Control of Immune Responses by Naturally Arising CD4+ Regulatory T Cells That Express Toll-like Receptors

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It is a key issue in immunology to understand how the immune system discriminates between self- and nonself, inhibiting autoimmune responses but allowing the host to mount immune responses against invading microbes (1–3). This is due to the fact that once antigen-specific lymphocytes are activated in adaptive immune responses, they exhibit essentially the same effector activity whether they respond to a microbe or to a self-constituent. That is, once aberrant immune responses (e.g., autoimmune, immunopathologic, or allergic responses) are triggered, serious damage to the host may ensue because of the power of potent effector activity, high antigen specificity, and immunologic memory of adaptive immunity. There is now accumulating evidence in humans and animals that the normal immune system harbors naturally arising CD4+ regulatory T (T_R) cells, which play key roles in controlling such aberrant immune responses (4, 5).

The majority, if not all, of naturally occurring CD4+ T_R cells constitutively express CD25 (IL-2 receptor α chain) in the physiologic state (4, 5). At least some of them are produced by the normal thymus as a functionally mature T cell subpopulation with a broad TCR repertoire recognizing self- and nonself antigens (4). They are engaged in the maintenance of immunologic self-tolerance by actively suppressing the activation and expansion of self-reactive lymphocytes that may cause autoimmune disease. This is illustrated by the finding that the removal of CD25+ CD4+ T cells, which constitute 5–10% of peripheral CD4+ T cells in mice, leads to spontaneous development of various autoimmune diseases in otherwise normal mice. Reconstitution of the depleted population prevents the autoimmunity (6). The removal of this population also triggers excessive or misdirected immune responses to microbial antigens, causing immunopathology. For example, depletion of T_R cells elicits inflammatory bowel disease in mice due to hyperreaction of the remaining T cells to commensal bacteria in the intestine (7). Their depletion similarly precipitates severe pneumonitis in mice that are opportu-

nistically infected with _Pneumocystis carinii_ (8). In contrast, such treatments can enhance protective immune responses against invading microbes including bacteria, viruses, fungi, and intracellular parasites, leading to their eradication from the host (9–13). Thus, naturally occurring CD25+ CD4+ T_R cells not only inhibit immune responses against self-antigens but also hamper or suppress those against microbes invading or cohabiting with the host. This raises a critical question: how do CD25+ CD4+ T_R cells manage to suppress autoimmune responses or excessive antimicrobial immune responses causing immunopathology, but allow protective responses against invading pathogenic microbes?

Expression of Toll-like Receptors (TLRs) on CD25+ CD4+ T_R Cells. A report by Caramalho et al. (14) in this issue provides some clues to the above question. It shows that naturally arising CD25+ CD4+ T_R cells in normal mice selectively express several members of the TLR family and that stimulation of CD25+ CD4+ T_R cells through TLRs can expand them and strengthen their suppressive activity. TLRs, homologues of Toll receptor in _Drosophila_, are germline-encoded receptors that recognize pathogen-associated molecular patterns shared by large groups of microbes or certain endogenous molecules released during inflammation (15, 16). The ten members of TLR (TLRs 1–10) that have been so far reported in humans and mice respond to different ligands. For example, TLR4 recognizes LPS, a major component of gram-negative bacteria, and is also implicated in the recognition of heat shock protein (HSP) 60/70 and extracellular matrix breakdown products derived from necrotic tissue in inflammation (15, 16). Different classes of TLRs are differentially expressed on immune cells such as dendritic cells (DCs), macrophages, B cells, and certain γδ T cells (15–17). Caramalho et al. (14) show that CD25+ CD4+ T cells in normal naive mice selectively express TLR4, 5, 7, and 8 (TLR1, 2, and 6 appear to be more broadly expressed on CD4+ T cells, and not confined to CD25+ CD4+ T cells). In vitro stimulation of CD25+ CD4+ T cells with LPS elicits their proliferation, prolongs their survival, and augments their in vitro suppressive activity even in the absence of APCs, indicating that LPS directly acts on TLR4 molecules expressed by T_R cells. On the other hand, it is known that LPS stimulation

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of DCs triggers their maturation, leading to enhanced antigen processing, increased MHC expression, migration to the lymph nodes, production of proinflammatory cytokines and chemokines, and most importantly, induction of costimulatory molecules such as CD80 and CD86 (18). Thus, stimulation of TLRs have different effects on immune responses, i.e., one is to trigger DC maturation and augment T cell–mediated adaptive immunity and another is to activate endogenous CD25$^+$ CD4$^+$ T$_R$ cells and thereby down-regulate immune responses. Although these effects are apparently conflicting, it is of note that the concentration of LPS required for evoking the proliferation of T$_R$ cells and augmenting their suppressive activity in vitro is several orders of magnitude higher than the concentration required for in vitro activation of DCs (14, 18). It is therefore likely that upon gram-negative bacterial infection, LPS stimulation of TLRs on DCs will trigger maturation of DCs, thereby evoking the activation, expansion, and differentiation of microbe-specific naive T cells to effector T cells. If so large an amount of LPS is produced, T$_R$ cells activated by LPS through TLRs may augment their suppressive activity and thereby prevent local or systemic immunopathology (such as septic shock) due to the production of large amounts of proinflammatory cytokines (such as TNFα and IL-1) by macrophages. IL-10 produced by activated T$_R$ cells may suppress activation of macrophages and their cytokine production (12). Furthermore, IL-2 in the local milieu and TCR stimulation by microbial antigens may synergistically augment the suppressive activity of TLR-stimulated T$_R$ cells (14). The T$_R$ cells thus activated may also suppress the activation and expansion of self-reactive T cells that are recruited to the infection site where APCs present self-peptides derived from damaged tissues.

Tuning of the Intensity of T Cell–mediated Immunoregulation. In addition to TLRs, other molecules expressed by endogenous CD25$^+$ CD4$^+$ T$_R$ cells transduce signals to T$_R$ cells upon interacting with particular ligands and thereby contribute to tuning the degree of their expansion and suppressive activity. Accessory molecules on T$_R$ cells, for example, interact with costimulatory molecules on APCs, DCs in particular, and thereby confer signals augmenting or attenuating the activation state of T$_R$ cells with consequences for their suppressive activity. Unlike other T cells, CD25$^+$ CD4$^+$ T$_R$ cells constitutively express CTLA-4 (CD152) and this appears to be required for their activation because specific blockade of the interaction between CTLA-4 on CD25$^+$ CD4$^+$ T$_R$ cells and CD80/CD86 on APCs abrogates suppression (4). In contrast, active signaling through CD28, along with TCR stimulation, neutralizes suppression (4). CTLA-4 has much higher (∼100-fold) affinity for CD80/CD86 molecules than CD28 (19). Based on these findings, the following scenario can be envisaged for the roles of T$_R$ cells in controlling immune responses in the normal state or upon infection (Fig. 1). In the absence of infection, APCs express basal levels of CD80/CD86 and a low level of class II MHC binding self-peptides. CD25$^+$ CD4$^+$ T$_R$ cells with self-specificity might

be activated by recognizing self-peptides (e.g., derived from apoptotic cells) and receiving a signal through CTLA-4, but not through CD28, and thereby suppress autoreactive T cells. Upon infection, APCs are activated and increase their expression of CD80/CD86 and class II MHC molecules bound to microbe-derived peptides. CD25$^+$ CD4$^+$ T$_R$ cells recognizing the peptides will receive a signal through CD28 in addition to CTLA-4, lessen their suppressive activity, and allow the activation/expansion of effector T cells capable of eliminating microbes. IL-2 from responding effector T cells and a signal through glucocorticoid-induced TNF receptor–related gene (GITR) also act to attenuate the suppressive activity of T$_R$ cells in synergy with the signal through CD28 (Fig. 1 and see below; reference 20). With the elimination of microbes, together with a reduction of the APCs presenting the microbial peptides and a decrease of CD80/CD86 expression, T$_R$ cells revert to their original state and reacquire the basal regulatory activity.

CD25$^+$ CD4$^+$ T$_R$ cells constitutively express GlTR (20, 21). Active signaling through GITR to CD25$^+$ CD4$^+$ T$_R$ cells attenuates their suppressive activity, leading to enhanced immune responses to self- and nonself antigens (20). Mere blockade of GITR on T$_R$ cells fails to abrogate the suppression (20). GITR-ligand (L) is induced on DCs upon maturation (unpublished data). Thus, GITR–GITR-L interaction may also contribute to the tuning of CD25$^+$ CD4$^+$ T$_R$ cell–mediated immunoregulation.

The expression patterns of other accessory molecules on CD25$^+$ CD4$^+$ T$_R$ cells are CD45RB^{low}, CD44^{high}, CD5^{high}, CD54 (intercellular adhesion molecule 1)^{high}, CD11a/CD18 (LFA-1)^{high}, partly CD62L^{low} (4). This pattern suggests that they might be in a "primed," "activated," "effector," or "memory" state probably due to constant stimulation by self-peptides/MHCs on APCs in the normal internal milieu (4). Upon infection, they can be more swiftly recruited to the infection site than other T cells as recently demonstrated by Belkaid et al. (12).

Besides accessory molecules on CD25$^+$ CD4$^+$ T$_R$ cells, cytokines also play key roles in tuning their expansion and activation. High dose IL-2 along with TCR stimulation, for example, triggers their proliferation and neutralizes the suppressive activity during their proliferation (22). Such expanded T$_R$ cells show more potent suppressive activity than before expansion (23). TLR4 stimulation with LPS in the presence of IL-2 also results in their acquisition of more potent suppressive activity than LPS stimulation alone (14). It is thus likely that T cell immune responses to microbes may locally produce a high IL-2 milieu, contributing to the expansion of microbe-specific T$_R$ cells at the site of infection. Other cytokines may also participate in the activation or expansion of endogenous T$_R$ cells (7, 12).

Collectively, these findings indicate that various signals contribute to the activation and expansion of endogenous CD25$^+$ CD4$^+$ T$_R$ cells and thereby to the fine tuning of the suppressive activity of T$_R$ cells, hence the magnitude of immune responses. Strong suppression may make the
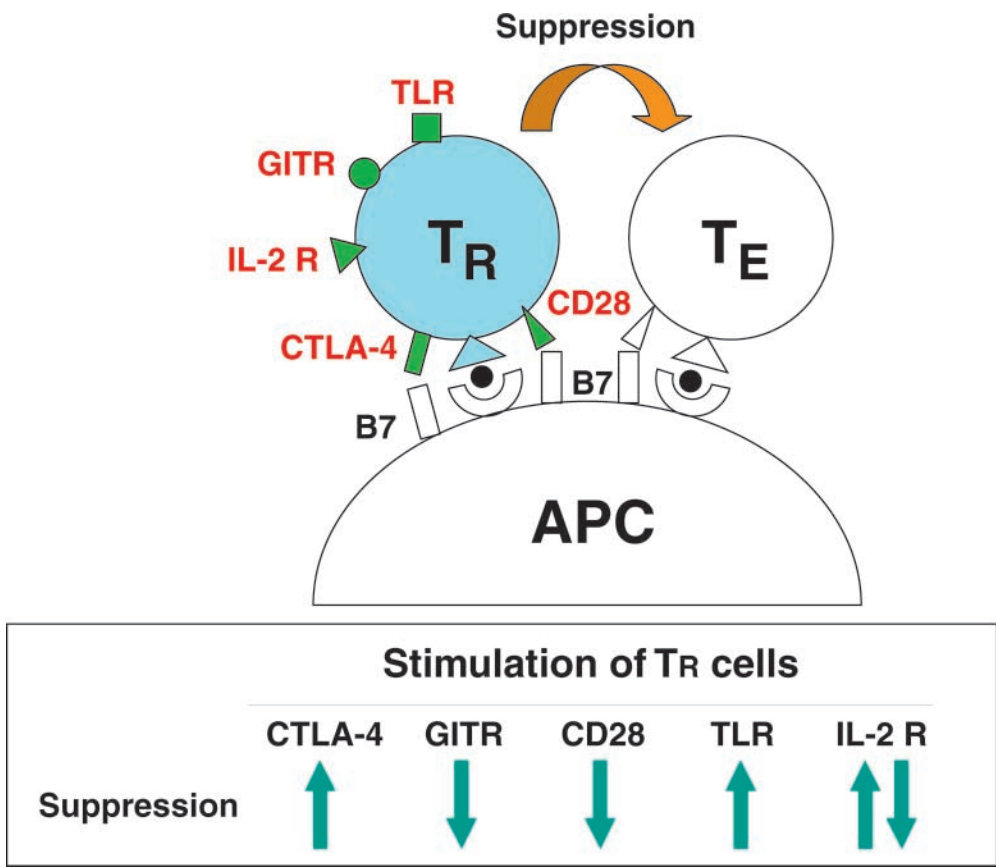
Figure 1. Signaling to CD25+ CD4+ T_R cells via several cell surface molecules including TCR, CD28, CTLA-4, GITR, IL-2 R (CD25), and TLRs results in augmentation or attenuation of their suppressive activity. During the proliferation of T_R cells in the presence of high dose IL-2 along with TCR stimulation, they lose suppressive activity whereas they show augmented suppressive activity upon withdrawal of IL-2 after expansion (references 22 and 23). TLR stimulation may also show a similar sequence of events, i.e., loss of suppressive activity during proliferation and augmented suppression after expansion.

host succumb to the infection whereas weak suppression may lead to complete eradication of the microbes but may lose concomitant immunity because of insufficient maintenance of memory T cells due to lack of microbe persistence (12). It is therefore required to elucidate how various signals, some of which are apparently in opposition (e.g., signals through CTLA-4 vs. CD28), are integrated in T_R cells to determine the degree of their expansion and suppression.

Adaptive Nature of CD25+ CD4+ T_R Cells and Their Roles in Immunological Diseases. There is now evidence not only for the presence of CD25+ CD4+ T_R cells in humans but also for their essential role in controlling autoimmunity, immunopathology, and allergy in human diseases. For example, an X-linked recessive autoimmune/inflammatory syndrome called IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) develops autoimmune diseases (such as type 1 diabetes), inflammatory bowel disease, and allergy similar to those produced in mice by depletion of CD25+ CD4+ T_R cells (24). The causative gene, *Foxp3*, which encodes a transcription factor, is specifically expressed in CD25+ CD4+ T cells in the thymus and periphery. Forced expression of the *Foxp3* gene in naive T cells converts them to T_R cells phenotypically and functionally similar to naturally arising CD25+ CD4+ T_R cells (25). The results indicate that a genetically determined abnormality in the generation or function of CD25+ CD4+ T_R cells can be a cause of im-

munological disease in humans (Fig. 2). It remains to be determined how *Foxp3*, a master regulatory gene for the development and/or function of CD25+ CD4+ T_R cells, controls the expression and functions of T_R-associated molecules such as CD25, CTLA-4, GITR, and TLRs. It is likely that other genetic abnormalities or environmental insults may also affect CD25+ CD4+ T_R cells and thereby cause autoimmune and other immunological diseases (26). Furthermore, defects or polymorphism of other genes encoding those molecules (including TLRs) that contribute to the control of T_R cells (see above) may determine the susceptibility or resistance to autoimmunity, allergy, and immunopathology.

As discussed above, one important feature of naturally arising CD25+ CD4+ T_R cells is that they are immunologically adaptive, i.e., they can expand and augment their suppressive activity when stimulated through TCR along with high dose IL-2 or through TLRs (Fig. 1). This means that repeated microbial infections may not only increase T cell immunity by generating memory T cells but also strengthen the suppressive activity of endogenous CD25+ CD4+ T_R cells. Another level of equilibrium set between the strengthened T_R cells and the memory T cells may ensure effective secondary responses without damage to the host upon reinfection. Furthermore, considering that CD25+ CD4+ T_R cells activated by a specific antigen exert antigen–nonspecific suppression, T_R cells stimulated by microbial antigens may suppress self-reac-
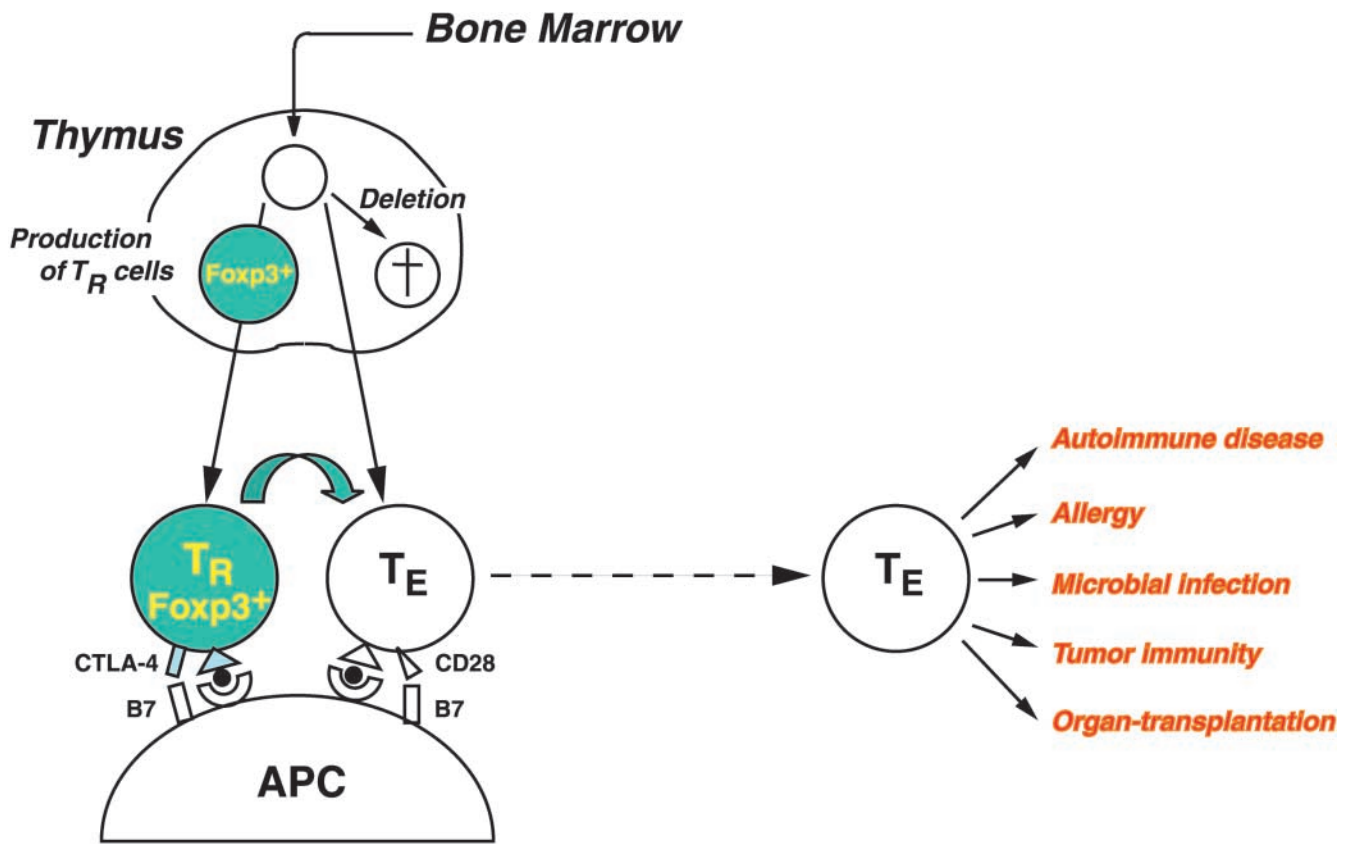
Figure 2. At least some of $CD25^+$ $CD4^+$ T_R cells are produced by the normal thymus as a functionally mature T cell subpopulation. They specifically express the *Foxp3* gene. Reduction of $CD25^+$ $CD4^+$ T_R cells or attenuation of their suppressive activity by manipulating various molecules shown in Fig. 1 may elicit autoimmunity or enhance autoimmunity, tumor immunity, microbial immunity, or allergy. In contrast, an increase of the number of T_R cells or augmentation of their suppressive activity can delay the rejection of organ grafts and induce transplantation tolerance.

tive T cells as well in a bystander manner (22, 23). For example, inoculation of complete Freund's adjuvant, which contains killed tubercle bacilli, inhibits the development of autoimmune disease in animal models (27, 28). It is likely in this finding that HSP of tubercle bacilli may activate endogenous T_R cells through stimulating their TLRs, thereby suppressing self-reactive T cells (29). Other TLR ligands including endogenous HSPs may also be able to strengthen the regulatory activity of endogenous T_R cells and prevent autoimmune disease.

Furthermore, assuming that endogenous T_R cells play key roles in preventing autoimmune diseases and allergy in humans as exemplified in IPEX syndrome, the recent increase of the incidence of autoimmune disease and allergy in developed countries could be attributed to insufficient adaptive expansion or strengthening of endogenous T_R cells due to less frequent opportunities for microbial infections in hygienic societies (26, 30).

Future Perspective. In addition to the control of T cell activation or nonactivation at the level of APCs that discriminate between "infectious nonself" and "noninfectious self" or sensing "danger" signals (2, 3), T_R cells mediate another mode of control of the activation and expansion of aberrant T cells. As shown by Caramalho et al. (14), $CD25^+$ $CD4^+$ T_R cells are an evolutionarily unique T cell subpopulation bearing two kinds of the receptor: TCRs that recognize a broad repertoire of various self- and nonself antigens and TLRs that sense conserved molecular patterns shared by microbial components or certain endogenous molecules (such as HSPs) released in inflammation. This dual signaling source for T_R cells, together with other signals via accessory molecules, may enable $CD25^+$ $CD4^+$ T_R cells to efficiently and finely tune their activity to control adaptive immune responses against self- and nonself antigens (Fig. 2; reference 4). Additional elucidation of the mechanisms by which endogenous T_R cells are generated, activated, or expanded and those by which they control other T cells will contribute to our better control of physiologic and pathologic adaptive immune responses.

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