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Cornerstone of peripheral tolerance: naturally occurring CD4^{+}CD25^{+} regulatory T cells

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Immunological tolerance involves maintaining a state of unresponsiveness to autoantigens while generating protective immunity against invading pathogens. Recently, a surprising resurgence in research on regulatory T cells has been noted, and few areas have enthralled immunologists and generated so many ardent debates. Naturally occurring CD4^{+}CD25^{+} regulatory T cells have become the cornerstone T-cell population mediating peripheral tolerance to autoantigens, as well as regulation of T-cell responses directed at foreign antigens. However, several fundamental questions concerning CD4^{+}CD25^{+} T cells remain unanswered. Here, we propose to critically re-evaluate some recent conclusions and provide a consensus overview.

A hallmark feature of autoimmune diseases is a breakdown in peripheral immunoregulatory mechanisms assuring self- and non-self-tolerance. Thymic clonal deletion of autoreactive T cells is the primary mechanism that leads to self-tolerance but, interestingly, autoreactive T cells are normally present in most individuals. However, autoimmunity manifests itself only infrequently in the population, suggesting that mechanisms for peripheral tolerance operate to silence potentially pathogenic T cells. Current evidence points to regulatory T (Treg) cells as an active mechanism to suppress autoreactive T cells that escape central tolerance.

The notion of Treg cells, originally termed suppressor T cells, transpired from experiments performed in the 1960–1970s describing the induction of suppressor T cells capable of downregulating antigen-specific T cell responses [1]. Conflicting findings rendered it difficult to characterize the phenotype and molecular pathway of the suppressor mechanism. It is now well accepted that Treg cells modulate immune responses to a variety of antigens.

Several types of Treg populations have been described, each with a specific surface phenotype and cytokine production potential after activation, and include cross-regulatory Th1 and Th2 cells, interleukin-10 (IL-10)-producing Tr1 cells and transforming growth factor-β (TGF-β)-secreting Th3 cells [2–4]. More recently, a seminal observation made by Sakaguchi and colleagues indicated that CD4^{+} T cells expressing CD25 [IL-2 receptor (R) α chain], which constitute 5–10% of the peripheral, naïve CD4^{+} T-cell repertoire of normal mice and humans, and thus termed naturally occurring, possess potent immunoregulatory functions [5] (Figure 1). Indeed, naturally occurring CD4^{+}CD25^{+} Treg (nTreg) cells have emerged as the dominant T-cell population governing peripheral self-tolerance in experimental animals and humans [6–16] because they prevent the development of multi-organ-specific autoimmunity induced by day 3 thymectomy, or transfer of CD4^{+} T-cell populations depleted of nTreg cells, and their removal from peripheral lymphoid compartments appears to increase tumor immunity, graft rejection and pathogen clearance [5,17–20]. Here, we propose to re-evaluate some recent findings and provide a consensus overview of the phenotype, development and functional properties of CD4^{+}CD25^{+} nTreg cells.

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CD4^{+}CD25^{+} mediated control of T-cell activation *in vitro*

Since the initial discovery of CD4^{+}CD25^{+} nTreg cells, research has centered on their phenotype, activation requirements and mechanism of suppression, the molecular basis of which is still not known. To date, no conclusive answer has been found, and the few studies that purport to resolve this question have generated controversy without leading to a resolution of the issue.

Requirements for activation in vitro*: how many signals?*

Freshly isolated CD4^{+}CD25^{+} nTreg cells do not manifest suppressive activity, and T-cell receptor (TCR) stimulation is required to induce suppressor function [20,21]. Once pre-stimulated with antigen and IL-2, they suppress both CD4^{+} and CD8^{+} T-cell responses in an antigen non-specific (bystander) fashion [22–24]. In a recent study, Yamazaki *et al.* reported that CD4^{+}CD25^{+} cells can be expanded with dendritic cells (DCs) in the absence of exogenous cytokines, and that expansion and induction of suppressive function require B7 co-stimulation [25]. Recently, we have examined the *in vitro* CTLA-4 or CD28 co-stimulatory requirements for the generation of nTreg cell-mediated suppression [26], and showed that when CD4^{+}CD25^{+} cells were pre-activated with either wild type (WT) or CD80$^{-/-}$ and CD86$^{-/-}$ antigen-presenting cells (APCs) or in the

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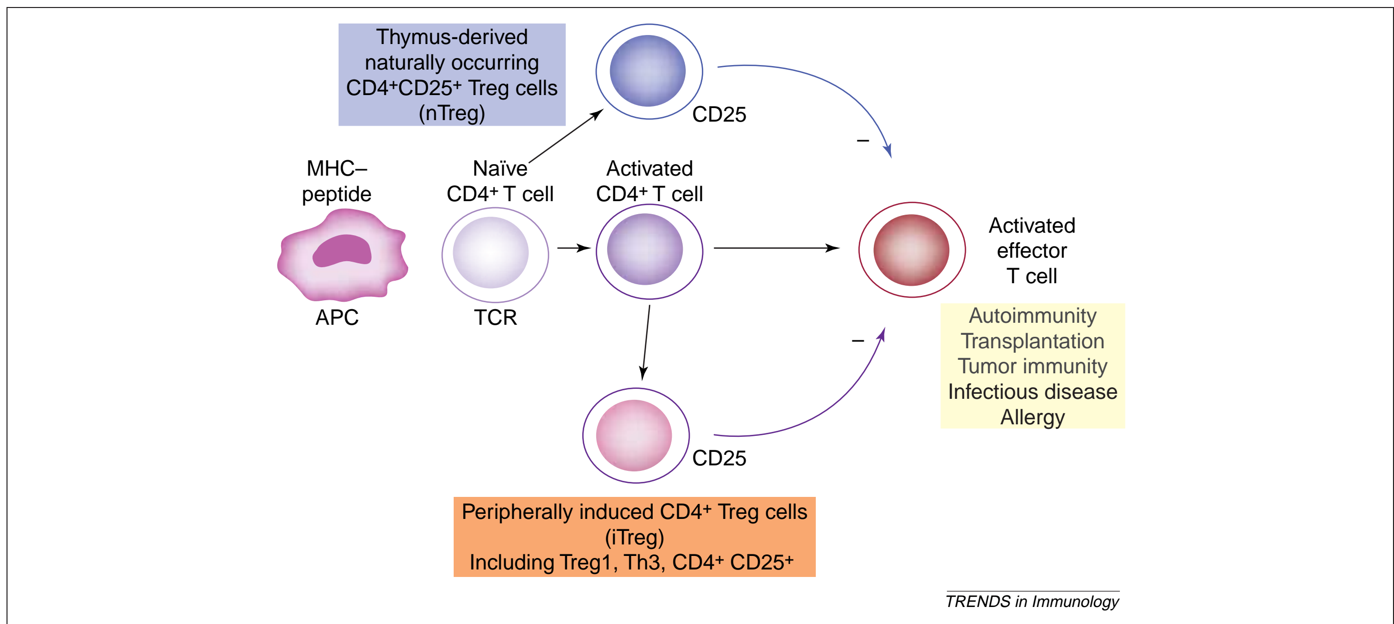

Figure 1. Naturally occurring and induced $CD4^+$ regulatory T cells control T cell responses. Both naturally occurring (nTreg) and induced (iTreg) $CD4^+$ regulatory T cell populations potentially downregulate the function of activated effector T cells in several immunological settings (yellow box). Whereas $CD4^+CD25^+$ nTreg cells differentiate in the thymus and are found in the normal, naïve $CD4^+$ T-cell repertoire, multiple iTreg cell subsets, possibly expressing CD25, emerge from conventional $CD4^+$ T cells, which are activated and differentiated in the periphery under unique stimulatory conditions. The relative contribution of each population in the overall regulation of immune responses is unclear but both conceivably can cooperate to achieve this goal. Abbreviations: APC, antigen-presenting cell; TCR, T-cell receptor.

presence of CTLA-4 Ig fusion protein, $CD4^+CD25^+$ cells were fully competent suppressors under each condition. Moreover, because the proliferation of $CD8^+$ cells stimulated in the presence of either WT or $CD80^{-/-}CD86^{-/-}$ APCs and anti-CTLA-4 was only partially inhibited by these reagents, we were able to directly assess the contribution of CD28 and CTLA-4 signaling to $CD4^+CD25^+$ activation. Consistently, $CD4^+CD25^+$ cells were fully capable of inhibiting the remaining response, despite the lack of CD28 or CTLA-4 co-stimulation, although co-stimulation through other members of the CD28 family has not been ruled out. Interestingly, recent studies indicate that non-specific signals, such as those generated by the lipopolysaccharide (LPS) and Toll-like receptor 4 (TLR4) pathway, might either directly activate nTreg cells [27] to promote suppression, or stimulate immature DCs to secrete IL-6 and a second factor that blocks the suppressive function of nTreg cells [28].

Role for immunosuppressive cytokines?

Most murine and human *in vitro* studies have concluded that nTreg-mediated suppression adopts a cytokine independent mechanism of suppression not involving the action of IL-4, IL-10 and TGF-β1, because *in vitro* neutralization of these cytokines, alone or in combination, failed to abrogate suppression [21] (Figure 2). In addition, $CD4^+CD25^+$ nTreg cells isolated from IL-4-, TGF-β1- or IL-10-deficient mice are as effective as WT $CD4^+CD25^+$ T cells in their ability to suppress T cell proliferation [21,29]. Supernatants derived from activated $CD4^+CD25^+$ T cells did not possess suppressive properties and suppression was not observed when $CD4^+CD25^+$ cells were separated from their target cells, supporting the notion that, rather than being cytokine mediated, $CD4^+CD25^+$-mediated suppression required cell contact.

Although most murine and human studies have failed to demonstrate an *in vitro* role for TGF-β1, one study reported that $CD4^+CD25^+$ T cells selectively expressed a membrane-bound, latent (inactive) form of TGF-β1, which mediated *in vitro* suppression [30]. However, TGF-β1RII dominant-negative transgenic or $Smad3^{-/-}$ T cells, both TGF-β1 unresponsive, remain completely susceptible to nTreg-cell-mediated regulation *in vitro* [29] and *in vivo* (pers. commun.). The possibility remains, however, that contact dependent suppression might be dominant *in vitro*, circumventing any requirements for long-range suppressive cytokines (Figure 2).

APCs: Treg cells battle for co-stimulation?

It was originally postulated that $CD4^+CD25^+$ cells could act upon the APC to either compete for or actively downregulate co-stimulation. We demonstrated, however, that suppression could not be abrogated by the addition of excess pre-activated, fixed APCs (expressing high levels of CD80 and CD86), and the expression of CD80 and CD86 on the B cell-enriched APC appeared to be unchanged in suppressive cultures, indicating that $CD4^+CD25^+$ cells do not compete or inhibit co-stimulation [23]. However, one study reported $CD4^+CD25^+$ T cells moderately decreasing the surface expression, but not the frequency, of CD86-expressing DCs [31].

We have addressed the functional relevance of Treg–APC interactions in $CD4^+CD25^+$-mediated inhibition. To this end, we used peptide–MHC I tetramers to stimulate transgenic $CD8^+$ T cells to assess whether activated $CD4^+CD25^+$ T cells mediate their suppressor function via a direct T–T-cell interaction or by modulating APC function. In this model, pre-activated $CD4^+CD25^+$ cells can suppress the proliferation and interferon-γ (IFN-γ) production by $CD8^+$ T cells in a T–T, APC independent

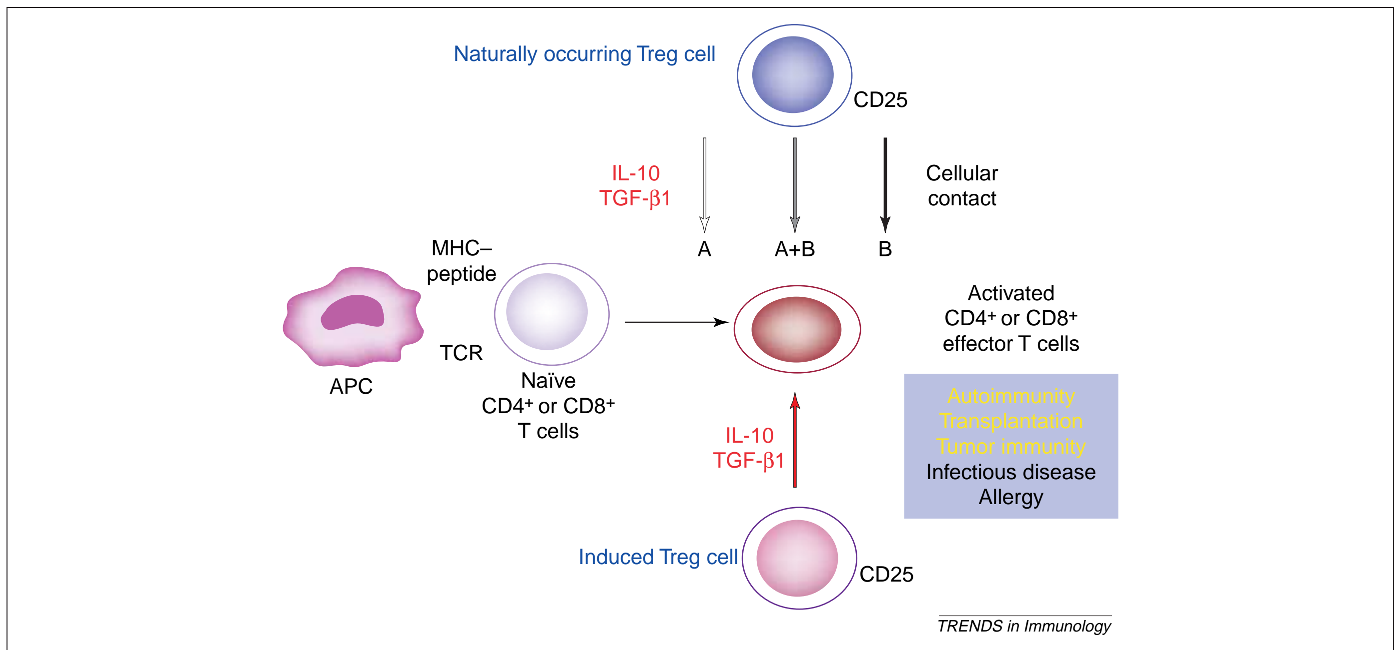

Figure 2. Possible mechanisms of action of $CD4^+$ regulatory T cells. Regulatory $CD4^+$ T cells (Treg) have crucial roles in suppressing the functions of activated $CD4^+$ or $CD8^+$ effector T cells in various types of immune responses (purple box), and the mechanisms for achieving this regulation are diverse. Whereas induced $CD4^+$ Treg cells operate via the secretion of immunosuppressive cytokines, including interleukin-10 (IL-10) and transforming growth factor-β1 (TGF-β1), naturally occurring Treg cells might opt for either cytokine dependent (A), cell contact dependent (B) or cytokine/cellular contact dependent (A + B) modes of action to control similar T-cell responses. Abbreviations: APC, antigen-presenting cell; TCR, T-cell receptor.

manner [24]. Our proposed T–T cell model of nTreg effector function does not negate a role for APC to serve as a stabilizing platform on which effector and Treg populations physically interact because the initial activation of Treg is undoubtedly APC dependent. Furthermore, this model does not exclude the possibility that Tregs can also target APC, B cells or natural killer (NK) cells.

Surface molecules on $CD4^+CD25^+$ Treg cells: any reliable biomarkers?

In addition to constitutively expressing CD25, several genetic studies have identified additional surface molecules that are unique to $CD4^+CD25^+$ cells when compared with $CD4^+$CD25- cells (Figure 3), including CD103, galectin-1, Ly6, OX-40, 4–1BB, CTLA-4 (CD152), glucocorticoid-induced tumor necrosis factor (TNF) receptor (GITR), TNFR2, TGF-βR1, programmed cell death 1 (PD1) and, more recently, neuropilin-1 [32–34]. A word of caution must be sounded regarding the use of these markers as faithful indicators of nTregs because most cell surface molecules, although unique to $CD4^+CD25^+$ cells, are, unfortunately, also expressed on $CD4^+$CD25- cells upon activation, with the exception of CD103. Thus, although these markers, including CD25, might distinguish resting $CD4^+CD25^+$ cells, no phenotypic markers can distinguish them from conventional activated $CD4^+$ T cells. Currently, none of these cell surface markers appear to be responsible for $CD4^+CD25^+$-mediated suppression, but questions remain as to the functional significance of these molecules.

The functional role of CTLA-4 in $CD4^+CD25^+$ T cell-mediated suppression remains ill-defined. Takehashi *et al.* reported that intracellular CTLA-4 was present in resting $CD4^+CD25^+$ cells, and that antibodies to CTLA-4 could reverse $CD4^+CD25^+$-mediated suppression, concluding that CTLA-4 signaling was required for the activation of $CD4^+CD25^+$ cells [35]. Read *et al.* further demonstrated that suppression of inflammatory bowel disease (IBD) by $CD4^+CD25^+$ cells could be reversed with anti-CTLA-4 [36]. However, the *in vitro* and *in vivo* effects of anti-CTLA-4 are difficult to interpret because anti-CTLA-4 can act upon $CD4^+$CD25- cells following TCR engagement. The idea that CTLA-4 delivers an activating signal is also contrary to the widely accepted downregulatory role of

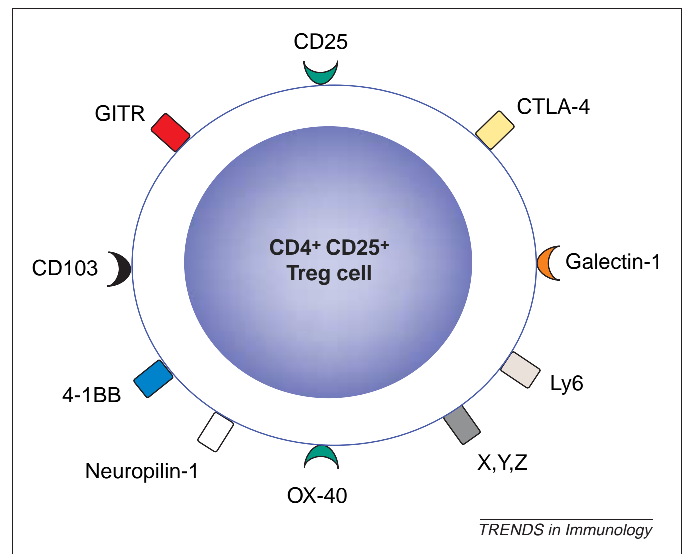

Figure 3. Surface molecules on $CD4^+CD25^+$ regulatory T (Treg) cells. Studies have reported several different molecules preferentially expressed on the surface of naturally occurring $CD4^+CD25^+$ Treg cells. However, the vast majority do not remain as faithful biomarkers after T-cell activation and none appear to be convincingly involved in the mechanism underlying T-cell suppression. Reliable identification and effector surface molecules are still ill-defined (X,Y,Z).

CTLA-4. The effects of anti-CTLA-4 are more consistent with the view that CTLA-4 engagement on $CD4^+CD25^+$ cells leads to an inhibition of signals required for induction of suppressor function. Interestingly, one study has shown that nTreg cells initiated tryptophan catabolism in DCs in a CTLA-4 dependent fashion [37]. Furthermore, $CD4^+CD25^+$ cells from CTLA-$4^{-/-}$ mice do possess suppressive activity (pers. commun.).

GITR has also been reported to play a role in the function of $CD4^+CD25^+$ cells. GITR is differentially expressed on $CD4^+CD25^+$ cells, but is expressed on $CD4^+$CD25- cells upon activation. Two recent studies demonstrated that anti-GITR mAb reversed $CD4^+CD25^+$-mediated suppression and it was concluded from both studies that GITR could play a functional role on $CD4^+CD25^+$ cells [32,38]. However, additional studies now indicate that anti-GITR might not affect $CD4^+CD25^+$ cells but, rather, alters the responsiveness of $CD4^+CD25^-$ target cells (pers. commun.).

Control of immune responses *in vivo*: role for cytokines?

The mechanism by which $CD4^+CD25^+$ nTreg cells regulate responses *in vivo* is unknown and the contribution of nTreg-derived cytokines in disease regulation remains controversial (Figure 2). Several immunosuppressive cytokines have been implicated in nTreg effector functions and their involvement might be affected by many context-dependent physiological factors, including the nature of the target organ and magnitude of inflammation. For instance, IL-$10^{-/-}$ nTreg cells can potently suppress autoimmune gastritis but are insufficient to suppress bacterial-driven IBD in the same recipient animal [39]. Recently, we demonstrated that nTreg cells control the persistence and concomitant immunity to re-infections of the intracellular pathogen *Leishmania major*. The control of pathogen persistence in sites of infection occurred independently of nTreg-derived IL-10 in the initial phase of disease establishment and appeared to be IL-10 dependent in chronic phases of disease, confirming the notion that nTreg can adapt to their inflammatory microenvironments for efficient and timely disease control [20]. This apparent requirement for IL-10 in chronic stages of infection might reflect possible roles for IL-10 in nTreg maintenance, or in the induction of IL-10 from other cellular sources for modulation of T cell and APC function. The regulatory role for TGF-β1 in $CD4^+CD25^+$ T cell-mediated suppression is more nebulous because the cellular sources *in vivo* are diverse and might include nTreg cells themselves, activated effector T cells or, possibly, non-lymphoid tissues that are targets of autoimmune attack, in the process of healing or induced by $CD4^+CD25^+$ nTreg cells. These experimental results might suggest that contact-mediated suppression of effector cells by nTreg is always necessary both *in vivo* and *in vitro* but that, under select circumstances *in vivo*, might be limiting (Figure 2). Alternatively, different nTreg-cell subsets might exist such that some inhibit by a cell contact-dependent mechanism, others are endowed with distinct cytokine production capabilities or, possibly, a combination of both mechanisms. Lastly, it is also reasonable to imagine that the $CD4^+CD25^+$ nTreg cells facilitate the generation of other types of Treg cells because co-culture of nTreg cells with responder T helper cells results in the differentiation of Treg-cell populations that can suppress *in vitro* Th1- or Th2-cell responses via the production of TGF-β1 and/or IL-10 [40].

In vivo development of $CD4^+CD25^+$ Treg cells

The thymic and peripheral signals required for nTreg generation and maintenance of cells are not thoroughly understood. Recently, the importance of CD28 co-stimulation in thymic development and peripheral homeostasis of the nTreg cells has been highlighted [41,42]. In addition to proper co-stimulation to developing immature thymic precursors, CD28 engagement might be required to sustain a stable peripheral pool of nTreg cells by promoting their survival and self-renewing potential, possibly through the expression of IL-2, Bcl-2-like anti-apoptotic molecules or through other cytokines that function as growth, survival or suppressor activity maintenance factors. Interestingly, the functional disruption of B7–CD28 or CD40–CD40L co-stimulatory pathways results in quantitative or qualitative defects in $CD4^+CD25^+$-mediated functions in peripheral lymphoid compartments, and a consequential induction of autoimmunity [43], as illustrated by $CD28^{-/-}$ and B7.1-$B7.2^{-/-}$ non-obese diabetic (NOD) mice which develop diabetes more rapidly than their NOD control littermates [41]. Moreover, mice deficient for IL-2, IL-2Rα or IL-2Rβ have few or no nTreg cells and die prematurely from a severe lymphoproliferative and autoimmune syndrome [44]. Furthermore, whereas *stat5* $^{-/-}$ mice have very few $CD4^+CD25^+$ cells, mice transgenic for the active form of STAT5 possess a greater frequency of these cells, thus confirming the requirement for IL-2 signaling in nTreg homeostasis [45]. Recently, Malek *et al.* have shown that nTreg cell numbers are restored and the induction of autoimmune disease is prevented when an IL-2Rβ transgene is expressed solely in the thymus of IL-$2R\beta^{-/-}$ mice, indicating that an intact IL-2–IL-2R pathway is required for thymic generation of nTreg [46]. Collectively, IL-2 is a crucial signal for the development and peripheral homeostasis of $CD4^+CD25^+$ nTreg cells.

Recent studies have shown that the forkhead transcription factor Foxp3 is a functional marker of nTreg cells, playing a central role in their generation. $CD4^+CD25^+$ nTreg cells account for the vast majority of Foxp3 expression within murine lymphoid cells, and *Foxp3* $^{-/-}$ mice suffer from an autoimmune pathology secondary to a loss of $CD4^+CD25^+$ nTreg cell function [47–50], a clinical outcome synonymous with what is believed to occur in Scurfy mice and X-linked inheritance patients [50,51]. Although primarily expressed in human $CD4^+CD25^+$ nTreg cells, conventional $CD4^+CD25^-$ T cells can also upregulate expression of this molecule [52]. Foxp3-overexpressing mice have more Treg cells, and can potently suppress the development of autoimmune disease [47]. Surprisingly, retroviral overexpression into non-regulatory $CD4^+CD25^-$ or $CD8^+$ T cells induces a functional phenotype similar to $CD4^+CD25^+$ nTreg cells possessing potent suppressive functions *in vitro* and *in vivo* [49]. Interestingly, induction of Foxp3 has also been described

in induced $CD4^+$ regulatory T (iTreg) cells [52]. It remains to be seen which genes are being modulated by Foxp3 and the impact they have on Treg function. Collectively, these studies suggest that Foxp3 has a determining role in the generation of $CD4^+CD25^+$ nTreg cells and illustrate how certain genetic pathways affect nTreg function, such that autoreactive T cells are left uncontrolled and allowed to induce autoimmunity.

Antigen specificity of $CD4^+CD25^+$ nTreg cells: self or non-self?

The antigen specificity directing thymic selection, differentiation and peripheral activation of nTreg cells still remains enigmatic. $CD4^+CD25^+$ nTreg cells have a polyclonal TCR repertoire, based on diverse gene expression of various TCR α/β elements, and could conceivably recognize a wide spectrum of self-antigens [53]. It is unclear whether they are biased towards recognition of a particular type or subset of self-antigens. In a double transgenic model in which TCR transgenic mice were crossed with mice expressing the corresponding cognate antigen in many cell types, Jordan *et al.* recently demonstrated that antigen specific Treg cells appear to require high affinity antigen recognition with thymic stromal cells to develop [54]. Conversely, the antigen specificity of nTreg cells in normal, non-transgenic animals is not known and it remains uncertain whether the requirements for normal nTreg development are identical to nTreg from double transgenic mice. Whether the repertoire of nTreg is biased toward self-antigens or is as diverse as that of $CD4^+CD25^-$ T cells is not known. Whereas nTreg cells exert their primary function by preventing the activation of autoreactive T cells, we would speculate that these cells similarly have a diverse TCR repertoire, which can respond to activation by both self and pathogen-derived antigens. These cells can also be expanded as a consequence of cross-reactivities with various foreign proteins, thus stimulating these cells in an inflammatory setting to mediate antigen non-specific suppression [7,8].

Different types of $CD4^+CD25^+$ regulatory T cells: induced or naturally occurring?

It is well established that $CD4^+CD25^+$ Treg cells control various types of immune responses [53]. A major problem relates to the failure to discriminate $CD4^+CD25^+$ nTreg cells from activated conventional $CD4^+$ T cells induced to express CD25 and to acquire suppressor activity. Indeed, $CD4^+$ iTreg cells can be generated by the activation of mature, peripheral $CD4^+CD25^-$ T cells, acquire CD25 following TCR activation and their regulatory functions might or might not resemble those associated with nTreg cells (Figure 1). These iTreg cells can be generated *in vitro* or *in vivo* under various stimulation regimens, including antigen in the presence of IL-10, TGF-β1, vitamin D3 or dexamethasone, CD40L blockade, allo- or pathogen-derived antigens, non-depleting anti-CD4 and anti-CD8 treatment, bivalent non-mitogenic anti-CD3 therapy or immature DC populations [55–62]. Induced Treg cells generally function in a cytokine-dependent manner *in vitro* and *in vivo* (Figure 2). Whereas nTreg cells differentiate intrathymically and are functional upon exit from the thymus, the peripheral development of iTreg cells might differ from nTreg cells by their requirement for further peripheral differentiation by antigen activation under unique immunological settings, including low affinity antigen or altered TCR signaling. It is not known whether nTreg and iTreg cells preferentially function alone or in synchrony at suppressing non-inflammatory or inflammatory T-cell responses to self/non-self proteins (Figures 1 and 2).

Conclusion: clinical significance of $CD4^+CD25^+$ regulatory T cells

A central question is whether the onset of autoimmunity reflects developmental or functional deficiencies in $CD4^+CD25^+$ nTreg cells, tipping the balance towards the excessive activation, expansion and recruitment of autoreactive T cells, pro-inflammatory cytokine production and disease establishment. A functional deficiency in nTreg cells might not be evident as a reduction in the cellular frequency in peripheral tissues, and might be resultant to selective gaps in antigen-specific specificities or various genetic polymorphisms modulating activation requirements, effector function, survival or trafficking properties. Further studies will be needed to understand the cellular basis of autoimmunity, and with the goal of developing novel strategies to potentiate nTreg function in autoimmunity prone individuals.

The tolerogenic functions of nTreg cells in the induction and establishment of autoimmune disease in several mouse models are well accepted [8,9,20]. To be of clinical significance, $CD4^+CD25^+$ cells must also be capable of inhibiting established disease. Recently, Mottet *et al.* reported that $CD4^+CD25^+$ cells could remarkably reverse disease in a well established model of IBD induced by injection of $CD4^+CD45Rb^{high}$ T cells in severe combined immunodeficient (SCID) mice, even 4 weeks after disease induction [63]. Thus, $CD4^+CD25^+$ cellular therapy could be beneficial in the treatment of autoimmunity, although a recent study showed delayed resolution of central nervous system tissue injury in the presence of nTreg [64]. In addition to autoimmunity, it is now evident that $CD4^+CD25^+$ nTreg cells also play a beneficial role in graft versus host disease (GvHD) models of transplantation tolerance induction, with the finding that depletion of $CD4^+CD25^+$ cells abrogates the protective effect [65,66]. Thus, an increase in their function and expansion is a desirable goal for inducing tolerance in GvHD and solid organ transplantation.

Given the immunosuppressive properties of $CD4^+CD25^+$ cells, their presence could be undesirable, not only during an immune response to pathogens, but also in vaccination. Suvas *et al.* demonstrated that $CD8^+$ T cell-mediated responses were subject to $CD4^+CD25^+$ cell regulation, in that the magnitude of the $CD8^+$ T-cell response in acute herpes simplex virus infection to the immunodominant epitope was enhanced after depletion of nTreg cells [67]. Similar findings in models of *Pneumosystis carnii* and *L. major* infections have also shown that the absence of nTreg cells could lead to a more robust immune response, resulting in the clearance of infection

rather than the establishment of chronic disease [19,68]. Furthermore, depletion of CD4+CD25+ cells before vaccination could potentially lead to protective immune responses to pathogens to which vaccines have not been successfully developed. Similarly, Shimuzu *et al.* demonstrated that tumor immunity, which is usually unsuccessful because of the poor immunogenicity of tumor specific antigens, can be induced in non-responding animals through the depletion of CD4+CD25+ cells [17], thus potentially enhancing responses and leading to successful tumor vaccines in humans.

Finally, more specific identification markers, and an accurate nomenclature that faithfully reflects the complexity of Treg cell populations, are much needed. 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. Thus, an array of unanswered questions regarding the role of nTreg populations in peripheral T-cell immunoregulation linger. Are there subsets of nTreg cells, and what are their antigen specificity, origins and specific activation requirements? What are the dynamics of the nTreg cells throughout the course of an immune response to self and non-self proteins? What are the specific signals required for their survival and self-renewal? Answers to these questions will inevitably reveal fundamental aspects of nTreg biology and potentially provide insights into ways of modulating nTreg function *in vivo*.

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