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Review Series

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CD4⁺ Tregs and immune control

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Recent years have seen Tregs become a popular subject of immunological research. Abundant experimental data have now confirmed that naturally occurring CD25⁺CD4⁺ Tregs in particular play a key role in the maintenance of self tolerance, with their dysfunction leading to severe or even fatal immunopathology. The sphere of influence of Tregs is now known to extend well beyond just the maintenance of immunological tolerance and to impinge on a host of clinically important areas from cancer to infectious diseases. The identification of specific molecular markers in both human and murine immune systems has enabled the unprecedented investigation of these cells and should prove key to ultimately unlocking their clinical potential.

Introduction

Naturally occurring CD25⁺CD4⁺ suppressor or Tregs cells play an active part in establishing and maintaining immunological unresponsiveness to self constituents (i.e., immunological self tolerance) and negative control of various immune responses to non-self antigens (1). Although not a new idea for immunologists, the existence of Tregs as a definite cellular entity has been of great controversy until recently because of the paucity of reliable markers for defining the cell, the ambiguity in the molecular basis of suppressive phenomena, the lack of ample evidence for their roles in immunological disease, and even the elusive nature of some suppressive phenomena themselves (2). Recent years, however, have witnessed increasing interest in Tregs in many fields of basic and clinical immunology. Among the several types of Tregs so far reported, naturally occurring CD25⁺CD4⁺ Tregs are the main focus of current research, because accumulating evidence indicates that this population plays a crucial role in the maintenance of immunological self tolerance and negative control of pathological as well as physiological immune responses. A prominent feature of CD25⁺CD4⁺ Tregs is that the majority, if not all, of them are naturally produced by the normal thymus as a functionally distinct and mature subpopulation of T cells and persist in the periphery with stable function, and that their generation is, at least in part, developmentally controlled (1). Congenital deficiency of this population, therefore, results in serious impairment of self tolerance and immunoregulation, leading to severe autoimmunity, immunopathology, and allergy in humans (3). On the other hand, their natural presence in the immune system as a phenotypically distinct population makes them a good target for designing ways to treat or prevent immunological diseases and to control pathological as well as physiological immune responses. In addition to this naturally arising "professional" Treg population, there are several other types of Tregs that can be induced from naive T cells by antigenic stimulation under specialized conditions in the periphery (4, 5). Although physiological roles for

these inducible or "adaptive" Tregs need to be fully established, they can still be exploited as a therapeutic tool (6). In this article, we shall review recent progress in our understanding of the roles of natural and adaptive CD4⁺ Tregs in immune tolerance and negative control of immune responses. We shall also touch briefly on their possible clinical applications.

Naturally occurring CD25⁺CD4⁺ Tregs in self tolerance and their production by the normal thymus

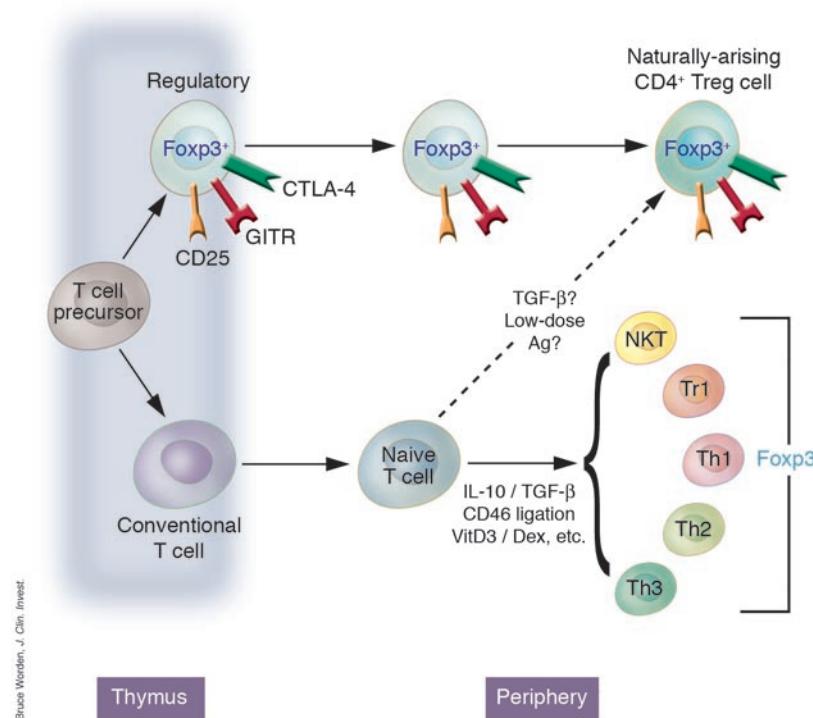
Experimental evidence for the existence of Tregs with autoimmune-inhibitory activity has been suggested in various animal models of autoimmune disease for many years (7, 8). Neonatal thymectomy, for example, leads to spontaneous development of autoimmune diseases including gastritis, thyroiditis, and oophoritis in selected strains of mice (7–9). Adult thymectomy and subsequent sublethal X-irradiation produced thyroiditis and type 1 diabetes (T1D) in selected strains of rats (10, 11). In NOD mice or Bio-Breeding (BB) rats, which spontaneously develop T1D and autoimmune thyroiditis, inoculation with CD4⁺ T cells from histocompatible normal animals effectively prevented T1D (12, 13). On the other hand, characterization of effector T cells mediating these organ-specific autoimmune diseases firmly documented that CD4⁺ Th cells destroy the target organs/tissues by helping B cells to form specific autoantibodies and by inducing cell-mediated immune responses to the target self antigens. Collectively, these findings suggested that normal individuals harbor 2 functionally distinct populations of CD4⁺ T cells, one capable of mediating autoimmune disease and the other capable of dominantly inhibiting it in the normal physiological state (8). To test this hypothesis directly, attempts were made from the mid-1980s onward to dissect these 2 CD4⁺ T cell populations by expression levels of particular cell surface molecules and to examine their potential correlation with autoimmune induction or inhibition. When CD4⁺ splenic T cell suspensions prepared from normal mice or rats were depleted of CD25⁺, RT6.1⁺, CD5^{high}, or CD45RB/RC^{low} cells and the remaining CD4⁺ T cells transferred to syngeneic T cell-deficient mice or rats, the recipients spontaneously developed various organ-specific autoimmune diseases (including T1D, thyroiditis, and gastritis) and systemic wasting disease in a few months; reconstitution of the eliminated population inhibited the development of autoimmune disease (1, 8, 14). A similar transfer experiment also induced inflammatory bowel disease (IBD), which appeared to result from an excessive immune response of T cells to com-

Nonstandard abbreviations used: GITR, glucocorticoid-induced TNF receptor family-related gene; GITRL, GITR ligand; IBD, inflammatory bowel disease; IDO, indoleamine 2,3-dioxygenase; IPEX, immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome; RAG, recombinase-activating gene; T1D, type 1 diabetes; Tr1, T regulatory cell type 1.

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mensal bacteria in the intestine (15). Currently CD25 is the most specific cell surface marker for such autoimmune- and IBD-preventive CD4⁺ T cells, because CD25⁺CD4⁺ T cells, which constitute 5–10% of CD4⁺ T cells in normal naive mice, are included in the CD5^{high} or CD45RB^{low}CD4⁺ population, and furthermore their depletion alone is sufficient to cause autoimmune disease/IBD, while their reconstitution is effectively able to inhibit autoimmune disease/IBD, in various models (1, 16). Additionally, the various immunological properties of natural Tregs, including their *in vitro* suppressive activity, are assigned to CD25⁺CD4⁺ T cells naturally arising in the immune system (1, 8, 14). It should be noted, however, that CD25 is not an absolute marker for naturally occurring Tregs, since it is also expressed at high levels on activated but otherwise conventional nonregulatory T cells. We shall discuss this caveat later in the article.

The normal thymus produces the majority, if not all, of CD25⁺CD4⁺ Tregs as a functionally mature T cell subpopulation, which appears to constitute a distinct cellular lineage and to be contiguous with those found in the periphery (17) (Figure 1). As shown with the transfer of CD25⁺CD4⁺ spleen cells described above, transfer of mature thymocyte suspensions depleted of CD25⁺ thymocytes produced various autoimmune diseases in syngeneic T cell-deficient mice (17). This indicates that the normal thymus is continuously producing pathogenic self-reactive CD4⁺ T cells as well as functionally mature CD25⁺CD4⁺ Tregs capable of controlling them. This centralized production of Tregs has been referred to as “the third function of the thymus” (18).

Accumulating evidence also indicates that thymic development of CD25⁺CD4⁺ Tregs requires unique interactions of their TCR with self-peptide/MHC complexes expressed by thymic stromal cells (19). In TCR transgenic mice, for example, a large number of CD25⁺CD4⁺ T cells express endogenous TCR α chains paired with transgenic β chains; recombinase-activating gene-2 (RAG-2) defi-

Figure 1

Regulatory CD4⁺ cells can develop in a number of ways, although the mechanisms by which these occur and the relationship of the resulting cells to one another are contestable. Thymically generated Treg cells, otherwise known as natural T_R cells or CD25⁺CD4⁺ T_R cells, develop intrathymically according to a specialized combination of TCR and costimulatory signals. Extrathymically generated T_R cells, e.g., Tr1 cells or Th3 cells, can be generated under a whole host of conditions. Whether a conventional naive CD4⁺ T cell can be converted in the periphery to a de facto Foxp3⁺ T_R cell remains controversial.

ciency, which blocks the gene rearrangement of the endogenous TCR α chain locus, abrogates the development of CD25⁺CD4⁺ Tregs in such TCR transgenic mice (17, 20). Furthermore, compared with thymic selection of other T cells, the development of CD25⁺CD4⁺ Tregs requires higher-avidity interactions of their TCRs with self peptide/MHC or class II MHC itself expressed on the thymic stromal cells (especially cortical epithelial cells), yet the required avidity must not be so high as to lead to their deletion (19–23). Accessory molecules, such as CD28, B7, and CD40, expressed on developing thymocytes and thymic stromal cells also contribute to the thymic generation of CD25⁺CD4⁺ Tregs (24, 25).

The naturally occurring CD4⁺ Treg phenotype

Naturally occurring CD4⁺ Tregs constitutively express a variety of cell surface molecules more commonly associated with activated/memory cells, most significantly CD25, CD45RB^{low}, CD62L, CD103, cytotoxic T lymphocyte antigen-4 (CTLA-4, or CD152), and glucocorticoid-induced TNF receptor family-related gene (GITR) (15, 16, 26–30). Neuropilin-1, a molecule more usually associated with axon guidance, was very recently reported to be constitutively expressed by natural Tregs and, interestingly, is *downregulated* on conventional T cells following activation (31). Even though none of these markers is *uniquely* expressed by naturally occurring CD4⁺ Tregs, their level of expression and constitutive nature have still made them useful as functional descriptors by enabling the consistent isolation and investigation of CD4⁺ T cells with regulatory properties. The naturally occurring Treg surface phenotype indicates that they are in an antigen-primed state and are, at least superficially, similar to memory-type T cells. Judging from the finding that CD25⁺CD4⁺ Tregs require a high-avidity interaction with self peptide/MHC for their thymic development and become functional within the thymus, one could speculate that they are broad in antigen specificity yet more capable of recognizing self antigens than other T cells are.



Although CD25 has so far proven to be the best surface marker for thymically produced CD4⁺ Tregs, it can be expressed on any T cell following activation (1). In the human system, where there are relatively large numbers of activated T cells, this is especially problematic. Currently, therefore, the best way to select natural human CD4⁺ Tregs is to sort the population that is very high in CD25 (32). The high constitutive expression of CD25 by Tregs begs the question of whether it is simply a convenient marker or a molecule essential for their function. Several lines of evidence indicate that CD25 is indispensable for the maintenance of natural CD25⁺CD4⁺ Tregs in the immune system. For example, it has been shown that mice deficient in IL-2, IL-2R α (CD25), or IL-2R β (CD122) develop lethal inflammatory disease, termed IL-2 deficiency syndrome, which can be prevented by inoculation of normal CD25⁺CD4⁺ T cells as long as a source of IL-2 is made available experimentally (33–35). Our own experiments indicated that neutralization of IL-2 selectively reduced numbers of CD25⁺CD4⁺ T cells in normal mice and consequently produced organ-specific autoimmune diseases similar to those produced by depletion of natural Tregs (R. Setoguchi et al., manuscript submitted for publication). Collectively, these results suggest that IL-2 is essential for the development, maintenance, and function of CD25⁺CD4⁺ Tregs.

GITR and its role in CD25⁺CD4⁺ Treg function is an interesting area. This molecule was identified as a constitutively expressed marker for naturally occurring Tregs, but, like most such candidate molecules involved in Treg identification, it is also upregulated on conventional activated CD4⁺ T cells (29, 30). An anti-mouse GITR mAb (DTA-1) is able to block CD25⁺CD4⁺ Treg suppression in vitro, and, furthermore, its injection leads to the induction of autoimmunity in vivo as well as enhancing the proliferation of CD25⁺CD4⁺ cells by transducing a costimulatory signal (29, 36). Since DTA-1 is nondepleting, it was originally presumed to primarily transmit a suppression-blocking signal to the CD25⁺CD4⁺ Treg. However, some very recent data instead suggest that ligation of GITR on *activated T cells*, not Tregs, renders them resistant to suppression (37). The natural ligand for GITR (GITRL) has now also been cloned and its distribution elucidated (38). GITRL is expressed on APCs (DCs, macrophages, and B cells) but is downregulated following maturation. Therefore the relative distribution patterns of GITR on activated T cells and Tregs, and of GITRL on APCs, suggest a complex dynamic of interaction, which is only just being elucidated.

Identification of an unambiguous surface marker for naturally occurring CD4⁺ Tregs remains something of a Holy Grail, especially where the isolation of human Tregs for clinical purposes is concerned. Efforts in this direction may well guide the progress of research in naturally occurring human Tregs.

FOXP3 as a master control gene for Treg development

A deeper understanding of the developmental processes of natural Tregs, as suggested by the neonatal thymectomy model of autoimmune disease, evolved out of studies on the Scurfy mouse and the human disease IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome). IPEX is an X-linked immunodeficiency syndrome associated with autoimmune disease in multiple endocrine organs (such as T1D and thyroiditis), IBD, atopic dermatitis, and fatal infections (3). The Scurfy mouse strain exhibits a fatal X-linked lymphoproliferation characterized by a multiorgan immunopathology very similar to the human disease IPEX (39–41). The causative gene, *Foxp3*

(*FOXP3* in humans), which underlies both syndromes, encodes a forkhead/winged-helix family transcriptional repressor called Scurfin (42–44). The striking similarities seen between mutations in *Foxp3*/*FOXP3* and depletion of CD25⁺CD4⁺ Tregs led several groups to investigate the relationship of this gene to Treg development and function. Experiments in mice indeed demonstrated *Foxp3* mRNA and Scurfin protein to be specifically expressed in CD25⁺CD4⁺ Tregs, and, in contrast to the cell surface markers used to date, they were never observed in non-Tregs following conventional activation or differentiation into Th1 and Th2, nor in natural killer T cells (45–47). Subsequent studies, also in mice, have further demonstrated the existence of a small population of CD25⁺CD4⁺ T cells that are nevertheless still *Foxp3*⁺ and have a regulatory function (ref. 1, and M. Ono et al., manuscript submitted for publication). Scurfy mutant mice, or those with a targeted deletion of *Foxp3*, were unable to support the development of natural CD25⁺CD4⁺ Tregs, although they contained large numbers of chronically activated CD25⁺ nonregulatory T cells (45, 47). By contrast, the number of CD25⁺CD4⁺ Tregs increased significantly in transgenic mice overexpressing *Foxp3* (45). A final critical observation showed that retroviral transduction of *Foxp3* into *Foxp3*[−] nonregulatory CD25⁺CD4⁺ T cells bestowed on them a fully functional Treg phenotype; e.g., cotransfer of *Foxp3*[−] transduced T cells with CD25⁺CD4⁺ T cells prevented autoimmune disease and IBD in SCID mice (see above) (46, 47).

Broadly speaking, an equivalent pattern of *FOXP3* expression has now also been reported in human cells, with Treg-like properties being similarly transferable by retroviral transduction (48–51). Already, however, some discrepancies are beginning to emerge between the behavior of human and that of mouse *FOXP3*/*Foxp3* expression. For instance, there is at least 1 example of *FOXP3* being apparently induced following standard antibody-mediated activation of normal CD25⁺ human T cells; this has not been observed thus far in the murine model (50). Similarly, some instances of CD25⁺ human T cell activation by DCs have also resulted in *FOXP3* upregulation (refs. 52, 53, and see below). The possibility remains, however, that the induction of *FOXP3* expression in human CD25⁺ cells may simply be a result of the expansion of the human counterpart to the murine CD25⁺CD4⁺ *Foxp3*⁺ population described above, as these studies all isolated their Tregs solely on the basis of CD25 (51).

Thus, *Foxp3*/*FOXP3* appears to be a master control gene for the development and function of natural CD25⁺CD4⁺ Tregs. Given that humans bear natural CD25⁺CD4⁺ Tregs with a phenotype and function comparable to those found in rodents (32), it is most likely that in IPEX, disruption of the *FOXP3* gene abrogates the development of thymic Tregs, leading to hyperactivation of T cells reactive with self antigens, commensal bacteria in the intestine, or innocuous environmental substances, and thus causing autoimmune polyendocrinopathy, IBD, or allergy, respectively. This has several implications for self tolerance and autoimmune/inflammatory disease in humans. First, this is so far the clearest example that an abnormality in naturally arising Tregs is a primary cause of human autoimmune disease, IBD, and allergy. Second, the development of natural Tregs is, at least in part, genetically and developmentally programmed. Third, hemizygous defects of the *FOXP3* gene in females illustrate that the mechanism of dominant self tolerance is physiologically operating in humans. Owing to random inactivation of the X chromosome during lyonization of individual Tregs, hemizygous females have *FOXP3*-defective Tregs



and *FOXP3*-normal ones as a genetic mosaic, yet they are nevertheless completely normal (54). This observation demonstrates that even reduced numbers of *Foxp3*⁺ Tregs are able to dominantly control pathogenic T cells, and, further, that even a partial restoration of Tregs could be sufficient to cure IPEX or, indeed, other autoimmune pathologies. Mechanistic data on *Foxp3* are thus far lacking; it is therefore currently unclear how it exerts its effects at a molecular level. The molecular interactions of *Foxp3*, and indeed the signals triggering its expression, are now an intensely investigated area, and unraveling them may well prove critical to exploiting natural Tregs in a therapeutic setting.

Functional characteristics of natural CD25⁺CD4⁺ Tregs and their mechanisms of suppression

Without question the most remarkable feature of CD25⁺CD4⁺ Tregs is their ability to dampen immune responses. They appear capable of suppressing a wide variety of immune cells, encompassing those of both the innate (55–57) and the adaptive immune systems (58–60). This suppressive ability can be modeled in vitro by mixing of titrated numbers of highly purified CD25⁺CD4⁺ Tregs and responder cells, typically CD25[−]CD4⁺ T cells plus a T cell stimulus. Under such conditions, the CD25⁺ population suppresses both the proliferation and, more fundamentally, the IL-2 production of the CD25[−] cells in a dose-dependent manner (58, 59, 61). CD25⁺CD4⁺ Tregs themselves require TCR stimulation, and, it now seems, IL-2, to actually trigger their suppressive effects, but once this condition has been satisfied their ensuing suppression can act non-antigen-specifically (58, 59, 61). Therefore, suppression is an active process and can be directed against bystander cells. Curiously, CD25⁺CD4⁺ Tregs themselves are anergic in vitro, i.e., they do not proliferate or produce IL-2 in response to conventional T cell stimuli such as plate- or bead-bound anti-CD3, concanavlin A (ConA), or splenic APCs. This anergy can, however, be broken by a sufficiently potent stimulus, e.g., the addition of high-dose exogenous IL-2 or anti-CD28, or the use of mature DCs as APCs (27, 58, 59, 61–63). Some of these strong stimuli, particularly mature DCs, also perturb CD25⁺CD4⁺ Treg suppression both in vitro and in vivo (63, 64). At least in vitro, anergy seems to be the default state of naturally occurring Tregs, since they revert back to it once potent stimulation is withdrawn (58). In vivo, however, CD25⁺CD4⁺ Treg anergy is not readily observed; instead they seem to have a highly active rate of turnover (33, 65). It seems likely, then, that CD25⁺CD4⁺ Treg anergy is an in vitro phenomenon, merely reflecting an exacting set of activation requirements generally absent from cell culture.

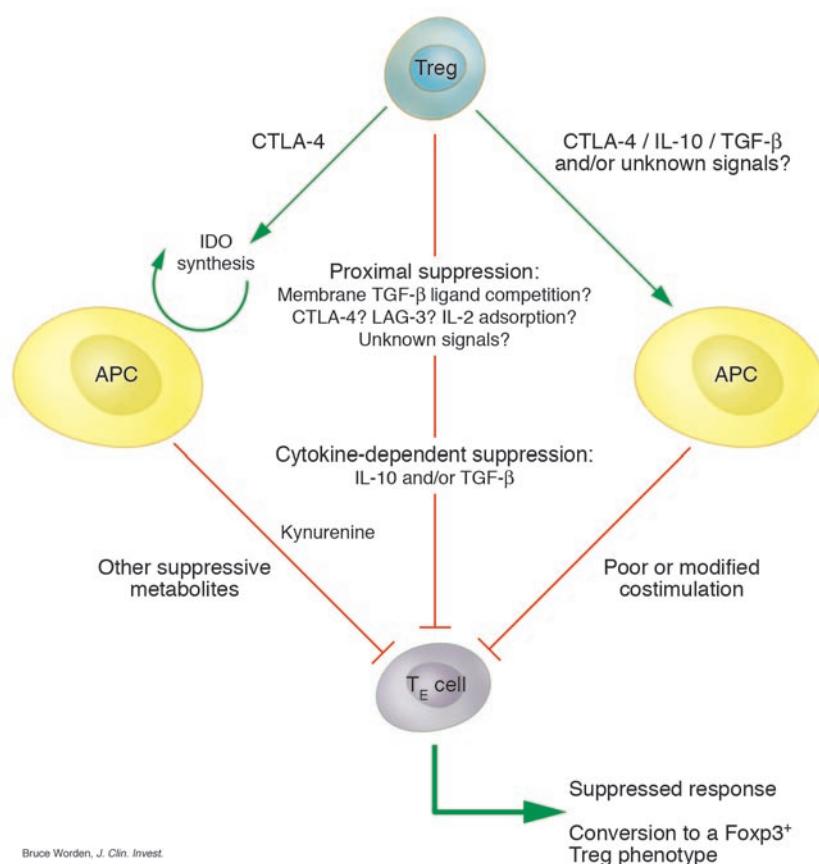
Given that the ability to control immune responses is the cardinal feature of CD25⁺CD4⁺ Tregs, it is surprising that their mechanism(s) of suppression remains elusive. Essentially, Treg suppression can be divided into those mechanisms mediated by relatively far-reaching soluble factors and those requiring intimate cell contact. In vivo experiments based chiefly on the IBD model mentioned previously have demonstrated the importance of the immunomodulatory cytokines IL-10 and TGF- β (66). By blocking IL-10 signaling in vivo with an anti-IL-10 receptor mAb, it was possible to abrogate the normal colitis-preventative action of CD45RB^{low} cells (66). Similarly, CD45RB^{low} T cells from IL-10^{−/−} mice lacked their otherwise intrinsic ability to protect from colitis and, moreover, were even colitogenic themselves when transferred alone (66). The importance of IL-10 is further underscored by the observation that IL-10^{−/−} mice spontaneously develop colitis (67, 68). Examination of the in vivo role

of TGF- β has generally painted a similar picture to that of IL-10, with Treg function being blocked by the presence of neutralizing anti-TGF- β mAbs (69). Some data also suggest that TGF- β may not necessarily act as a soluble factor but can also be found on the surface of activated CD25⁺CD4⁺ Tregs and may therefore act in a membrane-proximal manner (70). Interestingly, virtually all TGF- β ⁺ CD25⁺CD4⁺ Tregs also express thrombospondin, a factor capable of converting normally latent TGF- β into its active form (71). There should be a note of caution regarding these in vitro studies on TGF- β , since a comprehensive analysis by a second group failed to demonstrate any role for it in vitro (72).

The confusion over a definitive CD25⁺CD4⁺ Treg suppression mechanism is compounded when viewed in the context of the in vitro data, since here the overwhelming evidence highlights direct cell-cell interaction, and not cytokines, as being critical (58, 59, 73). Several lines of evidence lead to this conclusion: with the exception of the study on membrane-bound TGF- β alluded to above, both anti-IL-10 and anti-TGF- β fail to perturb CD25⁺CD4⁺ Treg suppression (58, 59, 70, 72). Similarly, supernatants from suppressed cultures or activated CD25⁺CD4⁺ Tregs show no inherent suppressive activity, nor can suppression be observed across a semipermeable membrane (58, 59). Collectively, these in vitro observations therefore appear to obviate a role not just for IL-10 and TGF- β but for soluble factors in general.

The actual membrane events occurring during suppression that depends on CD25⁺CD4⁺ Treg contact have yet to be clarified. The most simplistic models propose competition for APCs and specific MHC/peptide antigenic complexes. Additionally, the constitutive expression of the high-affinity IL-2 receptor could make naturally occurring Tregs into an effective IL-2 sink, depriving potential autoreactive T cells of this essential growth factor (74). However, given the relative physiological scarcity of naturally occurring Tregs, it is perhaps unlikely that a simple competitive-adsorptive model alone could account for their suppressive action in vivo. Other models of CD25⁺CD4⁺ Treg suppression propose a more proactive and antagonistic form of suppression that relies on the expression of specific “inhibitory” molecules. The identity and indeed even the very existence of such an inhibitory molecule are uncertain, but 1 potential molecule could be Treg-expressed CTLA-4. Aside from its well-established high affinity for the costimulatory molecules B7.1 and B7.2 (CD80 and CD86, respectively), CTLA-4 has also recently been shown to trigger the induction of the enzyme indoleamine 2,3-dioxygenase (IDO) when interacting with its ligands on DCs (75–78). IDO catalyzes the conversion of tryptophan to kynurenine and other metabolites, which have potent immunosuppressive effects in the local environment of the DC. In this way, CD25⁺CD4⁺ Tregs may exert their suppression by proxy through their action on APCs. Another APC-centric mode of suppression could be via the perturbation of antigen-presenting capacity. In support of this concept, one report has demonstrated that purified CD25⁺CD4⁺ Tregs are able to downregulate the expression of both CD80 and CD86 on DCs, converting them into inefficient APCs (57). At any rate, CD25⁺CD4⁺ Tregs need not act exclusively via the APC, since they are quite capable of suppressing in the context of “APC-free” systems such as plate- or bead-bound antibodies or MHC/peptide tetramers (58, 79). At least in vitro, *direct* suppression of the target cell is therefore still also possible.

A provocative investigation into the membrane events involved in CD25⁺CD4⁺ Treg suppression was recently reported (80). This

**Figure 2**

Possible CD25⁺CD4⁺ Treg suppression mechanisms in vivo. CD25⁺CD4⁺ Tregs may suppress their effector T cell targets (T_E) by a number of proposed mechanisms. In vivo CD25⁺CD4⁺ Tregs may act in a cell contact-dependent manner by competing directly for stimulatory ligands on the APC, by sinking essential growth factors such as IL-2, or by directly transmitting an as-yet uncharacterized negative signal. Alternatively, they may use longer-range suppressive mechanisms by means of the cytokines IL-10 and TGF- β . Finally, CD25⁺CD4⁺ Tregs may act through the APC either by triggering IDO activity, resulting in the generation of immunosuppressive metabolites, or by perturbing the APC's presenting capacity. Such mechanisms are not necessarily mutually exclusive, and more than 1 might operate in tandem.

study suggested that engagement of CD80, and to a lesser extent CD86, on the responder T cell and not the APC was responsible for the transmission of a negative signal, and therefore these were the molecular targets through which Tregs exert their function (80). In support of this, the authors demonstrated that B7^{-/-} responder cells were resistant to suppression in vitro and induced a fatal wasting disease refractory to cotransferred CD25⁺CD4⁺ Tregs (80). Again the obvious candidate Treg molecule for this inhibitory interaction would be CTLA-4, although this fails to explain the paradoxically intact suppression mediated by CTLA-4^{-/-} CD25⁺CD4⁺ Tregs (27). The presence of B7 on conventional T cells has been known for several years, and it would be interesting, then, if this hitherto puzzling expression pattern were shown to play a role in Treg-mediated suppression (81, 82). While the identification of a membrane-bound CD25⁺CD4⁺ Treg-specific inhibitory molecule remains inconclusive, some very recent work has suggested that the CD4-related molecule LAG-3 may be important (CD223), though this awaits independent confirmation (83, 84). Proving a negative hypothesis is always a difficult task, but it may yet be shown that there are no truly unique Treg-associated molecules responsible for inhibition. Rather, the specialized functions of Tregs could simply be the product of known molecules acting semi-redundantly, which together generate a suppressive phenotype. An integrated summary of CD25⁺CD4⁺ Treg suppressive mechanisms is shown in Figure 2.

Given the relative physiological scarcity of CD25⁺CD4⁺ Tregs, it seems likely that in vivo they would use mechanisms to amplify their suppressive action, and ones that are not normally fully appreciated under in vitro analysis. This could occur either by the modification

of APCs as outlined above or by the "infectious" spreading of tolerance to conventional T cells. In accordance with this, some recent work has demonstrated that human CD25⁺CD4⁺ Tregs can confer a suppressive phenotype to conventional CD4⁺ T cells in a contact-dependent manner (52). These newly generated regulatory-like cells then suppress by means of IL-10 or TGF- β . This would constitute a mechanism of not only spreading a suppressive phenotype but also making it more efficient on a per-cell basis by engaging the action of soluble mediators. Most satisfactorily, this scenario could also finally reconcile some of the disparities observed between the in vitro and the in vivo mechanisms of CD25⁺CD4⁺ Treg suppression.

Extrathymic generation of CD4⁺ Tregs

The possibility of extrathymic CD25⁺CD4⁺ Treg generation is currently contentious. An interesting recent study in this area readdressed so-called "low-zone" tolerance (85). This phenomenon was first observed decades ago and was described as the antigen-specific tolerance resulting from subimmunogenic doses of antigen given i.v. (86). Using an osmotic pump to deliver minute controlled quantities of antigenic peptide to TCR transgenic mice, the authors of this updated study demonstrated the appearance of CD25⁺CD4⁺ Tregs measurable by their function, surface phenotype, and *Foxp3* expression. Importantly, the study showed that Treg development could still occur in thymectomized TCR transgenic mice on a RAG knockout background, which are normally wholly lacking in CD25⁺CD4⁺ Tregs (17, 20, 46, 85). It therefore seems possible that CD25⁺CD4⁺ Treg development, as measured by *Foxp3* and suppressive function, can occur in conventional T cells under specific in vivo activation

**Table 1**Potential clinical applications of CD25⁺CD4⁺ Tregs

	Target condition	Potential therapeutic approach
Enhancement of CD25 ⁺ CD4 ⁺ Treg function	Organ transplantation, autoimmune disease, allergy	Transfer of Tregs or enhancement of their function allows specific suppression of immune responses; e.g., ex vivo gene transduction of <i>Foxp3</i> ; ex vivo generation of regulatory cells using cytokines, pharmacological agents, or modified DCs
Reduction of CD25 ⁺ CD4 ⁺ Treg function	Cancer, infectious disease	Removal of Tregs or blocking of their function boosts immune responses; anti-CTLA-4, -CD25, -GITR mAbs

conditions, therefore demonstrating a potentially clinically significant developmental plasticity.

The involvement of TGF- β in many facets of Treg behavior is well known (87), but a recent report has also suggested that its exogenous addition results in development of *Foxp3*⁺ Tregs, from conventional and even from RAG knockout CD4⁺ T cells (refs. 87, 88). However, it remains to be seen whether generation of CD25⁺CD4⁺ Tregs by the mechanisms described above can occur outside of the relatively artificial confines of a TCR transgenic system, and indeed whether the initial cell populations contain only "truly" naive T cells and no potential *Foxp3*⁺CD25⁺CD4⁺ Treg precursors.

In the original demonstration of extrathymically generated regulatory cells, the cells were termed T regulatory cell type 1 (Tr1) or Th3 cells (89, 90). It is likely that these cells form a cell type distinct from their thymically generated CD25⁺CD4⁺ Treg counterparts that have been elaborated on above. Tr1 and related cells have been generated using a variety of approaches, typically involving T cell activation in the presence of immunomodulating cytokines or repetitive stimulation with nonprofessional APCs. Tr1 cells were initially generated by chronic stimulation of normal nonregulatory T cells in the presence of IL-10 (89). Such cells secrete a pattern of cytokines distinct from that of the more usual Th1 or Th2 profile and are characterized by high levels of IL-10 and generally low levels of TGF- β and IL-5 (89). Moreover, Tr1 cells are functionally suppressive in vivo and able to prevent the development of Th1 autoimmune diseases such as colitis (89, 91). Th3 cells, on the other hand, were cloned from the mesenteric lymph nodes of mice orally tolerized with myelin basic protein (90). The majority of such cells produce TGF- β and varying levels of Th2 cytokines and suppress the induction of experimental autoimmune encephalitis (90). In vitro treatment of human and mouse T cells with a combination of the immunosuppressants vitamin D₃ and dexamethasone has also resulted in the generation of *Foxp3*⁺ regulatory cells, but with properties somewhat distinct from those reported for Tr1 or Th3 cells (92–94). Finally, there is also a study suggesting that signaling through the complement receptor (CD46) concomitant to more conventional TCR activation can trigger the peripheral induction of human CD4⁺ regulatory cells (95).

Much attention has also focused on the influence that DCs may have on the extrathymic development of regulatory cells. Stimulation with immature DCs (i.e., low levels of costimulatory molecules) and stimulation with DCs modified by pretreatment with IL-10 or TGF- β have both been shown to result in the induction of anergic cells with suppressive capabilities in vitro and in vivo (96–98). Current models of DC-based tolerance state that T cell antigen recognition on immature DCs results in tolerization whereas mature DCs elicit effector responses (99). A system structured in this way would

be effective at maintaining self tolerance in the physiological steady state, i.e., in the absence of inflammatory "danger signals," yet would support productive immune responses following DC maturation triggered by the presence of microbes. However, there would always be the potential danger that DCs matured during "sterile" inflammation, e.g., following mechanical injury, could elicit immunity to autoantigens. Similarly, self tolerance could theoretically also be broken by autoantigens presented on DCs matured during a contemporaneous microbial infection. It seems, though, that the immune system may have yet another level of control to protect against just the kind of scenarios outlined above. In support of this, it was recently reported that the response of conventional human CD4⁺ T cells to autologous peptides presented by *mature*, but not by immature, DCs results in the generation of regulatory-like T cells (53). If confirmed, this ability of the immune system to so dramatically alter the outcome of a response depending on the antigen being recognized is rather remarkable, especially given the apparently matured DC phenotype. Possibly the net response is attributable to the nature or source of ancillary signals, e.g., which toll-like receptors (TLRs) are being engaged, alone or in combination.

Extrathymically generated regulatory cells represent a heterogeneous assemblage whose ontogenetic relationship to naturally occurring Tregs is still being determined. The only really clear point of convergence between the 2 broad families of regulatory cells is that they share a suppressive capability. One interpretation would suggest that peripherally generated regulatory cells merely represent a specialized activation state of conventional CD4⁺ cells (i.e., "adaptive regulatory cells") whereas CD25⁺CD4⁺ Tregs are a de facto lineage by virtue of their distinctive *Foxp3* expression, although the most recent data stemming from the use of TGF- β or low-zone tolerance induction protocols are perhaps blurring even this distinction (85, 88). The use of *Foxp3*/FOXP3 to disentangle this conundrum has been only partially successful. As far as mice are concerned, *Foxp3* seems, by and large, to be a stable marker expressed only in naturally occurring CD25⁺CD4⁺ Tregs and thus far is not in most models of extrathymically generated regulatory cells (but see refs. 85, 88). In contrast, human FOXP3 expression appears far less stringent, with some reports already demonstrating upregulation in extrathymically generated regulatory cells following even basic activation (52, 53). Whether the apparent variability in human FOXP3 and, to a much lesser extent, mouse *Foxp3* expression undermines its importance as an unambiguous marker for naturally occurring Tregs remains to be seen.

Conclusion and clinical perspective

Abundant evidence now strongly supports the once controversial existence of Tregs as key controllers of self tolerance. It also now



seems that their roles can be expanded to many areas of immunology, in fact, potentially, to any scenario where the suppression and/or tuning of an immune response is required. A strategic manipulation of Tregs, either naturally occurring or extrathymically generated, to dampen or enhance their functions as appropriate may prove to have great clinical benefit (Table 1). Already manipulation of CD25⁺CD4⁺ Tregs in various animal models has provided encouraging results for both enhancement of tumor immunity and maintenance of allograft tolerance (100–104). In the case of organ transplantation in particular, CD25⁺CD4⁺ Tregs seem to offer a flexible and adaptive form of immunological control apparently not achievable with standard small-molecule immunosuppression (see for example refs. 102, 104, 105). These practical applications will be expanded upon in other Reviews in this series. Informed by the murine studies, recent experiments are also increasingly demonstrating the significant roles CD25⁺CD4⁺ Tregs can play in human pathologies as varied as rheumatoid arthritis, multiple sclerosis, HIV infection, and allergy (106–112). Recent advances in our understanding of CD25⁺CD4⁺ Treg development and important functional markers such as the association with *Foxp3/FOXP3* have permitted the accurate isolation and manipulation of these cells in mice and, importantly, their human counterparts. Understanding the events both upstream and downstream

of *Foxp3/FOXP3* may enable us to “tailor-make” large numbers of CD25⁺CD4⁺ Tregs to specifically suppress immune responses in autoimmunity and allergy or to antagonize them where a boost of immunity is required, e.g., in microbial and antitumor responses. The potential clinical focus, though, need not be solely on thymically produced CD25⁺CD4⁺ Tregs, since peripherally generated regulatory cells such as Tr1, with their potent cytokine-mediated suppressive capacity, may also hold great therapeutic promise.

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