autoimmunity, hamper tumor immunity and suppress graft rejection. Moreover, they undergo antigen-specific proliferation *in vivo* after strong antigenic stimulation or *in vitro* stimulation with antigen and high-dose IL-2, although to a lesser degree than the proliferation of effector T cells[40,54,55,80,81]. They are actually more proliferative than other T cells in the physiological steady state through the recognition of self antigens[33,82]. Finally, they are already specialized in suppressive function before antigen exposure and are functionally stable after *in vivo* or *in vitro* population expansion[54,55], indicating that simple antigen-specific population expansion is sufficient for the preparation of antigen-specific T_{reg} cells.

As mentioned above, depletion or dysfunction of natural $CD25^+CD4^+$ T_{reg} cells alone suffices to cause autoimmune disease in otherwise normal animals regardless of the physiological presence of other types of T_{reg} cells and the possible capacity of naive $CD4^+$ or $CD8^+$ T cells to differentiate to T_{reg} cells in certain conditions[17]. This means that any genetic abnormality or environmental insult could be a cause of or predisposing factor for autoimmune disease, especially organ-specific ones, if it reduces the number or affects the function of natural $CD25^+CD4^+$ T_{reg} cells or, more generally, tips the balance between natural T_{reg} cells and self-reactive T cells toward dominance of the latter[4,5,83,84]. As discussed above, deficiency or functional alteration of costimulatory or accessory molecules (such as CTLA-4 or GITR) on T cells or APCs, or a cytokine (such as IL-2), can indeed break self-tolerance and cause similar autoimmune disease by affecting natural T_{reg} cells. Polymorphisms of the genes encoding these molecules (for example, CTLA-4 and IL-2) have also been shown to affect autoimmunity in humans and rodents[85–87]. In a therapeutic context, there is accumulating evidence with animal models that natural $CD25^+CD4^+$ T_{reg} cell populations expanded *in vivo* or *ex vivo* can be used not only for preventing autoimmune disease but also for treating ongoing autoimmune responses by suppressing the population expansion and function of effector T cells[88,89]. Furthermore, it is well documented that the incidence of autoimmune and other inflammatory diseases, including inflammatory bowel disease and allergy, has increased recently in developed countries[90]. Assuming that natural $CD25^+CD4^+$ T_{reg} cells are key in the prevention of these diseases in humans, as best exemplified by IPEX, this could be attributed in part to insufficient adaptive population expansion or activation of natural T_{reg} cells because of less frequent opportunities for microbial infections in hygienic environments. This needs to be examined at the population level.

The fact that many tumor-associated antigens recognized by autologous T cells in cancer patients are antigenically normal self constituents indicates that natural $CD25^+CD4^+$ T_{reg} cells engaged in the maintenance of self-tolerance may concomitantly impede immunosurveillance against autologous tumor cells[91,92]. This possibility seems to be the case, because depletion of $CD25^+CD4^+$ T cells before tumor challenge elicits effective immune responses to syngeneic tumors in otherwise nonresponding mice[93–96]. Furthermore, T cell populations reactive with tumor-associated antigens can expand and become detectable in the peripheral blood of normal humans when T cell samples are first depleted of $CD25^+CD4^+$ T cells *in vitro* and then stimulated with the antigens[97]. Manipulation of natural T_{reg} cells also helps in the treatment of established tumors. For example, administration of monoclonal antibody to CTLA-4 or to GITR, either of which can cause autoimmune disease in mice, is able to elicit effective tumor immunity by both attenuating T_{reg} cell–mediated control of effector T cells and enhancing their effector activity[95,98] (K. Ko and S.S., unpublished data). It is possible that a combination of tumor vaccination plus T_{reg} cell manipulation, especially at the site of vaccination, may make cancer immunotherapy more efficacious, with the caveat that certain genetically autoimmune-susceptible people could develop autoimmunity when natural T_{reg} cells are systemically manipulated[98].

The ultimate goal of organ transplantation is to establish graft tolerance that is as stable as natural self-tolerance, without continuous general immunosuppression. Natural $CD25^+CD4^+$ T_{reg} cells show spontaneous alloantigen-specific population expansion *in vivo* when exposed to allografts (for example, allogeneic skin grafts)[40]. Their antigen-specific population expansion can also be achieved *in vitro* by antigenic stimulation in the presence of high-dose IL-2 (refs. 40,99,100).

By exploiting these properties of natural $CD25^+CD4^+$ T_{reg} cells, there are two possible ways of using them to induce graft-specific tolerance without hampering immune responses to other antigens (**Fig. 3**). One is to prepare an immunological condition facilitating antigen-specific spontaneous population expansion of natural T_{reg} cells by controlling graft-reactive effector T cells; for example, reducing the number or blocking the activation of non–T_{reg} cells as specifically as possible and meanwhile sensitizing the remaining $CD25^+CD4^+$ T_{reg} cells to alloantigens, allowing an alloantigen-specific T_{reg} cell population to expand to the extent that it can dominantly suppress graft-reactive T cells recovering from the reduction or blockade[40]. Certain monoclonal antibodies or drugs whose administration for a limited period can induce long-term T_{reg} cell–dependent graft tolerance may have this effect as a common mechanism of tolerance induction[101,102]. Another method of tolerance induction using natural T_{reg} cells is to isolate $CD25^+CD4^+$ T_{reg} cells from the recipient of the organ transplant, stimulate them *ex vivo* with donor stimulator cells and IL-2, and transfer the expanded antigen-specific T_{reg} cell populations back into the recipient, as is done in living donor transplantation or when donor stimulator cells can be preserved for T_{reg} cell stimulation. Antigen-specific *Foxp3*-transduced T cells or adaptive T_{reg} cells prepared from alloantigen-reactive T cells may also be instrumental in this cell therapy. These *in vivo* and *in vitro* ways of inducing immunological tolerance to organ transplants could in principle be applied to re-establish self-tolerance in autoimmune disease, control aberrant or excessive immune responses to non-self antigens (immunopathology and allergy) and maintain feto-maternal tolerance in pregnancy[103] (**Fig. 1**).

Conclusion and perspectives

Thymus-derived $Foxp3^+CD25^+CD4^+$ T_{reg} cells link central and peripheral mechanisms of self-tolerance; that is, thymic T cell selection is responsible for both negative selection of self-reactive T cells and production of natural T_{reg} cells, which act in the periphery to control self-reactive T cells that have escaped thymic negative selection. Control of immune responses to self, 'quasi-self' and non-self is also closely linked, as the same T_{reg} cell population is responsible for self-tolerance and hyporesponsiveness to tumor antigens and can be exploited to induce transplantation tolerance. $Foxp3^+CD25^+CD4^+$ T_{reg} cells are naturally present in the normal immune system as a phenotypically and functionally distinct T cell subpopulation. They are therefore a good target for designing ways to treat and prevent immunological diseases and to control pathological and physiological immune responses. Future searches for specific molecular markers, particularly cell surface molecules, that can reliably differentiate between T_{reg} cells and effector T cells is essential for the specific manipulation of T_{reg} cells. Further elucidation of the molecular basis of their development and function of T_{reg} cells, especially the molecular mechanism of T_{reg} cell-mediated suppression, is also needed for reliable control of their function in clinical settings.

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COMPETING INTERESTS STATEMENT

The author declares that he has no competing financial interests.

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