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-\(\beta\) (TGF-\(\beta\))-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) \(\alpha\) 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.

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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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−, IL-10− and 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 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.

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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\(^+\) T 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-\(\beta\)R1, 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
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 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.).

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, Bel-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-2Rβ⁻/⁻ 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 potentially 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 αβ 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+45RBhigh 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+ Tcell-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 *Pneumocystis carinii* 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 underlyng 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.

References
The WHO and six medical journal publishers have launched the Access to Research Initiative, which enables nearly 70 of the world’s poorest countries to gain free access to biomedical literature through the Internet.

The science publishers, Blackwell, Elsevier, the Harcourt Worldwide STM group, Wolters Kluwer International Health and Science, Springer-Verlag and John Wiley, were approached by the WHO and the British Medical Journal in 2001. Initially, more than 1000 journals will be available for free or at significantly reduced prices to universities, medical schools, research and public institutions in developing countries. The second stage involves extending this initiative to institutions in other countries.

Gro Harlem Brundtland, director-general for the WHO, said that this initiative was ‘perhaps the biggest step ever taken towards reducing the health information gap between rich and poor countries’.

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